Impact in Plasma Metabolome as Effect of Lifestyle Intervention for Weight-Loss Reveals Metabolic Benefits in Metabolically Healthy Obese Women

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23 **ABSTRACT**

24 Little is known regarding metabolic benefits of weight loss (WL) on the metabolically healthy obese
25 (MHO) patients. We aimed to examine the impact of a lifestyle weight loss (LWL) treatment on the
26 plasma metabolomic profile in MHO individuals. Plasma samples from 57 MHO women allocated
27 to an intensive LWL treatment group (TG, hypocaloric Mediterranean diet and regular physical
28 activity, n = 30) or to a control group (CG, general recommendations of a healthy diet and physical
29 activity, n = 27) were analyzed using an untargeted 1H NMR metabolomics approach at baseline,
30 after 3 months (intervention), and 12 months (follow-up). The impact of the LWL intervention on
31 plasma metabolome was statistically significant at 3 months but not at follow-up and included higher
32 levels of formate and phosphocreatine and lower levels of LDL/VLDL (signals) and trimethylamine
33 in the TG. These metabolites were also correlated with WL. Higher myo-inositol, methylguanidine,
34 and 3-hydroxybutyrate, and lower proline, were also found in the TG; higher levels of hippurate and
35 asparagine, and lower levels of 2-hydroxybutyrate and creatine, were associated with WL. The
36 current findings suggest that an intensive LWL treatment, and the consequent WL, leads to an
37 improved plasma metabolic profile in MHO women through its impact on energy, amino acid,
38 lipoprotein, and microbial metabolism.

39 **KEYWORDS:** metabolomics, NMR, hypocaloric diet, physical activity, metabolically healthy
40 obesity.

41

42 **INTRODUCTION**

43 Recent studies have shown that even within the obese phenotype, cardiometabolic risk may not
44 necessarily vary primarily in relation to weight or body mass index (BMI) but to other subclinical
45 alterations.1,2 In this sense, there is a subset of obese individuals with lower risk of CVD or all-cause
46 mortality,3,4 which has been referred to as the “obese healthy paradox”.5 Although there is currently
47 a lack of consensus on its definition, it has been suggested that metabolically healthy obese (MHO)
48 individuals have ≤ 1 of the metabolic syndrome (MetS) criteria in addition to waist circumference

(WC) ≥ 102 cm for men and ≥ 88 cm for women, that is, hypertension, hypertriglyceridaemia, hyperglycaemia or diagnosis of diabetes, and dyslipidaemia in comparison to their metabolically unhealthy obese (MUO) counterparts.6 In addition, other parameters, such as insulin sensitivity, and inflammatory markers, such as tumor necrosis factor alpha (TNF-α), have been suggested for inclusion in the definition of MHO.5,7 In the face of a lack of consensus for MHO definition, recent metabolomic studies have contributed with a better characterization of the metabolic signatures of this phenotype.8 The prevalence of MHO varies widely, partly depending on definition criteria, but seems to be higher in women and in people of younger ages and also dependent on region and lifestyle.9,10 On the other hand, long-term studies have suggested that MHO is a transient state toward MUO,11,12 indicating that without proper care, MHO individuals may increase their risk of developing T2D and CVD. Furthermore, since a decisive feature of MHO is the absence of visceral fat accumulation,13 the promotion of lifestyle interventions aimed at minimizing visceral fat accumulation is of fundamental importance from a public health perspective. Although diet and physical activity are well-known and modifiable CVD risk factors, their potential beneficial impact on MHO under controlled conditions has only recently received attention.14,15 Recent studies have demonstrated that intensive lifestyle weight loss (LWL) interventions based on calorie restriction and physical activity were effective as means of improving body composition and several cardiometabolic risk markers in obese individuals.16−18 In this sense, for instance, the adherence to healthy dietary patterns such as the Mediterranean diet (MedDiet) has been widely recommended for reducing the incidence and lowering the prevalence of MetS and its components.19 Currently, a better understanding of how a LWL intervention impacts on the metabolism of obese individuals has been little addressed by the use of metabolomics. For example, in a recent study, Khakimov and colleagues reported that as result of a LWL intervention study with a healthy New Nordic Diet, participants with greater weight loss differed in their plasma metabolite composition including metabolites related to energy metabolism and food intake.20 In the current study, we therefore aimed to (i) determine the impact of an intensive lifestyle treatment for weight loss (based on a hypocaloric Mediterranean diet and regular physical activity) in comparison to a control group (general recommendations for a cardiometabolic healthy diet and physical activity) on the plasma metabolome, measured by

untargeted 1H NMR metabolomics, in women defined as MHO and to (ii) investigate the associations of WL and changes in other cardiometabolic risk markers with changes in metabolome after following the intervention.

EXPERIMENTAL SECTION

Participants and Study Design

A total of 115 women aged 35−55 defined as MHO were recruited from four health centers in the Malaga District of the Andalusian Health Service (Spain).21 Diagnosis of metabolic health status was based on the general criteria proposed by the International Diabetes Federation (IDF).22 Besides obesity (BMI ≥ 30 kg/m2), participants were included if they had ≤1 of the following cardiovascular risk factors: elevated fasting glucose levels (plasma glucose ≥100 mg/dL), elevated blood pressure (systolic ≥135 or diastolic ≥85 mmHg or use of antihypertensive drugs), elevated triglycerides (≥150 mg/dL or treatment with lipid-lowering medication), or decreased high-density-lipoprotein cholesterol (HDL) (<50 mg/dL). The exclusion criteria were previous diagnosis of diabetes, pregnancy or planning to become pregnant during the study, CVD, presence of any severe chronic illness, alcohol or drug abuse, or undertaking a WL program that included physical activity or diet in the past three months. Participants were randomly allocated to either the control (n = 48) or the treatment group (n = 67). Participants assigned to the control group received general recommendations on a cardiometabolic healthy diet and physical activity. Participants in the treatment group received an intensive intervention program for losing weight, consisting of a hypocaloric MedDiet and regular physical activity. The MedDiet included the intake of extra virgin olive oil and nuts but with an overall energy restriction of about 600 kcal (approximately 30% of estimated energy requirements). The distribution of the target daily total caloric intake for the intervention group was: 35−40% fats (8− 10% saturated fatty acids; SFA), 40−45% carbohydrates with low glycemic index, and 20% protein. Adherence to the MedDiet was measured at baseline and after 12 months of follow-up by using a 16-item screener from the PREDIMED study23 and adapted to assess hypocaloric MedDiet. In the physical activity program, participants were encouraged to

practice a minimum of 150 min/wk of walking. Participants allocated to the treatment group attended visits with a certified nutritionist every week during the first 3 months, and then once at 12 months, whereas individuals in the control group attended these visits only after 3 and 12 months of the study. All participants provided written informed consent. The study was conducted in accordance with the guidelines set out in the Declaration of Helsinki, and all protocols were approved by an Ethics and Research Committee (Comite Coordinador de Etica de la Investigacion Biomedica de Andalucia). This study was registered at https://www.isrctn.com/ as ID ISRCTN88315555.

Clinical Measurements and Sampling

Anthropometric measurements, including weight, height, waist circumference (WC), and BMI, were taken by trained nurses at baseline and after 3 and 12 months, and blood pressure measurements were taken at baseline and at 12 months. Fasting blood samples were collected in tubes containing EDTA on the day of enrolment and after 3 and 12 months. Analyses of fasting glucose and lipid profile were conducted according to routine methods and within 12 h of sample collection. For metabolomics analysis, plasma samples were collected at the same time points, aliquoted, and immediately stored at −80 °C until analysis.

NMR Metabolomics

Sample Preparation. Plasma samples were thawed at 4 °C, briefly spun down and 150 μL was mixed with 150 μL of ultrapure water and 600 μL of pure cold (−20 °C) methanol in 96-deep-well plates. The mixtures were vortexed, incubated (first at 12 °C, 800 rpm for 10 min and then at −20 °C for 30 min), and centrifuged (2250g at 4 °C for 60 min) to precipitate proteins. Supernatants (600 μL) were transferred into clean deep-well plates and lyophilized at −4 °C for 16 h. Dried samples were washed in 50 μL of deuterated methanol (MeOD) and again lyophilized to remove the excess of non- MeOD. The new pellets were reconstituted in 200 μL of buffer (37.5 mM sodium phosphate, pH 6.95, 100% D20, 0.02% NaN3, 0.25 mM DSS-d6 and 1 mM imidazole) and shaken in an Eppendorf ThermoMixer at 800 rpm, 22 °C, for 30 min. Samples in buffer (180 μL) were transferred into 3 mm

SampleJet NMR tubes using a SamplePro L liquid handling robot (Bruker BioSpin, Rheinstetten, Germany).

1H NMR Spectroscopy. All 1H NMR experiments were performed on an Oxford 800 MHz magnet equipped with a Bruker Avance III HD console and a 3 mm TCI cryoprobe using a water suppression pulse program. Each spectrum was acquired at 298 K applying 128 scans, a spectral width of 20 ppm, a data size of 65 K points, an acquisition time of 2.05 s and a relaxation delay of 3 s. Spectra were processed using TopSpin 3.5pI6 (Bruker GmbH, Rheinstetten, Germany). Processed spectral data were imported into MatLab (Math- Works Inc., Natick, MA) using in-house written scripts. Alignment was achieved using a combination of an in-house peak reference picking function and the "speaq" R-package (version 1.2.1).24

Statistical Analyses. All statistical data analyses were performed within the R environment (version 3.3.1). Differences in anthropometric and clinical variables at baseline and after 3 and 12 months were assessed by independent or paired Student's t tests according to comparisons between or within groups, respectively. Data are expressed as mean ± SD, unless otherwise stated. To determine discriminant metabolites between control and treatment groups at 3 of intervention and at 12 months of follow-up, we used NMR data of differences in metabolome between baseline and each time point (3 or 12 months) and conducted a supervised analysis based on random forest (RF) modeling within an in-house-developed repeated double crossvalidation framework (rdCV).25,26 In brief, the in-house double CV procedure, which has been successfully used in untargeted metabolomics27 and microbiota analysis,28 consists of nested loops (outer "testing" and inner "calibration" loops) to reduce bias from overfitting models to experimental data.25 Feature ranking and selection are performed within the inner loop, to minimize statistical overfitting, by iteratively turning over successively fewer features, removing from each step in the loop the 10% least informative features.27 The rdCV procedure was subjected to 30 repetitions to improve modeling accuracy and with misclassification as the fitness function. The overall validity and degree of overfitting of models were assessed by permutation analysis, following the same rdCV procedure and by reporting the cumulative probability of actual model fitness within a population of fitness measures of randomly

permuted classifications (n = 200) based on the assumption of Student's t-distribution. The assumption was confirmed by visual inspection of the histograms of permuted distributions. Secondary analyses of associations of changes in metabolome with changes in weight or other clinical parameters were performed using both the control and treatment groups together, as well as in treatment group alone, by partial leastsquares (PLS) regression within a similar rdCV framework. The quality of each model was evaluated by the R2 (the proportion of the variance of the response variable that is explained by the model) and Q2 (the predictive ability) parameters. Permutation tests (n = 200) were performed similarly to the analysis above, but with Q2 as the fitness measure. Differences in changes of metabolites between groups after the intervention were calculated by fold change (FC), taking the control group as reference, and assessed by independent Student's t tests. The FC here was calculated as follows: $FC = \Delta Treament / \Delta Control$, where $\Delta Treament$ and $\Delta Control$ denote the differences between the NMR intensities of metabolites at either 3 or 12 months and at baseline, for treatment and control groups, repectively. Correlations between significant metabolites selected from multivariate modeling of weight change after the intervention were calculated by Spearman's rank correlation ("Hmisc" R-package version 4.0−2). A false discovery rate (FDR) test based on Benjamini−Hochberg procedure29 was applied to adjust the pvalue for multiple comparisons in univariate and correlation analyses.

Metabolite Identification. Identification of metabolites was achieved by matching experimental NMR spectra with those stored in Chenomx NMR Suite 8.2 software (Chenomx Inc., Canada) in combination with an in-house R script for statistical correlation spectroscopy30 and through searching in the Human Metabolome Database (HMDB) compound reference library.31

RESULTS

Baseline Characteristics of the Participants

Of the 115 participants recruited, 58 were excluded due to dropout or failure to show at all visits (n = 43), illness (n = 6), unavailable sample at some time point (at baseline, 3 or 12 months, n = 7), or change of residence (n = 2). Therefore, 57 participants were included in the present data analyses.

Anthropometric measures and clinical parameters at baseline and after 3 and 12 months are presented in Table 1. At baseline, MHO participants had a mean (±SD) age of 45.1 ± 3.45 y and a BMI of 35.8 ± 4.93 kg/m2. No differences between the control and treatment groups were observed at baseline regarding menopause, weight, waist circumference, blood pressure, glycaemia, or lipid profile (Table 1).

Changes in Anthropometric and Clinical Measurements

At both 3 and 12 months, the treatment group showed greater WL and more pronounced reductions in BMI and WC than the control group (Table 1). Compared to baseline, both groups showed a decrease in total cholesterol and changes in HDL at 3 and 12 months. In particular, at 3 months, the levels of HDL were decreased in the treatment group and increased in the control group. Moreover, at 3 months, only participants in the treatment group showed decreases in LDL cholesterol and at 12 months decreases in SBP, glucose, and triglycerides, whereas at 12 months, only the control group showed decreases in LDL (Table 1).

Multivariate Modeling of Intervention and Weight Change

The classification of participants as treatment or control group based on the changes in metabolome is shown in Figure 1. The rdCV-RF models resulted in a high correct classification rate (86%, permutation test $p < 0.001$) of the individuals at 3 months and lower correct classification rate (65%, permutation test $p < 0.05$) at 12 months (Figure 1; Supporting Information, Figure S-1). With the exception of weight change, changes in other clinical parameters were not significantly associated with changes in the metabolome (data not shown). The rdCVPLS regression of weight change based on changes in metabolome resulted in moderate associations when all participants ($R2 = 0.630$, $Q2 = 0.257$; permutation test $p < 0.001$), or only participants in the treatment group ($R2 = 0.744$, $Q2 = 0.298$; permutation test $p < 0.001$), were included in the analysis (Figure 2; Supporting Information, Figure S-2).

Modulatory Effect of Intervention on Plasmatic Metabolites

Changes in metabolome after 3 months of intervention included higher levels in the treatment group of 3- hydroxybutyrate (3-HB), formate, methylguanidine, myoinositol, and phosphocreatine, as well as lower levels of LDL/VLDL signals, proline, trimethylamine (TMA), and three unassigned compounds (U3.32, U4.35, and U6.40) (Table 2). Absolute FC in 3-HB, methylguanidine, phosphocreatine, myo-inositol, proline, U4.35, and U6.40 were ≥2 (two-times or more) higher in the treatment group than in the control group. Because of the poor multivariate classification between groups at 12 months, discriminant metabolites at 3 months were further investigated by t test at 12 months of follow-up (Table 2). From this analysis, differences between the treatment and control groups at 12 months were only observed in U3.32 ($p < 0.05$) and phosphocreatine ($p < 0.05$). However, compared to at 3 months, fold changes in these metabolites at 12 months indicated a more accentuated change in U3.32 and a change from upregulation to downregulation in phosphocreatine.

Changes in Metabolome Associated with Weight Loss

A total of 11 metabolites were moderately associated with a change in weight from baseline in both groups at 3 months (Table 3). Keeping in mind that an association of metabolites with WL was established as an inverse association with weight change (i.e., a positive association with weight change means an inverse association with WL), WL was inversely associated with 2-hydroxybutyrate (2-HB), creatine, LDL/VLDL signals, TMA, and three unknown compounds (U.sugar, U2.96, and U3.32) and directly associated with asparagine, formate, hippurate, and phosphocreatine. Interestingly, from this model, the changes in formate, phosphocreatine, LDL/VLDL signals, TMA, and U3.32 were found to be in the same direction as those observed in the previous model with treatment (Figure 3).

DISCUSSION

Using untargeted 1H NMR-based metabolomics and multivariate modeling, we were able to determine changes in the plasma metabolome associated with a LWL treatment based on a hypocaloric diet and physical activity in MHO women. Within this context, we further investigated the association of WL with changes in the metabolome. As expected, compared to the control group,

individuals in the treatment group underwent greater WL. It is important to highlight that participants of the current metabolomics study were a subpopulation of other larger study aimed to assess the effect of WL on cardiometabolic risk markers.21 Findings in the present study regarding changes in clinical parameters were similar to that larger study. Consequently, the discussion in the current work focuses on the impact of LWL intervention on the plasma metabolome. Differences in the plasma metabolome between the treatment and control groups were more pronounced at 3 than at 12 months (Figure 1; Supporting Information, Figure S-1). One reason could be the similar WL achieved during the period between the third and 12th months after beginning the intervention (Table 1) or compensatory mechanisms that attenuated the effects at 12 months. Among cardiometabolic risk markers, only weight change was associated with the changes in metabolome after 3 months of the study (Figure 2; Supporting Information, Figure S-2). We found distinct and common metabolites associated in the same direction with the intervention and WL (Figure 3), which together reflect a positive impact of an intensive LWL intervention on the metabolism of energy, amino acids, lipoproteins, and microbiota. For instance, the higher 3-HB observed in the treatment group than in the control group is consistent with previous studies on weight loss.32,33 High circulating levels of ketone bodies are observed under energyrestricted metabolic states caused by fasting and caloric restriction, through increased lipolysis of fatty acids in liver mitochondria.34 Therefore, the increase of 3-HB in treatment may reflect energy homeostasis through increased lipid oxidation.35 Interestingly, 2-HB, a well-known early biomarker of impaired glucose regulation in non-T2D individuals,36 was found to decrease with WL. We therefore speculate that as a result of WL, the MHO individuals may have decreased their risk of developing T2D.37 The association of proline and asparagine with treatment and WL, respectively, reflects impacts on amino acid metabolism. The lower levels of proline in the treatment group than in the control group are in line with previously reported data, indicating that both caloric restriction33 and increased physical activity38 result in lower circulating proline in obese individuals. Moreover, previous studies have also shown an association between long-term successful WL and lower plasma proline levels.39 However, this was not supported in the present study since proline was not directly associated with WL. The positive association between asparagine and WL found in our study is consistent with previous reports, which

have shown an inverse association between this amino acid and obesity.40,41 Circulating levels of asparagine can be obtained from dietary sources or synthesized from endogenous oxaloacetate via aspartate. Studies conducted in animal models have demonstrated that supplementation with aspartate and asparagine increased the glucose uptake and glycogen content in skeletal muscle, possibly through the incorporation of glucose transporters type 4 or vesicles into the glycogen complex.42 We therefore speculate that along with WL, an increase of asparagine may be associated, in part, with an improved glucose homeostasis. However, future studies are warranted to better determine the functional role of asparagine in WL. Taken together, the observed associations of 3-HB, 2- HB, and asparagine with the current LWL intervention and WL strongly suggest a positive impact on glucose homeostasis in the MHO phenotype, which could also be interpreted as a decreased risk of T2D. Also related to amino acid metabolism, we found that phosphocreatine increased with both treatment and WL, whereas creatine decreased with WL. Creatine is mainly produced in the liver and skeletal muscle from glycine and arginine and can further be phosphorylated to form phosphocreatine by the enzymatic action of creatine kinase (CK).43 We therefore speculate that the contrasting association of creatine and phosphocreatine with WL may be related to a modulatory effect of WL on CK activity. Recent studies showing significant associations of CK with obesity44,45 and weight loss46 may support this hypothesis. With regard to lipoprotein metabolism, we found lower intensity of signals corresponding to LDL/VLDL in the treatment than in the control group and this was also inversely associated with WL, which is consistent with previous studies47 and probably related to increased expression of LDL receptor and lipoprotein lipase.48 Similar findings regarding the profile of these lipoprotein fragments associated with WL were recently reported by Rodriguez-Garcia et al. in this cohort, albeit using a different analytical approach.21 The contribution of four microbial metabolites, that is, formate, hippurate, methylguanidine, and TMA, to either treatment or weight change models strongly supports previous reports that highlight the role of host-microbiota interactions in body weight composition and WL; the latter being promoted by either a LWL intervention based on diet49 or bariatric surgery.50 For example, TMA, an intermediate metabolite from the microbial metabolism of dietary carnitine and choline, decreased after LWL and WL in the present study. Trimethylamine is oxidized by hepatic flavin-containing monooxygenases to form trimethylamine-N-oxide (TMAO),

which has been shown to be both proatherogenic and associated with cardiovascular disease risk.51,52 We hypothesize that the lower levels of TMA associated with treatment and WL are related to either a lower intake of its dietary precursors (i.e., eggs and meat)52,53 or modulation of choline and carnitine metabolism, and consequently point to a lower risk of CVD due to a likely reduced synthesis of TMAO. The observed treatment-related changes in these microbial metabolites may be related with dietary intake. In the current study, however, data on food intake at 3 months that would have allowed us to better establish this relationship were unfortunately lacking. Other unidentified metabolites, including unassigned signals corresponding to sugars, were found to be related to treatment and WL (Tables 1 and 2). Of particular interest is the unknown U3.32, which was not only found to be related to treatment and WL at 3 months, but also remained significant in the treatment group at 12-month follow-up. Further research to identify this compound to understand its role in WL in the short and long-term is needed. The high number of subjects misclassified at 12 months suggests a larger similarity in the changes in metabolome between groups, presumably due to either loss of compliance or adaptation to changes after the first 3 months in the treatment group. Several factors, including physiological, behavioral, and environmental ones, are key to both compliance and dropout in long-term programs for WL.54 It is well documented that although lifestyle interventions can be effective for long-term WL and improvements on cardiometabolic markers, maximum WL is normally achieved between 1 and 6 months, followed by variable weight maintenance or weight regain.55 However, in the present study, no differences in WL could be observed within the groups from 3 to 12 months, thereby suggesting a maintenance phase. Therefore, based on the poor multivariate predictions at 12 months combined with the maintained WL between 3 and 12 months, we hypothesize that a metabolic adaptation occurs during this maintenance stage. To the best of our knowledge, however, there are no reports on this type of metabolic/metabolome adaptation as a result of longer-term WL interventions. Furthermore, although participants were defined a priori as belonging to MHO, the combined results at 3 months, in terms of changes in clinical parameters and metabolome, indicate that the current weight loss intervention caused shifts toward a healthier phenotype with reduced risk of CVD. However, the positive metabolic regulations appeared to be attenuated in the longer term, even though weight loss was maintained. The reasons for this

attenuation remain unclear. We recognize that our study has a number of limitations and strengths. For instance, the sample size is relatively small and the study participants were exclusively Caucasian, women, and middle-aged. Thus, we cannot extrapolate our conclusions to the general population. In this sense, it would be interesting, for example, to determine the effect of a LWL in MUO individuals as well as in men. Another limitation of our study was that compliance of physical activity practice during all study and of MedDiet at 3 months, in both groups, was not measured, thus leading to a lack of information regarding adherence to parts of the applied intervention. We, however, hypothesize that due to the larger WL at both 3 and 12 months, the practice of physical activity and intake of hypocaloric MedDiet were significantly higher in treatment than in control group, as expected. On the other hand, because we were not able to assign the identity of unknown compounds, potentially important information about metabolic perturbations in relation to intervention and WL was unavailable. Future research aimed at identifying these unknown compounds is warranted. As was also pointed out above, our study also had several strengths. The current findings demonstrate that even with a relative healthy condition, the adoption of LWL is always a recommended strategy to reduce the cardiovascular risk and complications in obese individuals. This would be supported, for instance, with the observed inverse association between WL and an early biomarker of impaired glucose regulation (2-HB), suggesting a modulatory effect of WL on the diabetes risk. Furthermore, the untargeted workflow employed peak picking instead of binning, thereby expanding and improving the available information content in the original data. The multivariate modeling procedure and validation framework employed a data-driven, robust approach to maximize information density while minimizing the likelihood of false-positive findings, thereby focusing automatically on the most relevant metabolic perturbations in relation to the WL intervention.27 Finally, our findings reinforce the utility of metabolomics in the identification of biomarkers (beyond clinical parameters) of LWL interventions in individuals with moderate risk of CVD. These biomarkers could be used in future research as additional targets of LWL interventions.

CONCLUSIONS

In conclusion, using untargeted 1H NMR metabolomics and multivariate modeling, we determined that the impact on plasma metabolome of MHO women after a lifestyle intervention for weight loss, based on hypocaloric Mediterranean diet and regular physical activity, was driven by changes in amino acid, lipoprotein and microbial metabolism. Furthermore, we found that changes in the metabolome were associated with weight loss within the frame of the same intervention. Taken together, the lifestyle intervention and weight loss regulated plasma metabolome of MHO toward a healthier phenotype. Such regulations were only observed at 3 months. Although weight loss was maintained at 12 months, the metabolic changes driven by intervention were substantially attenuated at 12 months, suggesting metabolic adaptation. The inverse association between WL and 2-HB, in conjunction with observed changes in other energy-related metabolites, could be interpreted as a decreased risk of T2D as an effect of the LWL treatment. Future research on metabolomic changes and adaptation in long-term studies is warranted.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.8b00042. Permutation test of repeated double cross-validated RF model for classification of individuals according to intervention group at 3 months; permutation tests of rdCV-PLS models for weight change from baseline to 3 months in control and treatment groups together and treatment group alone (PDF)

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Author Contributions

△These authors equally contributed to this work. M.R.B.-L., R.G.H., F.J.T., and C.A.L. conceived and designed the study. M.R.B.-L., F.J.T., and R.G.H. provided the clinical samples of study. E.A.A. and F.J.M. performed metabolomics analyses. E.A.A. performed statistical analyses and drafted the initial manuscript. C.B. and M.G.A. supervised the statistical analyses and drafting of the manuscript. C.B., M.G.A., R.L., and C.A.L. interpreted data and revised manuscript for important intellectual content. C.A.L. and M.R.B.-L. have the primary responsibility for the final content. All authors were involved in revising the manuscript and had final approval of the submitted version.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

LWL, lifestyle weight loss; MHO, metabolically healthy obese; 1H-NMR, proton nuclear magnetic resonance; LDL, lowdensity lipoprotein cholesterol; VLDL, very low-density lipoprotein cholesterol; WL, weight loss; BMI, body mass index; CVD, cardiovascular disease; MetS, metabolic syndrome; WC, waist circumference; MUO, metabolically unhealthy obese; TNF-α, tumor necrosis factor alpha; T2D, type 2 diabetes; MedDiet, Mediterranean diet; HDL, highdensity lipoprotein cholesterol; MeOD, deuterated methanol; FDR, false discovery rate; RF, random forest; PLS, partial leastsquares regression; rdCV, repeated double cross-validation; HMDB, Human Metabolome Database; 3-HB, 3-hydroxybutyrate;; TMA, trimethylamine; 2-HB, 2-hydroxybutyrate; CK, creatine kinase.

REFERENCES

(1) Sims, E. A. H. Are There Persons Who Are Obese, but Metabolically Healthy? Metab., Clin. Exp. 2001, 50 (12), 1499−1504.

(2) Primeau, V.; Coderre, L.; Karelis, A. D.; Brochu, M.; Lavoie, M.; Messier, V.; Sladek, R.; Rabasa-Lhoret, R. Characterizing the Profile of Obese Patients Who Are Metabolically Healthy. Int. J. Obes. 2011, 35 (7), 971−981.

(3) Calori, G.; Lattuada, G.; Piemonti, L.; Garancini, M. P.; Ragogna, F.; Villa, M.; Mannino, S.; Crosignani, P.; Bosi, E.; Luzi, L.; Ruotolo, G.; Perseghin, G.; et al. Prevalence, Metabolic Features, and Prognosis of Metabolically Healthy Obese Italian Individuals: The Cremona Study. Diabetes Care 2011, 34 (1), 210−215.

(4) Hamer, M.; Stamatakis, E. Metabolically Healthy Obesity and Risk of All-Cause and Cardiovascular Disease Mortality. J. Clin. Endocrinol. Metab. 2012, 97 (7), 2482−2488.

(5) Barbarroja, N.; Lo´pez-Pedrera, R.; Mayas, M. D.; García- Fuentes, E.; Garrido-Sa´nchez, L.; Macias-Gonzalez, M.; El Bekay, R.; Vidal-Puig, A.; Tinahones, F. J. The Obese Healthy Paradox: Is Inflammation the Answer? Biochem. J. 2010, 430 (1), 141−149.

(6) Alberti, K. G.; Eckel, R. H.; Grundy, S. M.; Zimmet, P. Z.; Cleeman, J. I.; Donato, K. A.; Fruchart, J.-C.; James, W. P. T.; Loria, C. M.; Smith, S. C. J. Harmonizing the Metabolic Syndrome: A Joint

417 Interim Statement of the International Diabetes Federation Task Force on Epidemiology and
418 Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart
419 Federation; International. Circulation 2009, 120 (16), 1640−1645.

420 (7) Phillips, C. M.; Perry, I. J. Does Inflammation Determine Metabolic Health Status in Obese and
421 Nonobese Adults? J. Clin. Endocrinol. Metab. 2013, 98 (10), E1610−9.

422 (8) Bagheri, M.; Farzadfar, F.; Qi, L.; Yekaninejad, M. S.; Chamari, M.; Zeleznik, O. A.; Kalantar,
423 Z.; Ebrahimi, Z.; Sheidaie, A.; Koletzko, B.; Uhl, O.; Djazayery, A. Obesity-Related Metabolomic
424 Profiles and Discrimination of Metabolically Unhealthy Obesity. J. Proteome Res. 2018, 17, 1452−
425 1462.

426 (9) Rey-Lo´pez, J. P.; de Rezende, L. F.; Pastor-Valero, M.; Tess, B. H. The Prevalence of
427 Metabolically Healthy Obesity: A Systematic Review and Critical Evaluation of the Definitions
428 Used. Obes. Rev. 2014, 15 (10), 781−790.

429 (10) Wang, B.; Zhuang, R.; Luo, X.; Yin, L.; Pang, C.; Feng, T.; You, H.; Zhai, Y.; Ren, Y.; Zhang,
430 L.; Li, L.; Zhao, J.; Hu, D.; et al. Prevalence of Metabolically Healthy Obese and Metabolically Obese
431 but Normal Weight in Adults Worldwide: A Meta-Analysis. Horm. Metab. Res. 2015, 47 (11), 839−
432 845.

433 (11) Tulipani, S.; Palau-Rodriguez, M.; Min˜arro Alonso, A.; Cardona, F.; Marco-Ramell, A.; Zonja,
434 B.; Lopez de Alda, M.; Munoz-Garach, A.; Sanchez-Pla, A.; Tinahones, F. J.; Andres-Lacueva, C.;
435 et al. Biomarkers of Morbid Obesity and Prediabetes by Metabolomic Profiling of Human Discordant
436 Phenotypes. Clin. Chim. Acta 2016, 463, 53−61.

437 (12) Kramer, C. K.; Zinman, B.; Retnakaran, R. Are Metabolically Healthy Overweight and Obesity
438 Benign Conditions?: A Systematic Review and Meta-Analysis. Ann. Intern. Med. 2013, 159 (11),
439 758− 769.

(13) Hwang, Y.; Hayashi, T.; Fujimoto, W. Y.; Kahn, S. E.; Leonetti, D. L.; McNeely, M. J.; Boyko, E. J. Visceral Abdominal Fat Accumulation Predicts the Conversion of Metabolically Healthy Obese Subjects to an Unhealthy Phenotype. Int. J. Obes. 2015, 39 (9), 1365−1370.

(14) Dalzill, C.; Nigam, A.; Juneau, M.; Guilbeault, V.; Latour, E.; Maurie ̀ge, P.; Gayda, M. Intensive Lifestyle Intervention Improves Cardiometabolic and Exercise Parameters in Metabolically Healthy Obese and Metabolically Unhealthy Obese Individuals. Can. J. Cardiol. 2014, 30 (4), 434−440.

(15) Arsenault, B. J.; Co ̂te ́, M.; Cartier, A.; Lemieux, I.; Despre ́s, J.; Ross, R.; Earnest, C. P.; Blair, S. N.; Church, T. S. Effect of Exercise Training on Cardiometabolic Risk Markers among Sedentary, but Metabolically Healthy Overweight or Obese Post-Menopausal Women with Elevated Blood Pressure. Atherosclerosis 2009, 207 (2), 530−533.

(16) Petelin, A.; Bizjak, M.; C ̌ernelic ̌-Bizjak, M.; Jurdana, M.; Jakus, T.; Jenko-Praz ̌nikar, Z. Low-Grade Inflammation in Overweight and Obese Adults Is Affected by Weight Loss Program. J. Endocrinol. Invest. 2014, 37 (8), 745−755.

(17) Abbenhardt, C.; McTiernan, A.; Alfano, C. M.; Wener, M. H.; Campbell, K. L.; Duggan, C.; Foster-Schubert, K. E.; Kong, A.; Toriola, A. T.; Potter, J. D.; Mason, C.; Xiao, L.; Blackburn, G.L.; Bain, C.; Ulrich, C.M.; et al. Effects of Individual and Combined Dietary Weight Loss and Exercise Interventions in Postmenopausal Women on Adiponectin and Leptin Levels. J. Intern. Med. 2013, 274 (2), 163−175.

(18) Bouchonville, M.; Armamento-Villareal, R.; Shah, K.; Napoli, N.; Sinacore, D. R.; Qualls, C.; Villareal, D. T. Weight Loss, Exercise or Both and Cardiometabolic Risk Factors in Obese Older Adults: Results of a Randomized Controlled Trial. Int. J. Obes. 2014, 38 (3), 423−431.

(19) Pe ́rez-Martínez, P.; Mikhailidis, D. P.; Athyros, V. G.; Bullo, M.; Couture, P.; Covas, M. I.; de Koning, L.; Delgado-Lista, J.; Diaz- Lopez, A.; Drevon, C. A.; Estruch, R.; Esposito, K.; Fito ́, M.;

464 Garaulet, M.; Giugliano, D.; Garcıa´ -Rıo´ s, A.; Katsiki, N.; Kolovou, G.; Lamarche, B.; Maiorino,
465 M. I.; Mena-Sa´nchez, G.; Mun˜oz-Garach, A.; Nikolic, D.; Ordova´s, J. M.; Pe´rez-Jime´nez, F.;
466 Rizzo, M.; Salas- Salvado´, J.; Schro¨der, H.; Tinahones, F. J.; de la Torre, R.; van Ommen, B.;
467 Wopereis, S.; Ros, E.; Lo´pez-Miranda, J.; et al. Lifestyle Recommendations for the Prevention and
468 Management of Metabolic Syndrome: An International Panel Recommendation. Nutr. Rev. 2017, 75
469 (5), 307−326.

470 (20) Khakimov, B.; Poulsen, S. K.; Savorani, F.; Acar, E.; Gu¨rdeniz, G.; Larsen, T. M.; Astrup, A.;
471 Dragsted, L. O.; Engelsen, S. B. New Nordic Diet versus Average Danish Diet: A Randomized
472 Controlled Trial Revealed Healthy Long-Term Effects of the New Nordic Diet by GC-MS Blood
473 Plasma Metabolomics. J. Proteome Res. 2016, 15 (6), 1939−1954.

474 (21) Rodriguez-Garcia, E.; Ruiz-Nava, J.; Santamaria-Fernandez, S.; Fernandez-Garcia, J. C.;
475 Vargas-Candela, A.; Yahyaoui, R.; Tinahones, F. J.; Bernal-Lopez, M. R.; Gomez-Huelgas, R.
476 Characterization of Lipid Profile by Nuclear Magnetic Resonance Spectroscopy (1H NMR) of
477 Metabolically Healthy Obese Women after Weight Loss with Mediterranean Diet and Physical
478 Exercise. Medicine (Baltimore) 2017, 96 (27), e7040.

479 (22) Alberti, K. G.; Zimmet, P.; Shaw, J. IDF Epidemiology Task Force Consensus Group. The
480 Metabolic Syndrome←a New Worldwide Definition. Lancet 2005, 366 (9491), 1059−1062. (23)
481 Schro¨der, H.; Fito, M.; Estruch, R.; Martinez-Gonzalez, M. A.; Corella, D.; Salas-Salvado, J. A
482 Short Screener Is Valid for Assessing Mediterranean Diet Adherence among Older Spanish Men and
483 Women. J. Nutr. 2011, 141, 1140. (24) Vu, T. N.; Valkenborg, D.; Smets, K.; Verwaest, K. A.;
484 Dommisse, R.; Lemie`re, F.; Verschoren, A.; Goethals, B.; Laukens, K. An Integrated Workflow for
485 Robust Alignment and Simplified Quantitative Analysis of NMR Spectrometry Data. BMC Bioinf.
486 2011, 12, 405.

487 (25) Filzmoser, P.; Liebmann, B.; Varmuza, K. Repeated Double Cross Validation. J. Chemom. 2009,
488 23 (4), 160−171. (26) Westerhuis, J. A.; Hoefsloot, H. C. J.; Smit, S.; Vis, D. J.; Smilde, A. K.; van

489 Velzen, E. J. J.; van Duijnhoven, J. P. M.; van Dorsten, F. A. Assessment of PLSDA Cross Validation.
490 Metabolomics 2008, 4 (1), 81−89.

491 (27) Hanhineva, K.; Brunius, C.; Andersson, A.; Marklund, M.; Juvonen, R.; Keski-Rahkonen, P.;
492 Auriola, S.; Landberg, R. Discovery of Urinary Biomarkers of Whole Grain Rye Intake in Free-
493 Living Subjects Using Nontargeted LC-MS Metabolite Profiling. Mol. Nutr. Food Res. 2015, 59 (11),
494 2315−2325.

495 (28) Buck, M.; Nilsson, L. K.; Brunius, C.; Dabire´, R. K.; Hopkins, R.; Terenius, O. Bacterial
496 Associations Reveal Spatial Population Dynamics in Anopheles Gambiae Mosquitoes. Sci. Rep.
497 2016, 6, 22806.

498 (29) Benjamini, Y.; Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful
499 Approach to Multiple Testing. J. R. Stat. Soc. B 1995, 289−300.

500 (30) Cloarec, O.; Dumas, M. E.; Craig, A.; Barton, R. H.; Trygg, J.; Hudson, J.; Blancher, C.;
501 Gauguier, D.; Lindon, J. C.; Holmes, E.; et al. Statistical Total Correlation Spectroscopy: An
502 Exploratory Approach for Latent Biomarker Identification from Metabolic 1H NMR Data Sets. Anal.
503 Chem. 2005, 77 (5), 1282−1289.

504 (31) Wishart, D. S.; Jewison, T.; Guo, A. C.; Wilson, M.; Knox, C.; Liu, Y.; Djoumbou, Y.; Mandal,
505 R.; Aziat, F.; Dong, E.; Bouatra, S.; Sinelnikov, I.; Arndt, D.; Xia, J.; Liu, P.; Yallou, F.; Bjorndahl,
506 T.; Perez-Pineiro, R.; Eisner, R.; Allen, F.; Neveu, V.; Greiner, R.; Scalbert, A.; et al. HMDB 3.0−
507 The Human Metabolome Database in 2013. Nucleic Acids Res. 2013, 41 (Database), D801−7.

508 (32) Brehm, B. J.; Spang, S. E.; Lattin, B. L.; Seeley, R. J.; Daniels, S. R.; D'Alessio, D. A. The Role
509 of Energy Expenditure in the Differential Weight Loss in Obese Women on Low-Fat and Low-
510 Carbohydrate Diets. J. Clin. Endocrinol. Metab. 2005, 90 (3), 1475− 1482.

511 (33) Gu, Y.; Zhao, A.; Huang, F.; Zhang, Y.; Liu, J.; Wang, C.; Jia, W.; Xie, G.; Jia, W. Very Low
512 Carbohydrate Diet Significantly Alters the Serum Metabolic Profiles in Obese Subjects. J. Proteome
513 Res. 2013, 12 (12), 5801−5811.

514 (34) Cotter, D. G.; Schugar, R. C.; Crawford, P. A. Ketone Body Metabolism and Cardiovascular
515 Disease. Am. J. Physiol. Heart Circ. Physiol. 2013, 304 (8), H1060−76.

516 (35) Matoulek, M.; Svobodova, S.; Vetrovska, R.; Stranska, Z.; Svacina, S. Post-Exercise Changes
517 of Beta Hydroxybutyrate as a Predictor of Weight Changes. Physiol. Res. 2014, 63 (Suppl 2), S321−
518 5.

519 (36) Gall, W. E.; Beebe, K.; Lawton, K. A.; Adam, K. P.; Mitchell, M. W.; Nakhle, P. J.; Ryals, J.
520 A.; Milburn, M. V.; Nannipieri, M.; Camastra, S.; Natali, A.; Ferrannini, E.; et al. Alpha-
521 Hydroxybutyrate Is an Early Biomarker of Insulin Resistance and Glucose Intolerance in a
522 Nondiabetic Population. PLoS One 2010, 5 (5), e10883.

523 (37) Penn, L.; White, M.; Lindstro¨m, J.; den Boer, A. T.; Blaak, E.; Eriksson, J. G.; Feskens, E.;
524 Ilanne-Parikka, P.; Keinanen- Kiukaanniemi, S. M.; Walker, M.; Mathers, J. C.; Uusitupa, M.;
525 Tuomilehto, J.; et al. Importance of Weight Loss Maintenance and Risk Prediction in the Prevention
526 of Type 2 Diabetes: Analysis of European Diabetes Prevention Study RCT. PLoS One 2013, 8 (2),
527 e57143.

528 (38) Morris, C.; Grada, C. O.; Ryan, M.; Roche, H. M.; De Vito, G.; Gibney, M. J.; Gibney, E. R.;
529 Brennan, L. The Relationship between Aerobic Fitness Level and Metabolic Profiles in Healthy
530 Adults. Mol. Nutr. Food Res. 2013, 57 (7), 1246−1254.

531 (39) Zheng, Y.; Ceglarek, U.; Huang, T.; Li, L.; Rood, J.; Ryan, D. H.; Bray, G. A.; Sacks, F. M.;
532 Schwarzfuchs, D.; Thiery, J.; Shai, I.; Qi, L.; et al. Weight-Loss Diets and 2-y Changes in Circulating
533 Amino Acids in 2 Randomized Intervention Trials. Am. J. Clin. Nutr. 2016, 103 (2), 505−511.

534 (40) Cheng, S.; Rhee, E. P.; Larson, M. G.; Lewis, G. D.; McCabe, E. L.; Shen, D.; Palma, M. J.;
535 Roberts, L. D.; Dejam, A.; Souza, A. L.; Deik, A. A.; Magnusson, M.; O'Donnell, C. J.; Vasan, R.

S.; Melander, O.; Clish, C. B.; Gerszten, R. E.; Wang, T. J.; et al. Metabolite Profiling Identifies Pathways Associated with Metabolic Risk in Humans. Circulation 2012, 125 (18), 2222−2231.

(41) Takashina, C.; Tsujino, I.; Watanabe, T.; Sakaue, S.; Ikeda, D.; Yamada, A.; Sato, T.; Ohira, H.; Otsuka, Y.; Oyama-Manabe, N.; Ito, Y. M.; Nishimura, M.; et al. Associations among the Plasma Amino Acid Profile, Obesity, and Glucose Metabolism in Japanese Adults with Normal Glucose Tolerance. Nutr. Metab. 2016, 13, 5.

(42) Lancha, A. H. J.; Poortmans, J. R.; Pereira, L. O. The Effect of 5 Days of Aspartate and Asparagine Supplementation on Glucose Transport Activity in Rat Muscle. Cell Biochem. Funct. 2009, 27 (8), 552−557.

(43) Wyss, M.; Kaddurah-Daouk, R. Creatine and Creatinine Metabolism. Physiol. Rev. 2000, 80 (3), 1107−1213.

(44) Haan, Y. C.; Oudman, I.; Diemer, F. S.; Karamat, F. A.; van Valkengoed, I. G.; van Montfrans, G. A.; Brewster, L. M. Creatine Kinase as a Marker of Obesity in a Multi-Ethnic Population. Mol. Cell. Endocrinol. 2017, 442, 24−31.

(45) George, M. D.; McGill, N. K.; Baker, J. F. Creatine Kinase in the U.S. Population: Impact of Demographics, Comorbidities, and Body Composition on the Normal Range. Medicine (Baltimore) 2016, 95 (33), e4344.

(46) Van Weyenberg, S.; Hesta, M.; Buyse, J.; Janssens, G. P. The Effect of Weight Loss by Energy Restriction on Metabolic Profile and Glucose Tolerance in Ponies. J. Anim. Physiol. Anim. Nutr. 2008, 92 (5), 538−545.

(47) Dattilo, A. M.; Kris-Etherton, P. M. Effects of Weight Reduction on Blood Lipids and Lipoproteins: A Meta-Analysis. Am. J. Clin. Nutr. 1992, 56 (2), 320−328.

(48) Patalay, M.; Lofgren, I. E.; Freake, H. C.; Koo, S. I.; Fernandez, M. L. The Lowering of Plasma Lipids Following a Weight Reduction Program Is Related to Increased Expression of the LDL Receptor and Lipoprotein Lipase. J. Nutr. 2005, 135 (4), 735−739.

(49) Remely, M.; Tesar, I.; Hippe, B.; Gnauer, S.; Rust, P.; Haslberger, A. G. Gut Microbiota Composition Correlates with Changes in Body Fat Content Due to Weight Loss. Benef. Benefic. Microbes 2015, 6 (4), 431−439.

(50) Liu, R.; Hong, J.; Xu, X.; Feng, Q.; Zhang, D.; Gu, Y.; Shi, J.; Zhao, S.; Liu, W.; Wang, X.; et al. Gut Microbiome and Serum Metabolome Alterations in Obesity and after Weight-Loss Intervention. Nat. Med. 2017, 23 (7), 859−868.

(51) Wang, Z.; Klipfell, E.; Bennett, B. J.; Koeth, R.; Levison, B. S.; Dugar, B.; Feldstein, A. E.; Britt, E. B.; Fu, X.; Chung, Y.-M.; et al. Gut Flora Metabolism of Phosphatidylcholine Promotes Cardiovascular Disease. Nature 2011, 472 (7341), 57−63.

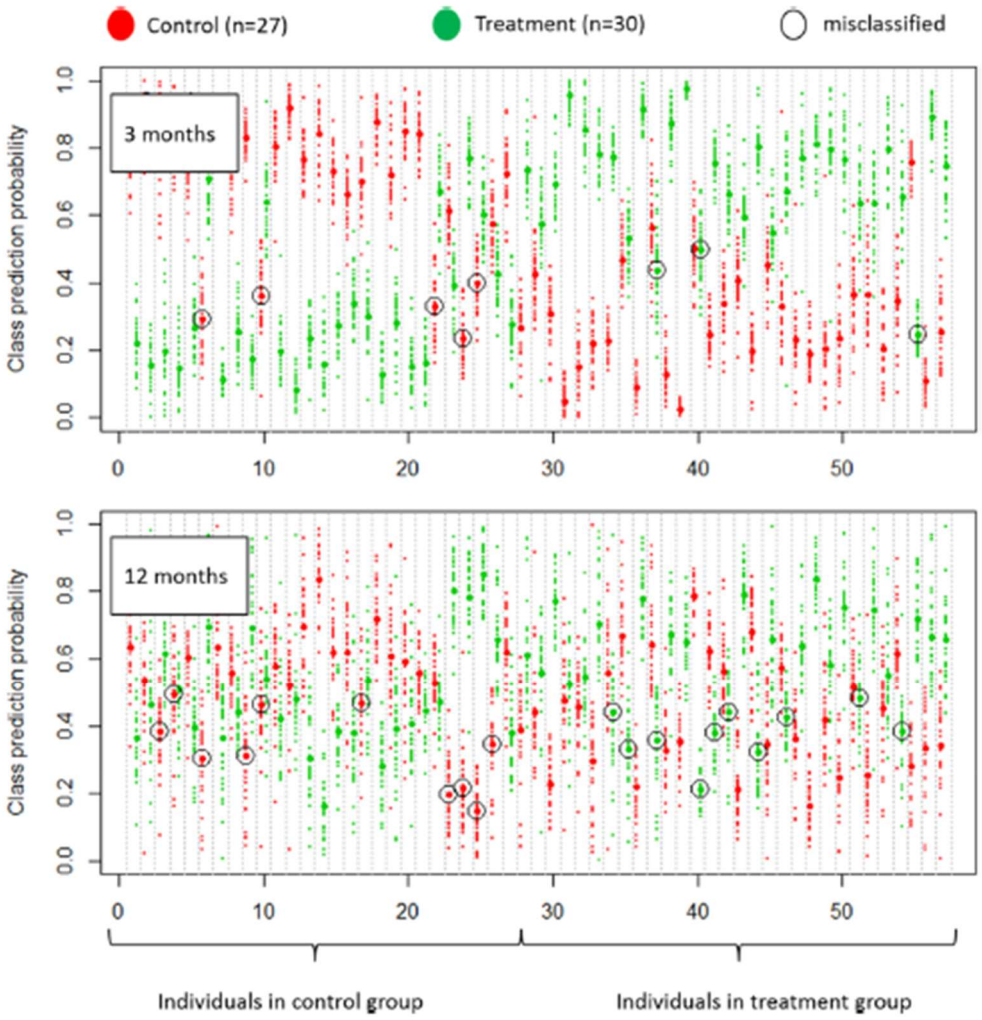
(52) Tang, W. H.; Wang, Z.; Levison, B. S.; Koeth, R. A.; Britt, E. B.; Fu, X.; Wu, Y.; Hazen, S. L. Intestinal Microbial Metabolism of Phosphatidylcholine and Cardiovascular Risk. N. Engl. J. Med. 2013, 368 (17), 1575−1584.

(53) Koeth, R. A.; Wang, Z.; Levison, B. S.; Buffa, J. A.; Org, E.; Sheehy, B. T.; Britt, E. B.; Fu, X.; Wu, Y.; Li, L.; et al. Intestinal Microbiota Metabolism of L-Carnitine, a Nutrient in Red Meat, Promotes Atherosclerosis. Nat. Med. 2013, 19 (5), 576−585.

(54) Greenberg, I.; Stampfer, M. J.; Schwarzfuchs, D.; Shai, I. DIRECT Group. Adherence and Success in Long-Term Weight Loss Diets: The Dietary Intervention Randomized Controlled Trial (DIRECT). J. Am. Coll. Nutr. 2009, 28 (2), 159−168.

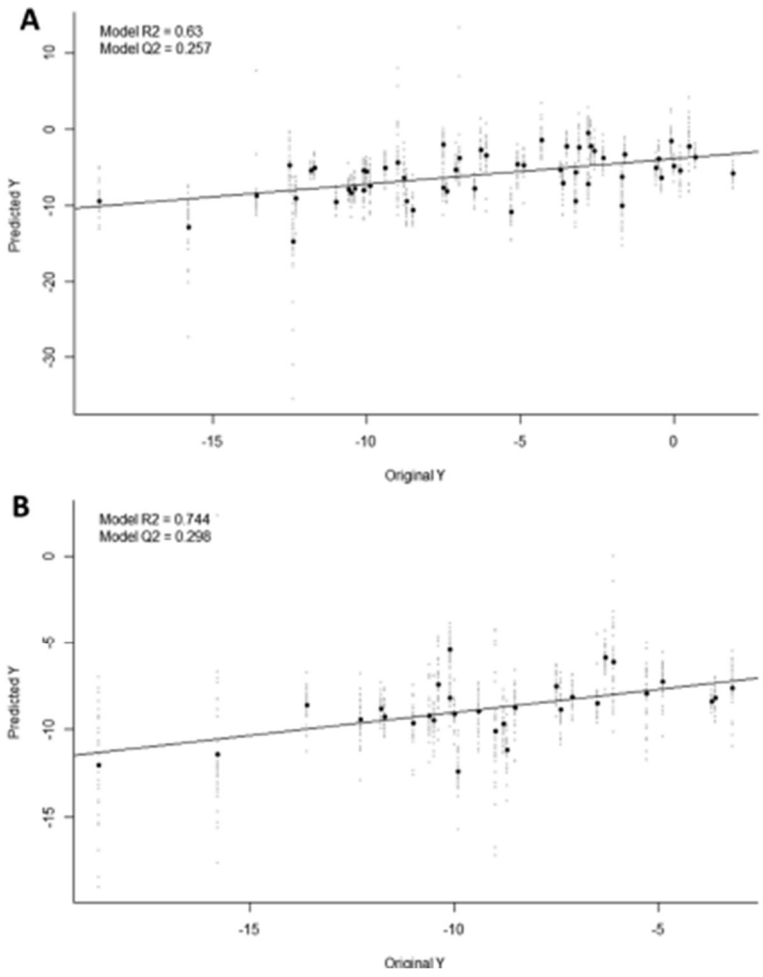
(55) Lean, M. E. Is Long-Term Weight Loss Possible? Br. J. Nutr. 2000, 83 (Suppl 1), S103−11.

581 **FIGURES**



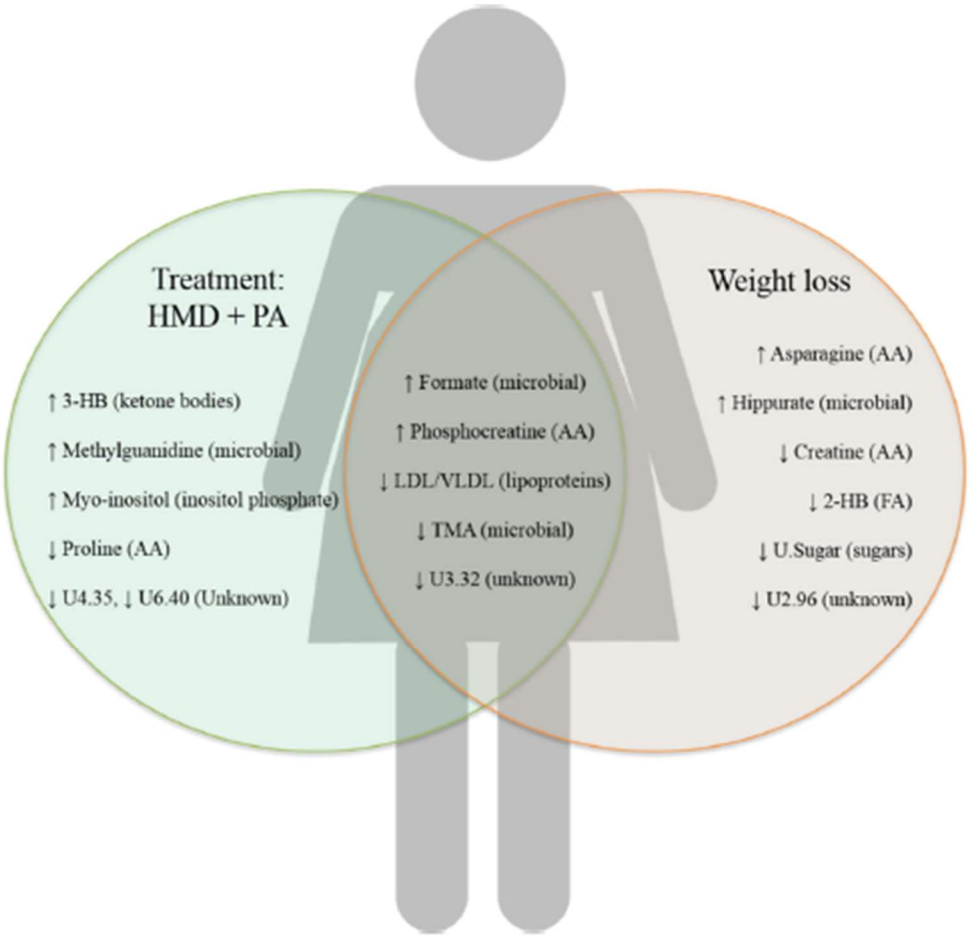
582

583 **Figure 1.** Predictive classification of individuals according to intervention group at 3 and 12 months.
584 Random forest modeling was conducted applying a repeated double cross-validation algorithm as
585 described in the Experimental section. Individual classification probability from each submodel (n =
586 30) is colored in red for control group (columns 1−27) and in green for treatment group (columns 28
587 −57). Averaged classification probability per individual according to group is shown in larger size
588 and similar color. Misclassified individuals are marked by a black circle.



589

590 **Figure 2**. Regression analyses between actual and predicted weight change from baseline to 3 months
591 according to rdCV-PLS modeling in control and treatment groups combined (A) and in treatment
592 group alone (B).



593

594 **Figure 3.** Distinct and common metabolic regulations caused by treatment or associated with weight
595 loss in MHO participants. Metabolite's class is enclosed in parentheses; ↑ and ↓ denote up- and
596 down-regulation, respectively. Abbreviations: 3-HB, 3-hydroxybutyrate; 2-HB, 2-hydroxybutyrate;
597 AA, amino acids; FA, fatty acids; HD, hypocaloric diet; LDL, low-density lipoprotein cholesterol;
598 PA, physical activity; VLDL, very low-density lipoprotein cholesterol; TMA, trimethylamine

599

TABLES

Table 1. Anthropometric and Clinical Characteristics of Study Participants at Baseline and after 3 months of Intervention and 2 Months of Follow-up[a]

	baseline				3 months			12 months		
	all	control	treatment	P^1	control	treatment	P^2	control	treatment	P^2
age, y	45.1 ± 3.45	44.4 ± 3.31	45.7 ± 3.51	0.16						
menopause, *n* (%)	12 (21.1)	3 (11.1)	9 (30.0)	0.11						
weight (kg)	90.3 ± 13.5	91.4 ± 15.6	89.3 ± 11.5	0.69	88.3 ± 13.8***	80.2 ± 10.5***	<0.001	86.7 ± 13.5**	79.3 ± 12.3***	0.03
BMI, kg/m^2	35.8 ± 4.93	36.3 ± 5.74	35.4 ± 4.12	0.49	35.1 ± 4.89***	31.7 ± 3.67***	<0.001	34.5 ± 4.84**	31.3 ± 4.19***	<0.01
waist circumference, cm	112 ± 11.2	110 ± 12.2	114 ± 10.1	0.19	105 ± 10.1***	104 ± 9.48***	<0.001	109 ± 12.5	103 ± 10.7***	<0.001
SBP, mmHg	114 ± 14.1	113 ± 15.0	115 ± 13.4	0.53	N/A	N/A		111 ± 12.1	110 ± 13.7*	0.35
DBP, mmHg	75.6 ± 9.29	74.7 ± 8.45	76.4 ± 10.1	0.50	N/A	N/A		74.9 ± 9.10	73.4 ± 10.4	0.25
glycaemia, mg/dL	88.0 ± 8.38	89.5 ± 9.30	86.4 ± 7.29	0.15	87.7 ± 6.55	80.9 ± 5.78	0.24	86.1 ± 7.12	80.4 ± 9.72**	0.28
total cholesterol, mg/mL	196 ± 28.6	195 ± 31.2	197 ± 26.5	0.72	184 ± 40.0*	175 ± 26.2***	0.16	186 ± 32.6*	189 ± 26.0*	0.93
LDL cholesterol, mg/mL	119 ± 27.5	123 ± 27.4	116 ± 27.7	0.43	135 ± 143	105.6 ± 21.8*	0.32	114 ± 27.1**	113 ± 21.6	0.98
HDL cholesterol, mg/mL	56.1 ± 12.1	52.9 ± 11.4	59.1 ± 12.2	0.05	57.0 ± 11.7**	53.2 ± 9.52***	<0.001	52.6 ± 11.0	57.8 ± 14.8	0.93
triglycerides, mg/mL	93.2 ± 38.7	95.7 ± 39.4	91.0 ± 38.6	0.67	95.3 ± 48.2	81.4 ± 28.1	0.29	98.9 ± 60.6	82.3 ± 29.5*	0.84
MedDiet adherence score	7.79 ± 1.93	8.22 ± 2.01	7.40 ± 1.81	0.11	N/A	N/A		8.89 ± 1.99	11.3 ± 1.55***	<0.001

[a]Data are presented as mean ± standard deviation or n (%), as indicated. Differences from baseline were assessed by paired *t* test (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). P^1, significance for comparisons of mean values between groups. P^2, significance for comparisons of mean change from baseline between groups. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; N/A, data not available; SBP, systolic blood pressure.

Table 2. Metabolites Selected from Multivariate Modelling for Optimum Discrimination between Treatment and Control Groups at 3 Months of Intervention, with Follow-up at 12 Months, Sorted in Order of Decreasing Variable Importance

		3 months			12 months		
chemical shift, ppm (multiplicity)	compound	change[a] TvsC	FC[b]	P^c	change[a] TvsC	FC[b]	P^c
3.32 (s), 3.61 (s)	unknown (U3.32)	↓	1.34	<0.001	↓	3.14	0.02
3.03 (s), 3.94 (s)	phosphocreatine	↑	2.07	<0.001	↓	0.12	0.01
1.27 (m), 5.32 (m)	LDL/VLDL[d]	↓	1.81	0.02	↓	3.81	0.33
6.40 (m)	unknown (U6.40)	↓	0.14	<0.01	↑	0.10	0.11
2.90 (s)	trimethylamine	↓	1.88	<0.01	↓	2.60	0.28
1.20 (d), 2.30 (m), 2.40 (m), 4.16 (m)	3-hydroxybutyrate	↑	2.21	0.04	↓	0.66	0.69
2.82 (s)	methylguanidine	↑	3.17	0.11	↓	1.02	0.97
4.35 (s)	unknown (U4.35)	↓	4.80	0.11	↓	1.10	0.93
2.0 (m), 2.06 (m), 2.34 (m), 3.33 (m), 3.41 (m), 4.12 (dd)	proline	↓	2.61	0.01	↓	2.44	0.14
3.28 (t), 3.52 (dd), 3.61 (t), 4.05 (t)	myo-inositol	↑	12.5	0.56	↓	1.32	0.77
8.45 (s)	formate	↑	0.63	0.73	↓	0.43	0.16

[a]Direction of change from baseline. [b]Fold change of treatment group/control group mean values (see Experimental Section). [c]Student's *t* test (2-tailed) between treatment and control groups (FDR-adjusted). [d]Methylene ($(CH_2)_n$) and olefinic ($-CH=CH-$) resonances at 1.27 and 5.32 ppm, respectively. Abbreviations: T, treatment; C, control; s, singlet; d, doublet; m, multiplet; dd, double of doublets; t, triplet;↑ and ↓ denote increased or decreased, respectively.

Table 3. Metabolites Selected from Multivariate Modelling of Weight Change from Baseline to 3 Months in Both Control and Treatment Groups, Sorted in Order of Decreasing Variable Importance

		correlation	
chemical shift, ppm (multiplicity)	compound	Spearman's rank correlation coefficient	P^a
3.03 (s), 3.93 (s)	phosphocreatine	−0.39	<0.01
3.32 (s), 3.61 (t)	unknown (U3.32)	0.42	<0.001
2.85 (m), 2.94 (m), 4.0 (dd)	asparagine	−0.27	0.04
8.44 (s)	formate	−0.31	0.02
0.89 (m), 1.64 (m), 1.74 (m)	2-hydroxybutyrate	0.32	0.01
3.39 (m), 3.42 (m), 3.68 (m)	unknown (U.sugar)	0.42	<0.001
0.90 (m), 1.25 (m)	LDL/VLDL[b]	0.40	<0.01
3.03 (s), 3.92 (s)	creatine	0.32	0.01
2.90 (s)	trimethylamine	0.28	0.04
2.96 (d)	unknown (U2.96)	0.27	0.04
7.62 (t), 7.84 (d)	hippurate	−0.29	0.03

[a]Significance (FDR-adjusted) for the corresponding correlations. [b]Methyl (CH_3) and methylene ($(CH_2)_n$) resonances at 0.90 and 1.25 ppm, respectively.