Effects of a long-term lifestyle intervention on metabolically healthy women with obesity: Metabolite profiles according to weight loss response

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Abbreviations

1,5-AG	1,5-anhydroglucitol
16OH-DHEA-S	16a-hydroxy DHEA 3-sulfate
3PG	3-phosphoglycerate
ADIOL-DS (1)	androstenediol (3beta,17beta) disulfate (1)
ADIOL-DS (2)	androstenediol (3beta,17beta) disulfate (2)
aHICA	alpha-hydroxyisocaproate
AMP	adenosine 50-monophosphate
BMI	body mass index
carnitine C24	lignoceroylcarnite
carnitine C26	cerotoylcarnitine
carnitine C3	propionylcarnitine
C-glyTrp	C-glycosyltryptophan
CHOL	cholesterol
cys-gly oxidized,	cysteine-glycine, oxidized
DAG (18:2/18:2)	linoleoyl-linoleoyl-glycerol (18:2/18:2)
DBP	diastolic blood pressure
ESI	electrospray ionization
FA	formic acid
FDR	false discovery rate
FM	fat mass
GCA-S	glycocholenate sulfate
glycosyl-ceramide (d18:1/16:0)	glycosyl-N-palmitoylsphingosine (d18:1/16:0)
GPC (18:1/18:2)	1-oleoyl-2-linoleoyl-GPC (18:1/18:2)
GPC (P-16:0/18:1)	1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1)
GPI (18:0)	1-stearoyl-GPI (18:0)
GPI (20:4)	1-arachidonoyl-GPI (20:4)
HbA1c	glycated hemoglobin A1
HDL	high-density lipoprotein cholesterol
HILIC	hydrophylic interaction liquid chromatography
Hip	hip circumference
HOMA-IR	insulin resistance calculated by homeostatic model assessment

73	HPLA	3-(4-hydroxyphenyl)lactate
74	HWL	high weight loss group
75	Insulin	fasting insulin
76	LDL	low-density lipoprotein cholesterol
77	LM	lean mass
78	LWL	low weight loss group
79	MedDiet	Mediterranenan diet
80	MG (18:2)	1-linoleoylglycerol (18:2)
81	MHO	metabolically healthy obesity
82	MS/MS	tandem mass spectrometry
83	NAA	N-acetylaspartate
84	non-HDL	non-high-density lipoprotein cholesterol
85	OEA	oleoyl ethanolamide
86	OGTT	oral glucose tolerance test
87	PEA	palmitoyl ethanolamide
88	PFPA	perfluoropentanoic acid
89	PLA	phenyllactate
90	rd-CV	repeated double cross-validation
91	RF	Random forest
92	RP	reverse phase
93	RSD	relative standard deviation
94	SBP	systolic blood pressure
95	SM	sphingomyelin
96	TG	triglycerides
97	UPLC	ultra-performance liquid chromatography
98	Waist	waist circumference
99		

SUMMARY

Background & aims: The benefits of weight loss in subjects with metabolically healthy obesity (MHO) are still a matter of controversy. We aimed to identify metabolic fingerprints and their associated pathways that discriminate women with MHO with high or low weight loss response after a lifestyle intervention, based on a hypocaloric Mediterranean diet (MedDiet) and physical activity. *Methods*: A UPLC-Q-Exactive-MS/MS metabolomics workflow was applied to plasma samples from 27 women with MHO before and after 12 months of a hypocaloric weight loss intervention with a MedDiet and increased physical activity. The subjects were stratified into two age-matched groups according to weight loss: <10% (low weight loss group, LWL) and >10% (high weight loss group, HWL). Random forest analysis was performed to identify metabolites discriminating between the LWL and the HWL as well as within-status effects. Modulated pathways and associations between metabolites and anthropometric and biochemical variables were also investigated.

Results: Thirteen metabolites discriminated between the LWL and the HWL, including 1,5-anhydroglucitol, carotenediol, 3-(4-hydroxyphenyl)lactic acid, N-acetylaspartate and several lipid species (steroids, a plasmalogen, sphingomyelins, a bile acid and long-chain acylcarnitines). 1,5-anhydroglucitol, 3-(4-hydroxyphenyl)lactic acid and sphingomyelins were positively associated with weight variables whereas N-acetylaspartate and the plasmalogen correlated negatively with them. Changes in very long-chain acylcarnitines and hydroxyphenyllactic levels were observed in the HWL and positively correlated with fasting glucose, and changes in levels of the plasmalogen negatively correlated with insulin resistance. Additionally, the cholesterol profile was positively associated with changes in acid hydroxyphenyllactic, sphingolipids and 1,5-AG.

Conclusions: Higher weight loss after a hypocaloric MedDiet and increased physical activity for 12 months is associated with changes in the plasma metabolome in women with MHO. These findings are associated with changes in biochemical variables and may suggest an improvement of the cardiometabolic risk profile in those patients that lose greater weight. Further studies are needed to

investigate whether the response of those subjects with MHO to this intervention differs from those with unhealthy obesity.

1. Introduction

Obesity comprises a variety of different metabolic profiles that diversify the risk of developing metabolic alterations that lead to diseases such as type 2 diabetes [1]. However, while obesity is usually associated with high cardiometabolic risk, it has been suggested that metabolically healthy obesity (MHO) has a different risk profile [2]. Subjects with MHO, despite having an excess of adipose tissue, present a propitious metabolic profile distinguished by higher insulin sensitivity, normal blood pressure, lower inflammatory parameters, lower visceral fat and more normal circulating lipid profiles than those with metabolically "unhealthy" obesity [1]. This may protect them from developing metabolic complications normally associated with obesity [3].

Men and womenwith obesity are advised or put on treatment to lose weight for better metabolic health [4]. However, it is as yet unclear whether subjects with an MHO phenotype will benefit from weight loss since they show a better cardiometabolic risk profile [1]. On the other hand, some studies have argued that the MHO condition may be a transient state towards a higher metabolic risk state. Therefore, it is important to investigate the effect of weight loss on cardiometabolic health intermediates in the MHO phenotype. Randomized controlled trials based on a Mediterranean diet (MedDiet) [5] and physical activity [6] have shown their beneficial effects on metabolic health per se. Moreover, when the two are combined even greater benefits have been demonstrated [7,8]. However, the impact of a lifestyle weight loss treatment on the MHO phenotype is poorly understood. Metabolomics is a powerful technique to define metabolic profiles through the comprehensive measurement of small molecule metabolites in a biological sample. The metabolome reflects the interaction of the exposome (i.e. the diet, gut microbiota and environmental agents to which an individual is exposed) with the gene cascade. Metabolomics can be used to identify biomarkers of prediction, progression or pathogenesis of conditions and diseases, as well as providing new clues regarding the mechanisms involved in metabolic deregulation [9,10]. Metabolomics may thus have

advantages over other omics techniques in the study of diseases with a major metabolic component. In the present study, we aimed to investigate how the plasma metabolite profiles would be affected according to weight loss response to a long-term lifestyle intervention based on a hypocaloric MedDiet and increased physical activity in women with metabolically healthy obesity. This could provide insights into affected metabolic pathways and potential consequences for cardiometabolic health.

2. Material and methods

2.1. Subjects and study design

Metabolically healthy women with obesity (BMI 30 kg/m2) aged 35e55 years were recruited by their family doctors between June 2013 and April 2014 from four primary health-care centers in the Malaga district of the Andalusian Health Service (Spain) [11]. A participant was considered to be metabolically healthy if they fulfilled 1 of the following criteria: elevated fasting plasma glucose (100 mg/dL); elevated blood pressure (135/85 mmHg or use of blood pressure-lowering agents); elevated triglycerides (150 mg/ dL or treatment with lipid-lowering medication); or decreased HDL cholesterol (<50 mg/dL). Exclusion criteria were: presence of diabetes or impaired glucose tolerance as detected on a 2-h, 75-g oral glucose tolerance test (OGTT); pregnancy or planning to become pregnant during the study; cardiovascular disease; presence of any severe systemic disease such as advanced organ failure, cancer or dementia; immobilized individuals; alcohol or drug abuse; having participated in a weight loss programme in the past three months; or having lost 5 kg of body weight in the last six months. Participants were enrolled into a lifestyle weight-loss intervention with a hypocaloric MedDiet and a recommendation of physical activity for 12 months. The hypocaloric dietwas based on a reduction of about 600 kcal in the energy intake with a calorie distribution as follows: 35e40% fats (8e10% saturated fatty acids), 40e45% carbohydrates and 20% protein. Additionally, participants were recommended to practice daily exercise, which involved walking on average for 150 min every week throughout the study.

The Rapid Assessment of Physical Activity questionnaire was used to determine the activity of the participants [12]. The dietary and physical intervention involved individual appointments with a nutritionist every week during the first two months, followed by monthly visits during the next four months and then once every three months up to 12 months. The study was conducted in accordance with the Declaration of Helsinki, all protocols were approved by the institutional ethical committee (Comite Coordinador de Etica de la Investigacion Biomedica de Andalucía) and all participants provided written informed consent. The clinical trial was registered at the ISRCTN registry (https://www.isrctn. com/ISRCTN88315555). Clinical measurements were taken at baseline and after 12 months of intervention by trained health-careworkers, and included anthropometry (weight, height, waist and hip circumference, and bodycomposition), blood pressure and the collection of fasting blood samples. Biochemical analyses were performed in the laboratory of the reference hospital and conducted using routine methods. Energy and nutrient intakes were determined using a previously validated semi-quantitative 137-item food frequency questionnaire [13] and Spanish food composition tables [14,15]. Adherence to the MedDiet was measured using the 14-item screener from the PREDIMED study [16]. For the present study, participants were classified in two groups according to the percentage of weight loss after 12 months of intervention: <10% (low weight loss group, LWL) and >10% (high weight loss group, HWL).

2.2. Metabolomics analysis

All samples were kept at -80 ºC until analysis using the Metabolon analytical system (Metabolon Inc., Durham, North Carolina, USA) [17]. Briefly, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills Geno/Grinder 2000). One aliquot of the resulting supernatant was analyzed using an approach based on hydrophilic interaction liquid chromatography -ultra-performance liquid chromatography (UPLC, Waters ACQUITY) coupled to a Thermo Scientific Q-Exactive tandem mass spectrometer (MS/MS) using negative ion mode electrospray ionization (ESI-). Three aliquots were analyzed by reverse phase (RP)- UPLC-ESI-MS/MS, two of them using positive ion mode electrospray ionization and the other using ESI-. The UPLC system was equipped

with a UPLC C18 BEH (2.1 100 mm,1.7 mm) or UPLC BEH Amide (2.1 150 mm, 1.7 mm) column (Waters). The Q-Exactive system was interfaced with a heated ESI source and an Orbitrap mass analyzer operating at a 35,000 mass resolution and covering 70e1000 m/z was used. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. Instrument variability was determined by calculating the relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the MS. In parallel, overall process variability was determined by calculating the RSD for all endogenous metabolites present in all of the quality control samples created from a large pool of human plasma that were analyzed at the beginning and at the end of the experimental run and evenly throughout the run. The median RSD of the analytical platform instrumentation was 3%, whereas the median RSD overall process variability was 6%. These values reflected acceptable levels of variability for both instrument and overall process variability. Peaks were quantified using the area under the curve and metabolites were identified by comparing them to library entries of purified standards, according to retention time, accurate mass and MS/MS spectral data [18].

2.3. Statistical analysis

All the statistical analyses and graphics were computed in R (version 3.3.3), unless otherwise specified. General characteristics of study participants, as well as anthropometric, clinical and dietary data, were examined through univariate statistical analyses. Fisher's exact test was used to compare categorical variables. For quantitative variables, data were analyzed using a non-parametric permutation test (n = 1000) of a mixed (within and between groups) factorial design using the ez package [19] to assess, respectively: i) between-group differences at baseline; ii) withingroup differences between before and after the intervention; and iii) between-group differences in the changes during the intervention. Quantitative data are expressed as median (interquartile range), whereas qualitative data are expressed as number of individuals (percentage). For metabolomics data, a multi-step process was carried out. Firstly, metabolites not found in at least 80% of the samples in either of the classes were removed (considering the time point and the weight loss group for the

definition of classes). Missing values were imputed with the k-nearest neighbors method (k = 5) [20]. Data were scaled to set the median equal to 1 and log-transformed. Finally, the differences in metabolites between baseline and 12 months after the intervention periodwere calculated. Random forest analysis with repeated double cross-validation (RF-rdCV) was used to select metabolites that discriminated between the LWL and the HWL during the intervention process, as well as those metabolites that discriminated between baseline and the 12-month intervention within each group [19,21]. Briefly, RF-rdCV was performed using a procedure developed in-house [22]: the double cross-validation separated the cross-validation into an outer "testing" loop (n = 8 CV segments) and inner "tuning" (or validation) loop (n = 7 CV segments) to reduce bias from overfitting models [19,21]. The rdCV was repeated 30 times for between-group analysis and 20 times for within-group analysis and misclassification was used for the fitness of the model tuning. Metabolite selectionwas performed within the inner loop by iteratively turning over successively fewer features, keeping in the subsequent inner loop iterations the 80% most informative metabolites. The validity of the models was assessed using two-tailed permutation tests (n = 1000). Additionally, the p values of within- and between-group differences in the changes in metabolites selected by each RF-rdCV model were also calculated through intra- and inter-group permutation tests (n = 1000) using the above-mentioned ez package [19]. Finally, Spearman correlation coefficients were calculated to estimate the associations among the selected metabolites and with clinical variables. Metabolite-clinical correlations were represented as a heat map and metabolite-metabolite-clinical correlations as a network. These p values were adjusted by false discovery rate (FDR) multiple testing, based on the Benjamini-Hochberg procedure, with the significant threshold set at $p < 0.1$ for the adjusted p values [23]. The Hmisc and ggplot packages were used for the analysis of correlation and the creation of the heat map, respectively. The correlation network was performed using Cytoscape 3.3.0.

3. Results

A total of 115 women with MHO were enrolled in the study. Of these, 43 dropped out during the intervention, six were excluded due to the presence of an illness, two for personal reasons and six for

not having completed the food frequency questionnaires at both baseline and 12 months. Finally, 27 women were randomly selected for metabolomics analysis based on previous experience from nutritional metabolomics experiments in order to achieve a resource-efficient proof-of-concept study. From the 27 women, 15 (55.6%) and 12 (44.4%) lost <10% and >10% of their body weight, respectively (Fig. S1). Table 1 presents the characteristics of these participants. In brief, the participants of both groups were of similar ages, and therewere similar proportions of menopause state, a high education level and smokers among them. Table 2 shows changes in clinical variables between the two groups. No baseline differences were observed in any of these variables between the groups. After 12 months, anthropometric and body composition parameters had improved in the HWL but remained unchanged in the LWL. The HWL also presented decreases in the OGTT and in glucose levels, but no changes in HbA1c, HOMA-IR or fasting insulin, whereas these three parameters increased in the LWL. Systolic blood pressure, as well as total, LDL and non-HDL cholesterol, also decreased in the HWL but did not do so in the LWL. These differences were also significant between the groups for most of the mentioned variables. At the beginning of the study the reported energy intake of the HWL was higher than that of the LWL (Table 3). Both groups showed decreases in energy intake through the study, but the women in the HWL presented significantly larger reductions, together with decreases in fat consumption, especially saturated and monounsaturated, and increases in protein intake. In general, the subjects in the HWL had a higher level of adherence to the treatment and also more of them followed the recommendations for physical activity in the HWL than in the LWL during the programme (Table 3).

3.1. Impact of weight loss on plasma metabolomic profile

Accurate classification predictions were obtained both between and within groups using the random forest classification scheme (Figs. 1, S2 and S3). While in between-group analysis, 24 out of 27 (88.9%) individuals were correctly classified, in within-HWL analysis, 11 out of 12 (91.7%) were correctly classified, and in within- LWL analysis, all subjects were correctly classified (p values of permutation test <0.05).

Thirteen metabolites were identified as determinants of the classification between weight loss groups, i.e. differences in 1,5- anhydroglucitol (1,5-AG), 3-(4-hydroxyphenyl)lactate (HPLA), Nacetylaspartate (NAA), the exogenous compound carotenediol, and nine lipids: 1 bile acid, 1 plasmalogen, 1 phospholipid, 2 sphingolipids, 2 steroids and 2 acylcarnitines (Fig. 2). Twenty and 33 metabolites, respectively, were selected from within-HWLandwithin-LWL analyses.1,5-AGwas also selected from within-LWL analysis,with higher levels at the 12-month intervention than at baseline. Sevenmetabolites fromthe between-group analysis were also selected from the within-HWL analysis (Fig. 3 and Fig. S4). Among these metabolites were the plasmalogen 1-(1-enyl-palmitoyl)- 2-oleoyl-sn-glycero-3-phosphocholine (P-16:0/18:1) (GPC (P- 16:0/18:1)) and the exogenous compound carotenediol, which increased during the within-HWL intervention, and significantly more so than in the LWL (Fig. S5). The levels of HPLA and some lipids (two steroids and two sphingolipids) decreased after the intervention in the HWL and more so in the HWL than in the LWL.

3.2. Correlation and pathway analysis

Metabolites that were identified as discriminating between weight loss groups were correlated with changes in clinical variables (Fig. 4A), and their intercorrelations were mapped in an organic metabolic network (Fig. 4B). Positive correlations were presented between changes in the levels of 1,5-AG, HPLA, SMs and carnitine C26 and weight variables, whereas changes in the levels of NAA, carotenediol, GPC (P-16:0/18:1) and GPC (18:1/18:2) correlated negatively with them. Furthermore, GPC (P-16:0/18:1) and carotenediol also correlated inversely with glycemic variables. In addition, several metabolites, including 1,5-AG, both SMs and HPLA, correlated positively with lipid biochemistry. Correlations between the selected metabolites in the within-group analyses and clinical variables are presented in Fig. S6. Finally, and in order to identify the most important metabolic pathways involved in these changes, pathway analyses were performed taking into account the discriminant metabolites selected from between-group analysis (Fig. 2) and from within-HWL (Fig. S4) and within-LWL (Fig. S5) analyses. No specific pathways were statistically significant using the

metabolites selected as discriminant in the between-group RF model (Fig. S7). Statistically significant pathways altered in the HWL and in the LWL are shown in Fig. S8.

4. Discussion

Although several studies report the metabolic benefits of weight loss in subjects with obesity [7,24], the benefits of a lifestyle intervention for subjects with MHO are not clear. The present study demonstrated differences in the modulation of the plasma metabolome, stratified by weight loss, after a lifestyle weight loss programme based on a hypocaloric MedDiet and physical activity in metabolically healthy women with obesity. This study shows greater differences in carotenediol levels in the HWL after the intervention and these levels were also observed to increase in this group of women. Carotenediols are vitamin A precursors found mainly in vegetables and fruit-rich diets such as the MedDiet. An increase in their levels could reflect a higher intake of such foods, which is also reflected in their greater adherence to the Mediterranean pattern [25]. 1,5-AG discriminated between the HWL and the LWL and was observed to increase in women with lower weight loss. Similar results were observed in a 6-month intervention based on the New Nordic Diet, which is rich in vegetables, whole grains, nuts and seafood products [26,27]. This metabolite has in fact been proposed as a biomarker of short-term glycemic control and for screening undetected type 2 diabetes in saliva [28]. In line with our results, Lipsky et al. (2016) also observed a higher association of 1,5-AG with BMI and adiposity indicators [29]. Small differences in the HWL likely reflect improved glycemic control as a result of a more successful intervention as measured by weight loss. Lipid metabolism has been extensively studied in obesity [30e33]. This study showed that lipid metabolism was altered, particularly in steroids, glycerophosphatidylcholines and sphingolipid metabolism. The steroids pathway was regulated differently in the two groups. Although androgen steroid sulfates decreased in the LWL, a higher decline was observed in the HWL. Similar behavior was observed by Ernst et al. (2013) when weight was lost after bariatric surgery [34]. 16a-hydroxy DHEA 3-sulfate (16OH-DHEA-S) and androstenediol (3beta,17beta) disulfate (ADIOL-DS) seem to be the major players in these changes. DHEA and ADIOL are interconverting molecules through the action of 17-

hydroxysteroid dehydrogenase [35]. However, while several studies have attributed an antiobesity role to DHEA-S [36], others have observed an inverse association between DHEA-S and the leptin hormone and satiety [37]. In addition, DHEA-S could play a role in the regulation of energetic balance in a fasting state or caloric restriction [38]. Steroid sulfation and desulfation are fundamental pathways for endocrine balance, specifically for fat mass distribution and glucose metabolism [39] regulated by sulfotransferases and sulfatase enzymes, respectively. These results could reflect an effect of modulation of the endocrine metabolism, especially in women of the HWL. SM (d18:0/22:0) and SM (d18/0/20:0, d16:0/22:0) were chosen by the multivariate model to discriminate between the HWL and the LWL. In addition, the overall sphingolipid profile decreased in both groups. Sphingolipids are the most prevalent class of lipid found in circulating LDL and activate inflammatory pathways [40]. Higher levels of sphingolipids are associated with obesity and related co-morbidities [30]. We observed a general decrease in these lipid species, which is in line with results reported after a lifestyle intervention in adolescents [41]. These two were selected and correlated with LDL, nHDL and CHOL. Thus, a downregulation of the sphingolipid pathway could indicate a better LDL profile and consequently potentially a reduction in the risk of developing cardiometabolic diseases. The plasmalogen GPC (P-16/18:0) discriminated between the HWL and the LWL and correlated negatively with adiposity variables. Plasmalogens act as an endogenous antioxidant produced by peroxisome. The production of plasmalogens is explained as a compensatory mechanism to protect the organism against higher oxidative stress such as in the development of metabolic syndrome [42]. Thus, an increase in GPC (P-16/18:0) levels in the HWL may indicate major protection of this group in the face of obesity complications. Strikingly, our study also reflects changes in lipid metabolism through changes in very long-chain acylcarnitines. We pointed out lower changes in the HWL than in the LWL. However, little is known about the role of very long-chain acylcarnitines in obesity and associated co-morbidities. Zhang et al. (2014) found higher concentrations of the carnitine C24, but not C26, in newly diagnosed type 2 diabetes subjects, and even in those with pre-diabetes, than in subjects with normal glucose tolerance [43]. Interestingly, higher levels of the C26 carnitine were detected in patients with a peroxisomal biogenesis disorder

and it has been proposed as a biomarker in neurodegenerative disorders [44]. However, the implications of our findings are still uncertain. We also observed that NAA, a marker of neuronal density [45] in the central nervous system, discriminated between the HWL and the LWL. This is in line with previous research, which showed that subjects with overweight and diabetes presented lower levels of NAA in the hippocampus [46] and that NAA in the cortex was positively correlated with physical fitness in elderly adults [47]. Finally, the significant decrease in the HWL of HPLA and PLA, lactobacillus breakdown products of phenylalanine and tyrosine, respectively [48], could reflect either a decreased protein intake or a possible modulation of gut microbiota from the intervention. Subjects with obesity present higher levels of phenylalanine, tyrosine and leucine, among other amino acids [49,50]. In addition, higher levels of microbial product of HPLA were found in children with obesity [46]. HPLA has been proposed as a potential biomarker of a higher percentage of lean mass in young and healthy adults, though with an unknown mechanism [51]. Furthermore, positive correlations between changes in HPLA and weight loss, dyslipidemia parameters and OGTT and fasting glucose may suggest a possible global metabolic improvement in those subjects that benefited more from of lifestyle intervention. A major limitation of this work, inherent to the study design, is that findings cannot be conclusively attributed to weight loss per se, a better adherence to a MedDiet and/or physical activity due to confounding. In addition, at the beginning of the study, the HWL had a greater energy intake than the LWL. In addition to this, the sample size was small and a validated cohort and prospective study is needed to corroborate our results. Moreover, our results are gender dependent and therefore we cannot extrapolate our findings to the general population. However, this limitation also contributed to a strength of this study: the fact that all participantsweremiddle-aged women from a singlemetabolic phenotype reduced other sources of variability. Moreover, our findings have been obtained using a robust multivariate modeling procedure to acquire the most relevant biomarkers of high weight loss. These results show the potential of metabolomics for metabolic profiling and the identification of potential biomarkers in the onset of diseases. Overall, our results reveal that weight loss after a lifestyle intervention is associated with the modulation of lipid metabolism, sulfation activation and microbiota metabolism likely associated with a metabolic

protective effect. Therefore, this study reinforces the idea that a healthy lifestyle, increased physical activity and weight loss lead to an improved metabolic health status in women with obesity, irrespectively of their initial metabolic state. Further studies are needed to investigate whether the response of those subjects with MHO to this intervention differs from that of those with unhealthier obesity.

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Conflicts of interest

The authors declare no competing interests.

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Magali Palau-Rodriguez: Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing - original draft, Writing - review & editing. **Mar Garcia-Aloy**: Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing - original draft, Writing - review & editing. **Antonio Miñarro**: Data curation, Supervision, Writing - review & editing. **M. Rosa Bernal-Lopez**: Conceptualization, Resources, Validation, Writing - review & editing. **Carl Brunius**: Data curation, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2019.01.018.

References

[1] Primeau V, Coderre L, Karelis AD, Brochu M, Lavoie M-E, Messier V, et al. Characterizing the profile of obese patients who are metabolically healthy. Int J Obes (Lond) 2011;35:971e81. https://doi.org/10.1038/ ijo.2010.216.

[2] Wildman RP, Muntner P, Reynolds K, Mcginn AP. The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering. Arch Intern Med 2008;168:1617e24. https://doi.org/10.1001/archinte.168.15.1617.

[3] Spatola L, Badalamenti S. Metabolic healthy overweight/obese individuals: not just a restricted group. Clin Nutr 2018;37:2301. https://doi.org/10.1016/ j.clnu.2018.08.027.

[4] García-Unciti M, Izquierdo M, Idoate F, Gorostiaga E, Grijalba A, Ortega- Delgado F, et al. Weight-loss diet alone or combined with progressive resistance training induces changes in association between the cardiometabolic risk profile and abdominal fat depots. Ann Nutr Metab 2012;61:296e304. https://doi.org/10.1159/000342467.

[5] Estruch R, Ros E, Salas-Salvado J, Covas M-I, Corella D, Aros F, et al. Primary prevention of cardiovascular disease with a mediterranean diet. N Engl J Med 2013;368:1279e90. https://doi.org/10.1056/NEJMoa1200303.

[6] Palmnas MSA, Kopciuk KA, Shaykhutdinov RA, Robson PJ, Mignault D, Rabasa- Lhoret R, et al. Serum metabolomics of activity energy expenditure and its relation to metabolic syndrome and obesity. Sci Rep 2018;8:3308. https:// doi.org/10.1038/s41598-018-21585-6.

[7] Elliot CA, Hamlin MJ. Combined diet and physical activity is better than diet or physical activity alone at improving health outcomes for patients in New Zealand's primary care intervention. BMC Public Health 2018;18:230. https:// doi.org/10.1186/s12889-018-5152-z.

[8] Almanza-Aguilera E, Brunius C, Bernal-Lopez MR, Garcia-Aloy M, Madrid- Gambin F, Tinahones FJ, et al. Impact in plasma metabolome as effect of lifestyle intervention for weight-loss reveals metabolic benefits in metabolically healthy obese women. J Proteome Res 2018;17:2600e10. https://doi.org/ 10.1021/acs.jproteome.8b00042.

[9] Neveu V, Moussy A, Rouaix H, Wedekind R, Pon A, Knox C, et al. Exposome- Explorer: a manually-curated database on biomarkers of exposure to dietary and environmental factors. Nucleic Acids Res 2017;45:D979e84. https:// doi.org/10.1093/nar/gkw980.

[10] Newgard CB. Metabolomics and metabolic diseases: where do we stand? Cell Metab 2017;25:43e56. https://doi.org/10.1016/j.cmet.2016.09.018.

[11] Rodriguez-Garcia E, Ruiz-Nava J, Santamaria-Fernandez S, Fernandez- Garcia JC, Vargas-Candela A, Yahyaoui R, et al. Characterization of lipid profile by nuclear magnetic resonance spectroscopy (1H NMR) of metabolically healthy obese women after weight loss with Mediterranean diet and physical exercise. Medicine (Baltimore) 2017;96:e7040. https://doi.org/10.1097/ MD.0000000000007040.

[12] Topolski TD, LoGerfo J, Patrick DL, Williams B, Walwick J, Patrick MB. The Rapid assessment of physical activity (RAPA) among older adults. Prev Chronic Dis 2006;3:A118.

[13] Fernandez-Ballart JD, Pi~nol JL, Zazpe I, Corella D, Carrasco P, Toledo E, et al. Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. Br J Nutr 2010;103:1808e16.

458 https://doi.org/10.1017/S0007114509993837.

459 [14] Mataix Verdú FJ. Tabla de composicio´ n de alimentos. Instituto de Nutricion y Tecnología de
460 Alimentos, Universidad de Granada; 2003.

461 [15] Cervera P, Farran A, Zamora-Ros R. Tablas de composicio´ n de alimentos CESNID = Taules
462 de composicio´ d'aliments del CESNID. Universitat de Barcelona; 2004.

463 [16] Schroder H, Fito M, Estruch R, Martínez-Gonzalez MA, Corella D, Salas- Salvado J, et al. A
464 short screener is valid for assessing mediterranean diet adherence among older Spanish men and
465 women 1e3. J Nutr 2011;141: 1140e5. https://doi.org/10.3945/jn.110.135566.

466 [17] Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E. Integrated, nontargeted ultrahigh
467 performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for
468 the identification and relative quantification of the small-molecule complement of biological systems.
469 Anal Chem 2009;81:6656e67. https://doi.org/10.1021/ac901536h.

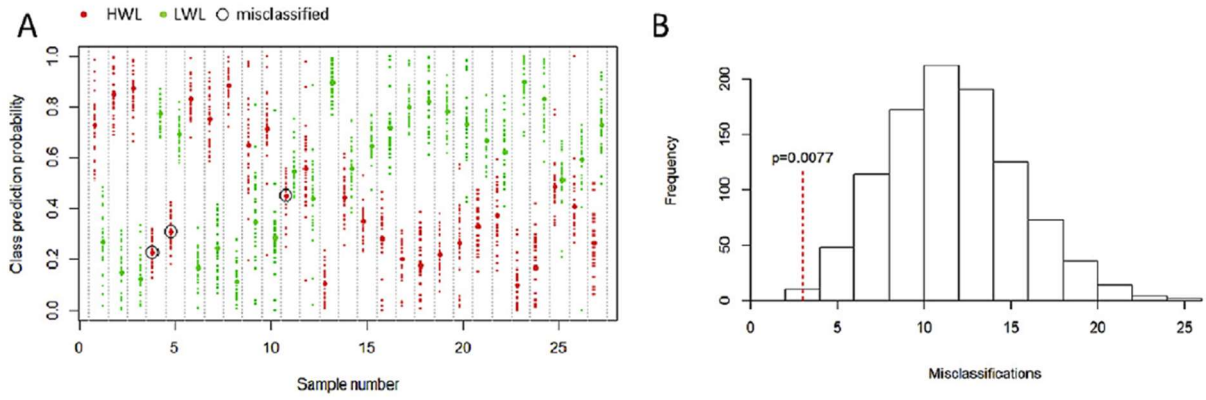
470 [18] Dehaven CD, Evans AM, Dai H, Lawton KA. Organization of GC/MS and LC/MS
471 metabolomics data into chemical libraries. J Cheminform 2010;2:9. https:// doi.org/10.1186/1758-
472 2946-2-9.

473 [19] Filzmoser P, Liebmann B, Varmuza K. Repeated double cross validation. J Chemom
474 2009;23:160e71. https://doi.org/10.1002/cem.1225.

475 [20] Gromski PS, Xu Y, Kotze HL, Correa E, Ellis DI, Armitage EG, et al. Influence of missing
476 values substitutes on multivariate analysis of metabolomics data. Metabolites 2014;4:433e52.
477 https://doi.org/10.3390/metabo4020433. [21] Breiman L. Random forests. Mach Learn
478 2001;45:5e32. https://doi.org/ 10.1023/A:1010933404324.

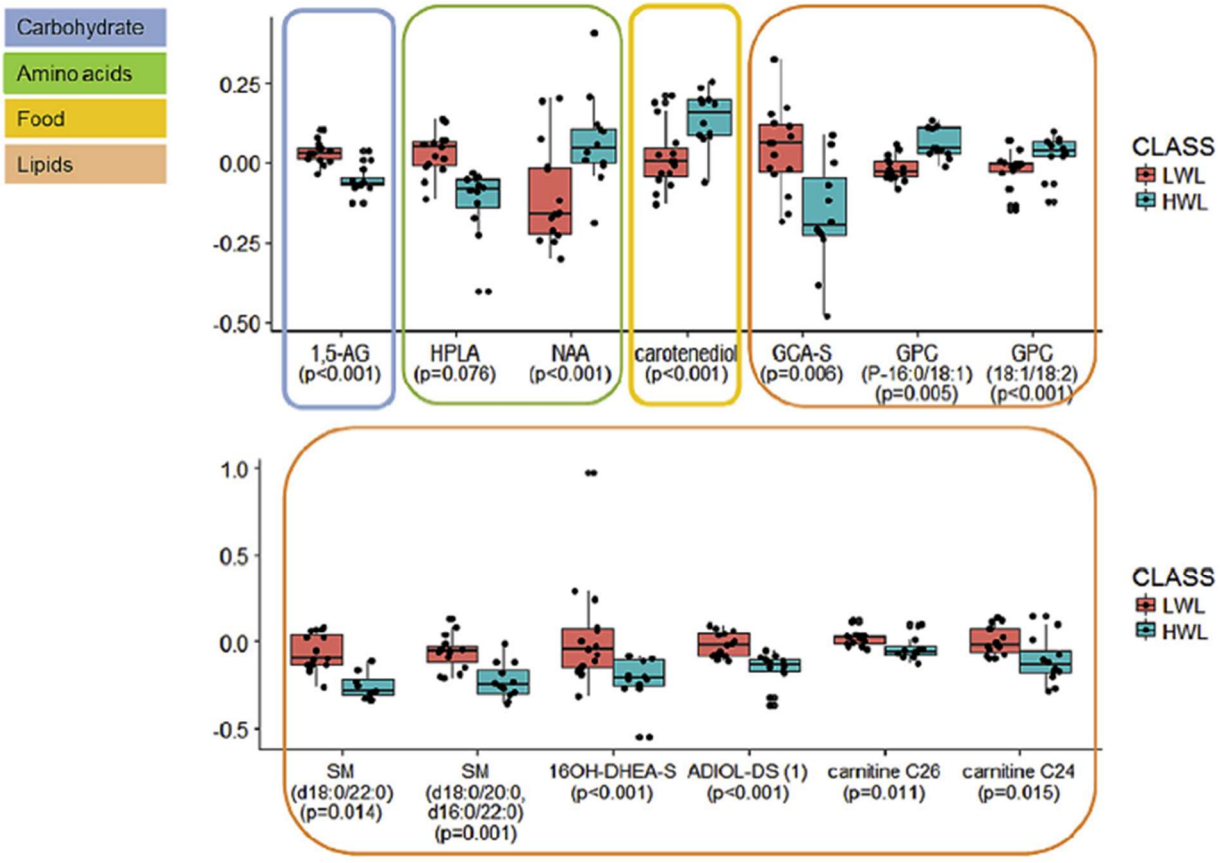
479

480 **FIGURES**



481

482 **Fig. 1.** Results from repeated double cross-validated random forest analysis (rdCV-RF) to classify
483 between weight loss groups: (A) Predictive classification of subjects according to weight loss group
484 (misclassified individuals are highlighted with a circle); (B) Histograms for permutation tests (n =
485 1000) of the rdCV-FR classification of subjects according to weight loss group.



486

487 **Fig. 2**. Differences between weight loss groups in metabolites selected by repeated double-cross
488 validated random forest model. p values were obtained by permutation test (n = 1000) of the
489 differences of the changes between groups. 1,5-AG, 1,5-anhydroglucitol; 16OH-DHEA-S, 16a-
490 hydroxydehydroepiandrosterone 3-sulfate; ADIOL-DS (1), androstenediol (3b,17b) disulfate;

carnitine C24, lignoceroylcarnitine; carnitine C26, cerotoylcarnitine; GCA-S, glycocholenate sulfate; GPC, glycerophosphocholine; HPLA, 3-(4- hydroxyphenyl)lactate; NAA, N-acetylaspartate; SM, sphingomyelin.

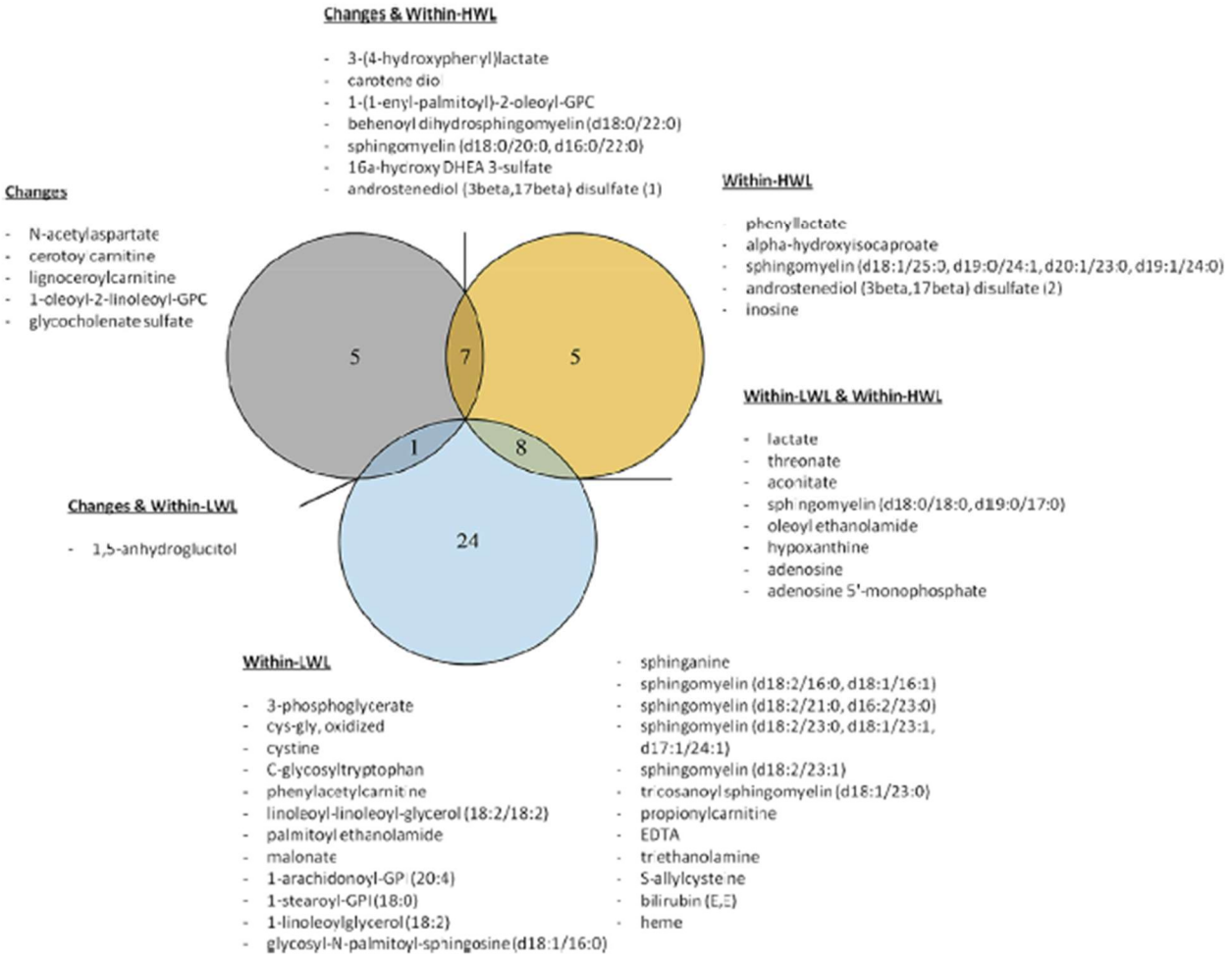


Fig. 3. Venn Diagram of metabolites discriminating between LWL and HWL, within-HWL and within-LWL, selected by repeated double-cross validated random forest modeling.

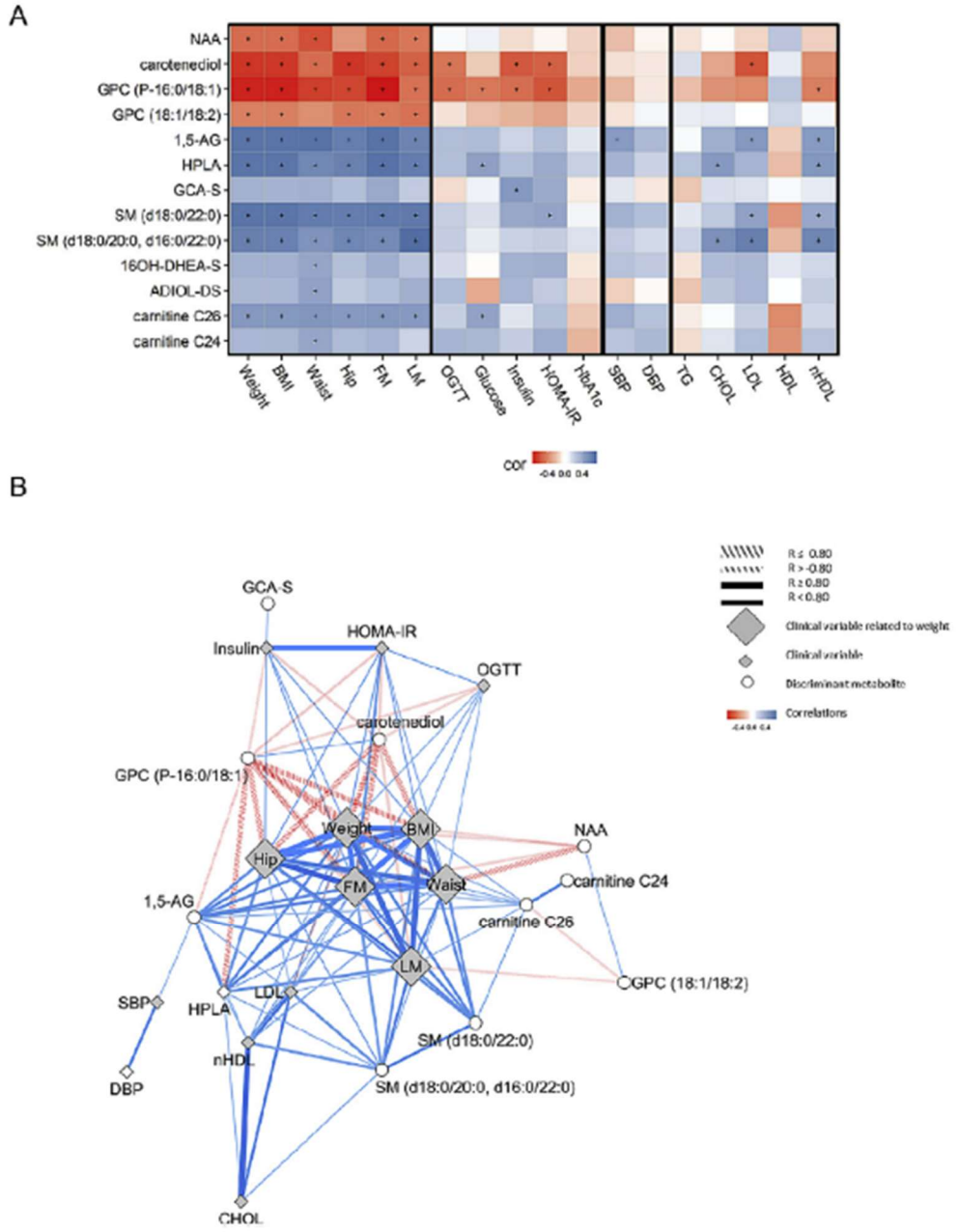


Fig. 4. Correlations of changes between metabolites and clinical parameters during the intervention program: (A) Metabolite-clinical correlations; (B) Metabolite-metaboliteclinical significant correlations. Associations determined by Spearman correlations adjusting p values by FDR, with significant threshold set at $p < 0.1$. Negative correlations are colored in red and positive correlations are colored in blue. 1,5-AG,1,5-anhydroglucitol; 16-OH-DHEA-S, 16a-hydroxy DHEA 3-sulfate; ADIOL- DS, androstenediol (3beta,17beta) disulfate (1); BMI, body mass index; carnitine C26, cerotoylcarnitine (C26); carnitine C24, lignoceroylcarnitine (C24); CHOL, total cholesterol; DBP, diastolic blood pressure; FM, fat mass; GCA-S, glycocholenate sulfate; GPC (18:1/18:2), 1-oleoyl-2-linoleoyl-GPC (18:1/18:2); GPC (P-16:0/18:1), 1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1); HbA1c, glycated hemoglobin A1c; HDL, high-density lipoproteins cholesterol; Hip, hip circumference; HOMA-IR, insulin resistance calculated by homeostatic model assessment; HPLA, 3-(4- hydroxyphenyl)lactate; LDL, low-density lipoproteins cholesterol; LM, lean mass; NAA, N-acetylaspartate; OGTT, oral glucose tolerance test; non-HDL, non-high-density lipoproteins cholesterol; SBP, systolic blood pressure; SM sphingomyelin; TG, triglycerides; Waist, waist circumference. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

TABLES

Table 1
Characteristics of the study participants.

Variable	All	LWL	HWL	p[a]
n	27	15	12	–
Age, median (Q1,Q3)	45.0 (42.0,48.0)	45.0 (42.0,46.5)	47.0 (40.7,49.5)	0.520
Menopause, n (%)	6 (22.2)	3 (20.0)	3 (25.0)	1.000
High education level, n (%)[b]	21 (77.8)	12 (80.0)	9 (75.0)	1.000
Smokers, n (%)	9 (33.3)	4 (26.7)	5 (41.7)	0.448

[a] *p* value was calculated by permutation test (n = 1000) for quantitative variables or Fisher's exact test for categorical variables.
[b] High educational level was considered when subjects had university or high school studies.

Table 2
Anthropometric and clinical variables at baseline and after 12 months of intervention according to weight loss group.[a]

	LWL (<10% weight loss)			HWL (>10% weight loss)			Treatment effect		
	Baseline	12 months	p[b]	Baseline	12 months	p[b]	Differences LWL	Differences HWL	p[c]
Weight, kg	95.3 (82.9, 98.2)	93.3 (83.1, 98.8)	0.694	85.4 (80.4, 105.1)	71.8 (65.9, 82.9)	0.002	−0.2 (−3.5, 2.6)	−15.0 (−17.4, −12.1)	<0.001
BMI, kg/m^2	36.0 (33.6, 37.8)	35.6 (32.7, 37.9)	0.722	34.7 (31.8, 38.1)	28.3 (26.8, 33.2)	0.002	0.0 (−1.5, 1.1)	−5.8 (−6.7, −4.7)	<0.001
Waist circumference, cm	111.0 (105.5, 118.5)	114.5 (106.5, 117.8)	0.441	114.3 (109.1, 126.3)	97.0 (94.5, 107.5)	<0.001	−0.5 (−2.5, 5.0)	−13.8 (−18.1, −9.9)	<0.001
Hip circumference, cm	123.0 (111.3, 126.5)	118.0 (111.3, 122.0)	0.142	121.5 (118.9, 128.0)	111.5 (105.5, 114.6)	<0.001	−1.0 (−4.3, 0.5)	−11.3 (−14.3, −6.8)	<0.001
Fat mass, %	40.3 (33.6, 46.0)	42.6 (31.9, 44.4)	0.824	36.9 (34.6, 45.4)	25.9 (21.4, 33.7)	<0.001	1.1 (−2.3, 2.1)	−10.2 (−13.4, −8.6)	<0.001
Lean mass, %	51.7 (47.7, 53.7)	50.8 (48.4, 52.6)	0.265	49.8 (46.6, 55.6)	44.6 (44.1, 50.9)	0.002	−0.6 (−1.6, 0.7)	−4.0 (−4.9, −3.0)	<0.001
OGTT	100.0 (83.0, 112.0)	89.0 (71.0, 104.0)	0.665	100.0 (91.8, 109.0)	62.5 (57.5, 85.5)	0.018	−8.0 (−14.5, 0.5)	−27.0 (−42.3, −5.5)	0.029
Glycemia, LWL/dL	90.0 (86.0, 92.5)	85.0 (79.5, 93.5)	0.064	88.5 (82.0, 93.3)	77.5 (72.0, 81.5)	0.005	−3.0 (−7.5, 0.0)	−8.5 (−13.0, −5.5)	0.076
Fasting insulin, uU/mL	9.7 (8.7, 14.7)	15.2 (11.6, 19.1)	<0.001	9.0 (8.4, 9.8)	8.7 (8.3, 9.7)	0.854	5.0 (1.2, 7.0)	0.0 (−0.7, 0.5)	0.390
HOMA-IR index	2.1 (1.9, 3.2)	3.2 (2.5, 3.9)	0.004	2.0 (1.8, 2.1)	1.7 (1.5, 2.2)	0.990	0.7 (0.1, 1.4)	−0.1 (−0.5, 0.0)	0.249
HbA1c %	5.4 (5.2, 5.5)	5.4 (5.2, 5.7)	0.043	5.4 (5.2, 5.6)	5.4 (5.3, 5.6)	0.833	0.1 (0.0, 0.2)	0.0 (−0.1, 0.1)	0.156
SBP, mmHg	105 (101, 117)	112 (105, 122)	0.823	114 (109, 124)	108 (100, 114)	0.041	4.0 (−4.5, 12.5)	−6.0 (−13.3, 1.3)	0.176
DBP, mmHg	71 (69, 79)	75 (68, 83)	0.351	75 (67, 87)	74 (67, 76)	0.404	2.0 (−4.8, 6.8)	0.0 (−11.3, 5.5)	0.183
TG, mg/mL	92.0 (67.5, 95.0)	79.0 (60.0, 114.5)	0.593	85.5 (57.3, 106.0)	76.0 (65.3, 96.3)	0.474	−1.0 (−17.0, 20.5)	0.0 (−26.3, 10.3)	0.420
CHOL, mg/mL	184.0 (176.0, 207.0)	187.0 (170.0, 200.0)	0.182	196.0 (163.8, 211.5)	172.0 (159.5, 178.3)	0.013	−3.0 (−11.0, 3.5)	−12.5 (−29.8, −5.0)	0.039
LDL, mg/mL	124.2 (101.6, 135.2)	118.6 (106.2, 124.7)	0.117	123.3 (97.0, 131.0)	98.4 (88.0, 106.3)	0.007	−9.2 (−14.0, 4.9)	−18.2 (−23.1, −6.2)	0.031
HDL, mg/mL	51.0 (45.0, 56.5)	48.0 (41.5, 58.5)	0.628	55.0 (50.0, 62.0)	54.5 (52.0, 65.5)	0.572	−1.0 (−5.5, 2.0)	2.5 (0.0, 6.0)	0.456
non-HDL, mg/mL	136.0 (123.0, 148.5)	136.0 (119.0, 146.0)	0.218	141.5 (112.5, 149.0)	115.0 (101.0, 126.3)	0.009	−1.0 (−14.0, 4.0)	−17.0 (−30.0, −10.3)	0.012

BMI, body mass index; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin A1c; HDL, high-density lipoproteins cholesterol; HOMA-IR, insulin resistance calculated by homeostatic model assessment; HWL, high weight loss group; LDL, low-density lipoproteins cholesterol; LWL, low weight loss group; OGTT, oral glucose tolerance test; SBP, systolic blood pressure; CHOL, total cholesterol; TG, triglycerides.

[a] Data are presented as median (interquartile range). There were no statistically significant between-group differences at baseline (p values obtained by permutation test, n = 1000).

[b] p values obtained by permutation test (n = 1000) for within-group differences.

[c] p values obtained by permutation test (n = 1000) for between-group differences of the changes during the intervention.

Table 3
Baseline energy, nutrient intake and adherence assessment of the Mediterranean diet and 12-week changes according to weight loss group.[a]

	LWL (<10% weight loss)			HWL (>10% weight loss)			Treatment effect		
	Baseline	12 months	p[c]	Baseline	12 months	p[c]	Differences LWL	Differences HWL	p[d]
Energy (kcal/day)	2179.2 (1895.6, 2465.1)	1705.9 (1432.1, 1972.0)	0.013	2691.4 (2304.7, 2855.5)*	1655.4 (1378.3, 1754.8)	0.002	−341.6 (−670.6, −73.1)	−1014.6 (−1534.4, −607.4)	0.022
Carbohydrate (%)	38.1 (32.4, 40.4)	38.0 (33.4, 39.9)	0.848	37.6 (34.6, 41.4)	39.4 (35.3, 42.7)	0.255	−0.5 (−2.9, 1.9)	3.4 (−0.7, 5.7)	0.497
Protein (%)	21.4 (17.6, 23.7)	23.6 (20.2, 25.0)	0.152	17.5 (16.4, 21.5)	27.5 (25.6, 30.3)	<0.001	2.1 (−0.3, 3.5)	9.2 (6.4, 10.6)	<0.001
Total fat (%)	42.0 (38.4, 44.6)	41.5 (37.5, 42.5)	0.338	42.2 (37.9, 45.6)	33.0 (29.8, 35.3)	0.003	−1.1 (−5.7, 1.4)	−12.0 (−13.9,−5.4)	0.006
Saturated (%)	11.7 (9.9, 13.4)	10.9 (10.0, 11.5)	0.060	12.6 (10.8, 14.2)	7.9 (6.6, 10.0)	<0.001	−0.8 (−2.7, 0.4)	−3.5 (−5.1, −2.4)	0.018
Monounsaturated (%)	19.0 (15.1, 21.9)	19.3 (15.9, 20.7)	0.892	19.5 (16.0, 20.6)	15.1 (10.6, 17.2)	0.014	0.6 (−1.4, 1.7)	−5.8 (−7.1, −0.9)	0.029
Polyunsaturated (%)	7.0 (6.0, 8.2)	6.9 (6.0, 7.4)	0.385	6.5 (5.4, 8.4)	5.8 (5.1, 6.5)	0.252	−0.2 (−1.6, 0.2)	−0.2 (−3.3, 0.6)	0.826
Cholesterol (g/day)	406.2 (369.2, 456.4)	308.2 (280.0, 373.6)	0.014	439.3 (363.1, 473.3)	375.2 (290.4, 404.0)	0.003	−78.7 (−118.2, 20.1)	−78.8 (−115.9, −41.9)	0.379
Ethanol (g/day)	1.2 (0.0, 3.4)	0.4 (0.0, 2.1)	0.059	1.6 (0.5, 4.2)	1.0 (0.0, 2.5)	0.075	−0.1 (−1.2, 0.0)	−0.7 (−2.6, 0.2)	0.109
Fiber (g/day)	24.4 (20.7, 30.5)	20.9 (18.2, 27.1)	0.283	31.9 (25.0, 34.7)	28.5 (23.7, 31.5)	0.763	−4.6 (−8.2, 2.5)	−2.5 (−7.1, 5.7)	0.606
MedDiet score	9 (7, 10)	9 (7, 9.5)	0.878	7.50 (6.75, 8.25)	12 (12, 12)	0.002	0 (−1, 1)	5 (3, 5.2)	<0.001
Physically Active[b]	3 (20.0%)	4 (26.7%)	1.00	1 (8.3%)	10 (83.3%)	0.008	13.3% (6.7%)	75.0% (0.0%)	0.004

*p values of statistical differences between groups at baseline, obtained by permutation test for quantitative variables or Fisher test for categorical variables $p < 0.05$.
HWL, high weight loss group; LWL, low weight loss group.

[a] Data are presented as median (interquartile range) for quantitative variables, or number of subjects (%) for categorical variables.

[b] Women physically active were considered if they reported to perform at least 150 min of moderate physical activity per week or 60 min of intense physical activity per week, measured using Rapid Assessment of Physical Activity questionnaire. Treatment effect on physical activity is presented as: % of subjects that increased physical activity as recommended (% of subjects that decreased physical activity as sedentary).

[c] p values were obtained by permutation test ($n = 1000$) within-groups for quantitative variables or McNemar's test for categorical variables.

[d] p values were obtained by permutation test ($n = 1000$) between-group for quantitative variables or repeated measures logistic regression (interaction term) for categorical variables.