

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

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33 **Keywords:**

34 Metabolomics

35 Metabolically healthy obese

36 LC-MS

| | | |
|----|--------------------------------|---|
| 37 | Mediterranean diet | |
| 38 | Lifestyle intervention | |
| 39 | Obesity | |
| 40 | | |
| 41 | Abbreviations | |
| 42 | 1,5-AG | 1,5-anhydroglucitol |
| 43 | 16OH-DHEA-S | 16 α -hydroxy DHEA 3-sulfate |
| 44 | 3PG | 3-phosphoglycerate |
| 45 | ADIOL-DS (1) | androstenediol (3 β ,17 β) disulfate (1) |
| 46 | ADIOL-DS (2) | androstenediol (3 β ,17 β) disulfate (2) |
| 47 | aHICA | alpha-hydroxyisocaproate |
| 48 | AMP | adenosine 50-monophosphate |
| 49 | BMI | body mass index |
| 50 | carnitine C24 | lignoceroylcarnite |
| 51 | carnitine C26 | cerotoylcarnitine |
| 52 | carnitine C3 | propionylcarnitine |
| 53 | C-glyTrp | C-glycosyltryptophan |
| 54 | CHOL | cholesterol |
| 55 | cys-gly oxidized, | cysteine-glycine, oxidized |
| 56 | DAG (18:2/18:2) | linoleoyl-linoleoyl-glycerol (18:2/18:2) |
| 57 | DBP | diastolic blood pressure |
| 58 | ESI | electrospray ionization |
| 59 | FA | formic acid |
| 60 | FDR | false discovery rate |
| 61 | FM | fat mass |
| 62 | GCA-S | glycocholenate sulfate |
| 63 | glycosyl-ceramide (d18:1/16:0) | glycosyl-N-palmitoylsphingosine (d18:1/16:0) |
| 64 | GPC (18:1/18:2) | 1-oleoyl-2-linoleoyl-GPC (18:1/18:2) |
| 65 | GPC (P-16:0/18:1) | 1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1) |
| 66 | GPI (18:0) | 1-stearoyl-GPI (18:0) |
| 67 | GPI (20:4) | 1-arachidonoyl-GPI (20:4) |
| 68 | HbA1c | glycated hemoglobin A1 |
| 69 | HDL | high-density lipoprotein cholesterol |
| 70 | HILIC | hydrophylic interaction liquid chromatography |
| 71 | Hip | hip circumference |
| 72 | 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 | Mediterranean 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 | | |

100 **SUMMARY**

101 *Background & aims:* The benefits of weight loss in subjects with metabolically healthy obesity
102 (MHO) are still a matter of controversy. We aimed to identify metabolic fingerprints and their
103 associated pathways that discriminate women with MHO with high or low weight loss response after
104 a lifestyle intervention, based on a hypocaloric Mediterranean diet (MedDiet) and physical activity.

105 *Methods:* A UPLC-Q-Exactive-MS/MS metabolomics workflow was applied to plasma samples from
106 27 women with MHO before and after 12 months of a hypocaloric weight loss intervention with a
107 MedDiet and increased physical activity. The subjects were stratified into two age-matched groups
108 according to weight loss: <10% (low weight loss group, LWL) and >10% (high weight loss group,
109 HWL). Random forest analysis was performed to identify metabolites discriminating between the
110 LWL and the HWL as well as within-status effects. Modulated pathways and associations between
111 metabolites and anthropometric and biochemical variables were also investigated.

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

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

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

127 **1. Introduction**

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

136 Men and women with obesity are advised or put on treatment to lose weight for better metabolic health
137 [4]. However, it is as yet unclear whether subjects with an MHO phenotype will benefit from weight
138 loss since they show a better cardiometabolic risk profile [1]. On the other hand, some studies have
139 argued that the MHO condition may be a transient state towards a higher metabolic risk state.
140 Therefore, it is important to investigate the effect of weight loss on cardiometabolic health
141 intermediates in the MHO phenotype. Randomized controlled trials based on a Mediterranean diet
142 (MedDiet) [5] and physical activity [6] have shown their beneficial effects on metabolic health per
143 se. Moreover, when the two are combined even greater benefits have been demonstrated [7,8].
144 However, the impact of a lifestyle weight loss treatment on the MHO phenotype is poorly understood.
145 Metabolomics is a powerful technique to define metabolic profiles through the comprehensive
146 measurement of small molecule metabolites in a biological sample. The metabolome reflects the
147 interaction of the exposome (i.e. the diet, gut microbiota and environmental agents to which an
148 individual is exposed) with the gene cascade. Metabolomics can be used to identify biomarkers of
149 prediction, progression or pathogenesis of conditions and diseases, as well as providing new clues
150 regarding the mechanisms involved in metabolic deregulation [9,10]. Metabolomics may thus have

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

157 **2. Material and methods**

158 *2.1. Subjects and study design*

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

176 The Rapid Assessment of Physical Activity questionnaire was used to determine the activity of the
177 participants [12]. The dietary and physical intervention involved individual appointments with a
178 nutritionist every week during the first two months, followed by monthly visits during the next four
179 months and then once every three months up to 12 months. The study was conducted in accordance
180 with the Declaration of Helsinki, all protocols were approved by the institutional ethical committee
181 (Comite Coordinador de Etica de la Investigacion Biomedica de Andalucía) and all participants
182 provided written informed consent. The clinical trial was registered at the ISRCTN registry
183 (<https://www.isrctn.com/ISRCTN88315555>). Clinical measurements were taken at baseline and after
184 12 months of intervention by trained health-careworkers, and included anthropometry (weight,
185 height, waist and hip circumference, and bodycomposition), blood pressure and the collection of
186 fasting blood samples. Biochemical analyses were performed in the laboratory of the reference
187 hospital and conducted using routine methods. Energy and nutrient intakes were determined using a
188 previously validated semi-quantitative 137-item food frequency questionnaire [13] and Spanish food
189 composition tables [14,15]. Adherence to the MedDiet was measured using the 14-item screener from
190 the PREDIMED study [16]. For the present study, participants were classified in two groups
191 according to the percentage of weight loss after 12 months of intervention: <10% (low weight loss
192 group, LWL) and >10% (high weight loss group, HWL).

193 *2.2. Metabolomics analysis*

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

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

216 *2.3. Statistical analysis*

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

228 definition of classes). Missing values were imputed with the k-nearest neighbors method ($k = 5$) [20].
229 Data were scaled to set the median equal to 1 and log-transformed. Finally, the differences in
230 metabolites between baseline and 12 months after the intervention period were calculated. Random
231 forest analysis with repeated double cross-validation (RF-rdCV) was used to select metabolites that
232 discriminated between the LWL and the HWL during the intervention process, as well as those
233 metabolites that discriminated between baseline and the 12-month intervention within each group
234 [19,21]. Briefly, RF-rdCV was performed using a procedure developed in-house [22]: the double
235 cross-validation separated the cross-validation into an outer “testing” loop ($n = 8$ CV segments) and
236 inner “tuning” (or validation) loop ($n = 7$ CV segments) to reduce bias from overfitting models
237 [19,21]. The rdCV was repeated 30 times for between-group analysis and 20 times for within-group
238 analysis and misclassification was used for the fitness of the model tuning. Metabolite selection was
239 performed within the inner loop by iteratively turning over successively fewer features, keeping in
240 the subsequent inner loop iterations the 80% most informative metabolites. The validity of the models
241 was assessed using two-tailed permutation tests ($n = 1000$). Additionally, the p values of within- and
242 between-group differences in the changes in metabolites selected by each RF-rdCV model were also
243 calculated through intra- and inter-group permutation tests ($n = 1000$) using the above-mentioned ez
244 package [19]. Finally, Spearman correlation coefficients were calculated to estimate the associations
245 among the selected metabolites and with clinical variables. Metabolite-clinical correlations were
246 represented as a heat map and metabolite-metabolite-clinical correlations as a network. These p
247 values were adjusted by false discovery rate (FDR) multiple testing, based on the Benjamini-
248 Hochberg procedure, with the significant threshold set at $p < 0.1$ for the adjusted p values [23]. The
249 Hmisc and ggplot packages were used for the analysis of correlation and the creation of the heat map,
250 respectively. The correlation network was performed using Cytoscape 3.3.0.

251 **3. Results**

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

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

274 *3.1. Impact of weight loss on plasma metabolomic profile*

275 Accurate classification predictions were obtained both between and within groups using the random
276 forest classification scheme (Figs. 1, S2 and S3). While in between-group analysis, 24 out of 27
277 (88.9%) individuals were correctly classified, in within-HWL analysis, 11 out of 12 (91.7%) were
278 correctly classified, and in within- LWL analysis, all subjects were correctly classified (p values of
279 permutation test <0.05).

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

292 *3.2. Correlation and pathway analysis*

293 Metabolites that were identified as discriminating between weight loss groups were correlated with
294 changes in clinical variables (Fig. 4A), and their intercorrelations were mapped in an organic
295 metabolic network (Fig. 4B). Positive correlations were presented between changes in the levels of
296 1,5-AG, HPLA, SMs and carnitine C26 and weight variables, whereas changes in the levels of NAA,
297 carotenediol, GPC (P-16:0/18:1) and GPC (18:1/18:2) correlated negatively with them. Furthermore,
298 GPC (P-16:0/18:1) and carotenediol also correlated inversely with glycemic variables. In addition,
299 several metabolites, including 1,5-AG, both SMs and HPLA, correlated positively with lipid
300 biochemistry. Correlations between the selected metabolites in the within-group analyses and clinical
301 variables are presented in Fig. S6. Finally, and in order to identify the most important metabolic
302 pathways involved in these changes, pathway analyses were performed taking into account the
303 discriminant metabolites selected from between-group analysis (Fig. 2) and from within-HWL (Fig.
304 S4) and within-LWL (Fig. S5) analyses. No specific pathways were statistically significant using the

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

307 **4. Discussion**

308 Although several studies report the metabolic benefits of weight loss in subjects with obesity [7,24],
309 the benefits of a lifestyle intervention for subjects with MHO are not clear. The present study
310 demonstrated differences in the modulation of the plasma metabolome, stratified by weight loss, after
311 a lifestyle weight loss programme based on a hypocaloric MedDiet and physical activity in
312 metabolically healthy women with obesity. This study shows greater differences in carotenediol
313 levels in the HWL after the intervention and these levels were also observed to increase in this group
314 of women. Carotenediols are vitamin A precursors found mainly in vegetables and fruit-rich diets
315 such as the MedDiet. An increase in their levels could reflect a higher intake of such foods, which is
316 also reflected in their greater adherence to the Mediterranean pattern [25]. 1,5-AG discriminated
317 between the HWL and the LWL and was observed to increase in women with lower weight loss.
318 Similar results were observed in a 6-month intervention based on the New Nordic Diet, which is rich
319 in vegetables, whole grains, nuts and seafood products [26,27]. This metabolite has in fact been
320 proposed as a biomarker of short-term glycemic control and for screening undetected type 2 diabetes
321 in saliva [28]. In line with our results, Lipsky et al. (2016) also observed a higher association of 1,5-
322 AG with BMI and adiposity indicators [29]. Small differences in the HWL likely reflect improved
323 glycemic control as a result of a more successful intervention as measured by weight loss. Lipid
324 metabolism has been extensively studied in obesity [30e33]. This study showed that lipid metabolism
325 was altered, particularly in steroids, glycerophosphatidylcholines and sphingolipid metabolism. The
326 steroids pathway was regulated differently in the two groups. Although androgen steroid sulfates
327 decreased in the LWL, a higher decline was observed in the HWL. Similar behavior was observed by
328 Ernst et al. (2013) when weight was lost after bariatric surgery [34]. 16 α -hydroxy DHEA 3-sulfate
329 (16OH-DHEA-S) and androstenediol (3 β ,17 β) disulfate (ADIOL-DS) seem to be the major
330 players in these changes. DHEA and ADIOL are interconverting molecules through the action of 17-

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

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

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

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401 **Conflicts of interest**

402 The authors declare no competing interests.

403 **CRedit authorship contribution statement**

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414 Writing - review & editing.

415 **Appendix A. Supplementary data**

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

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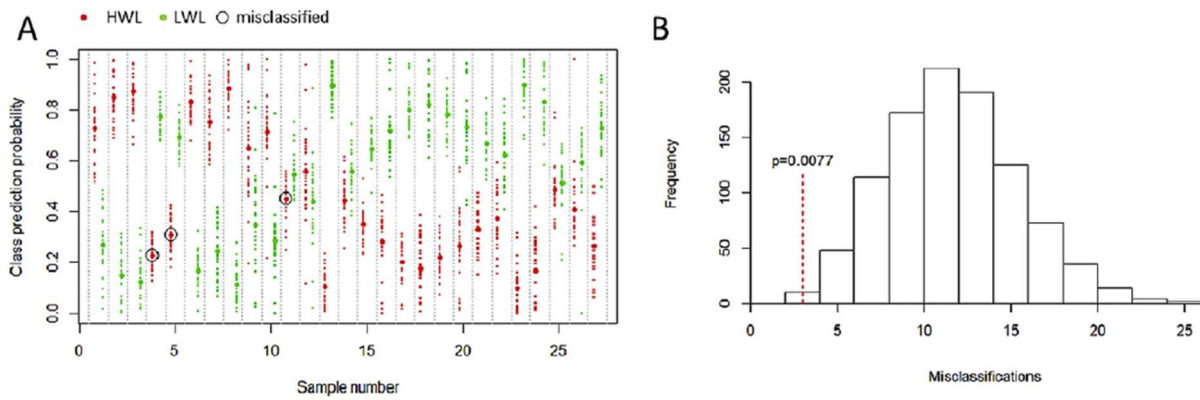
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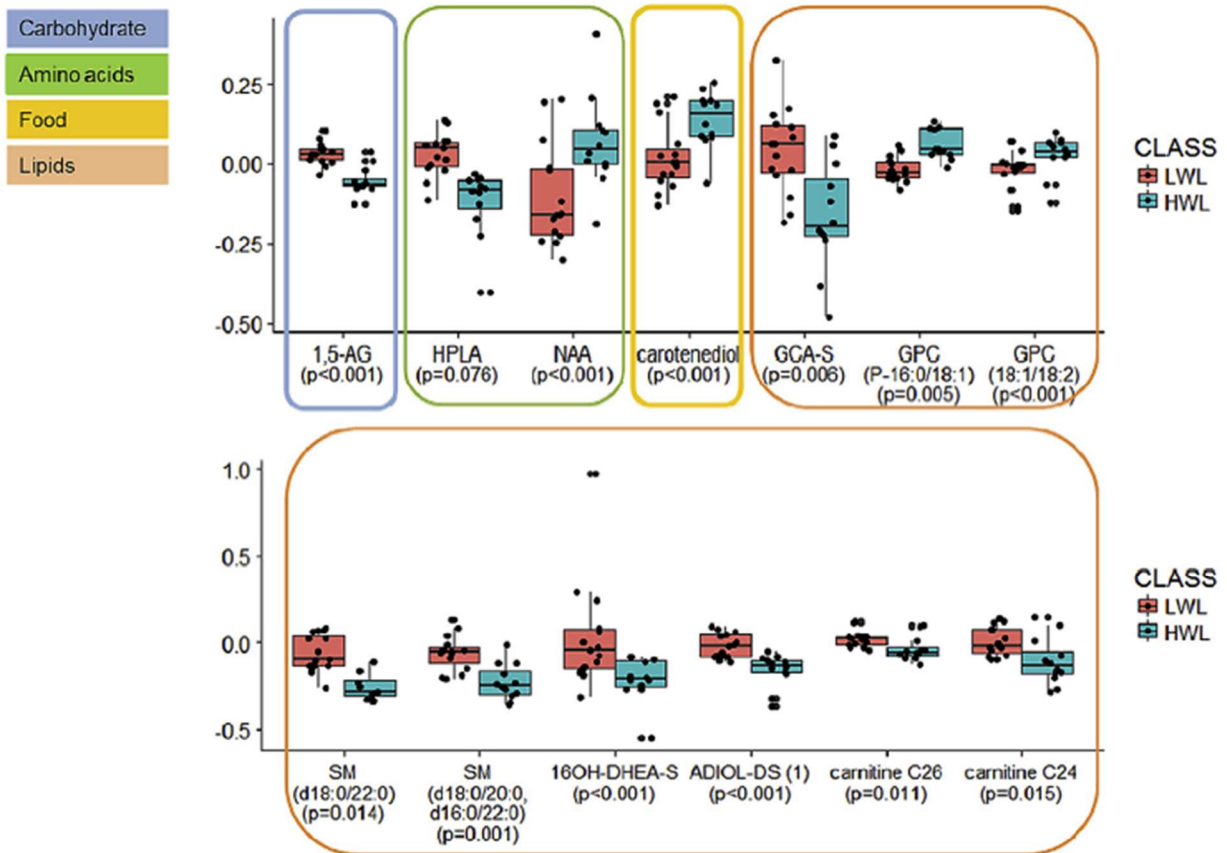
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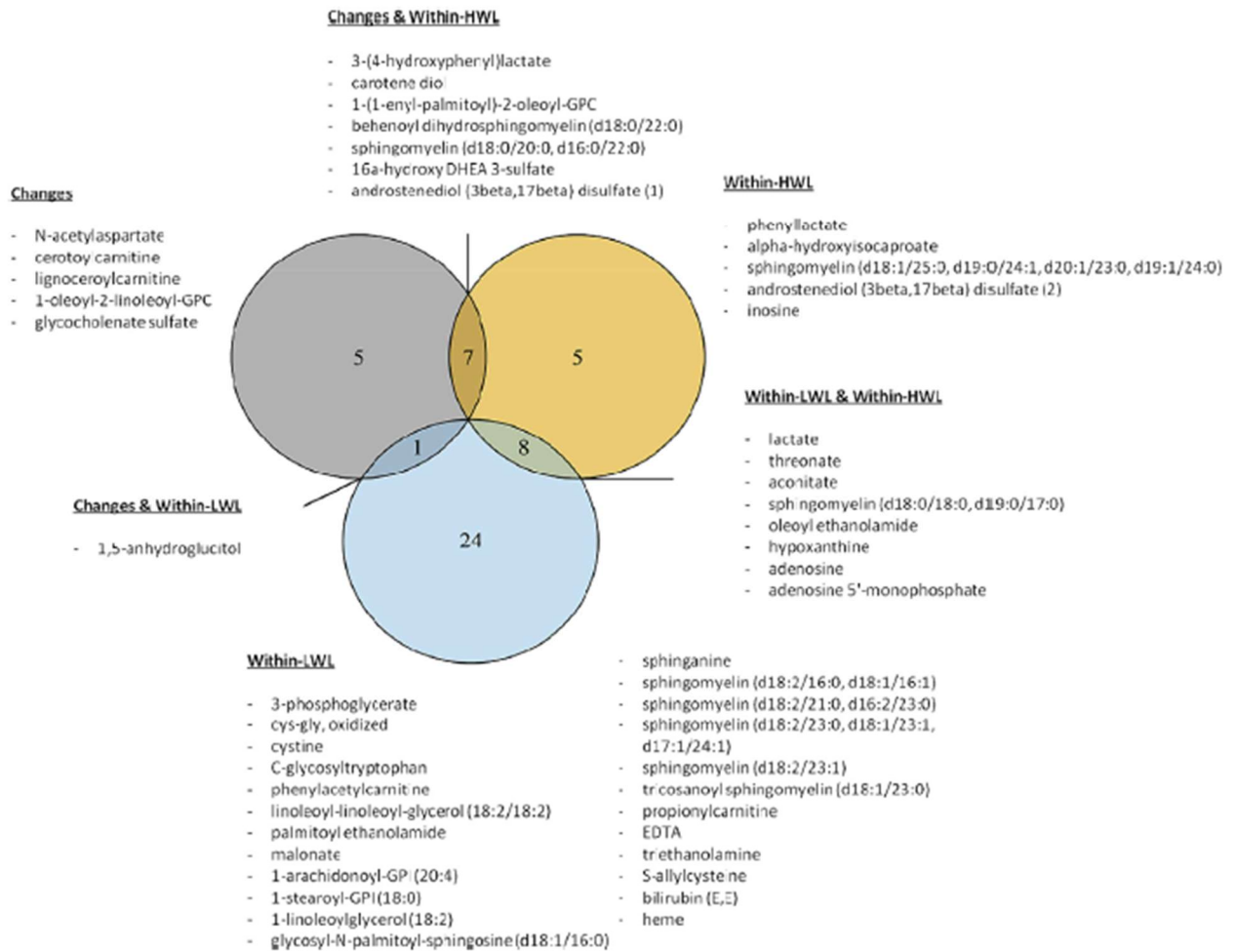
482 **Fig. 1.** Results from repeated double cross-validated random forest analysis (rdCV-RF) to classify
 483 between weight loss groups: (A) Predictive classification of subjects according to weight loss group
 484 (misclassified individuals are highlighted with a circle); (B) Histograms for permutation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



486

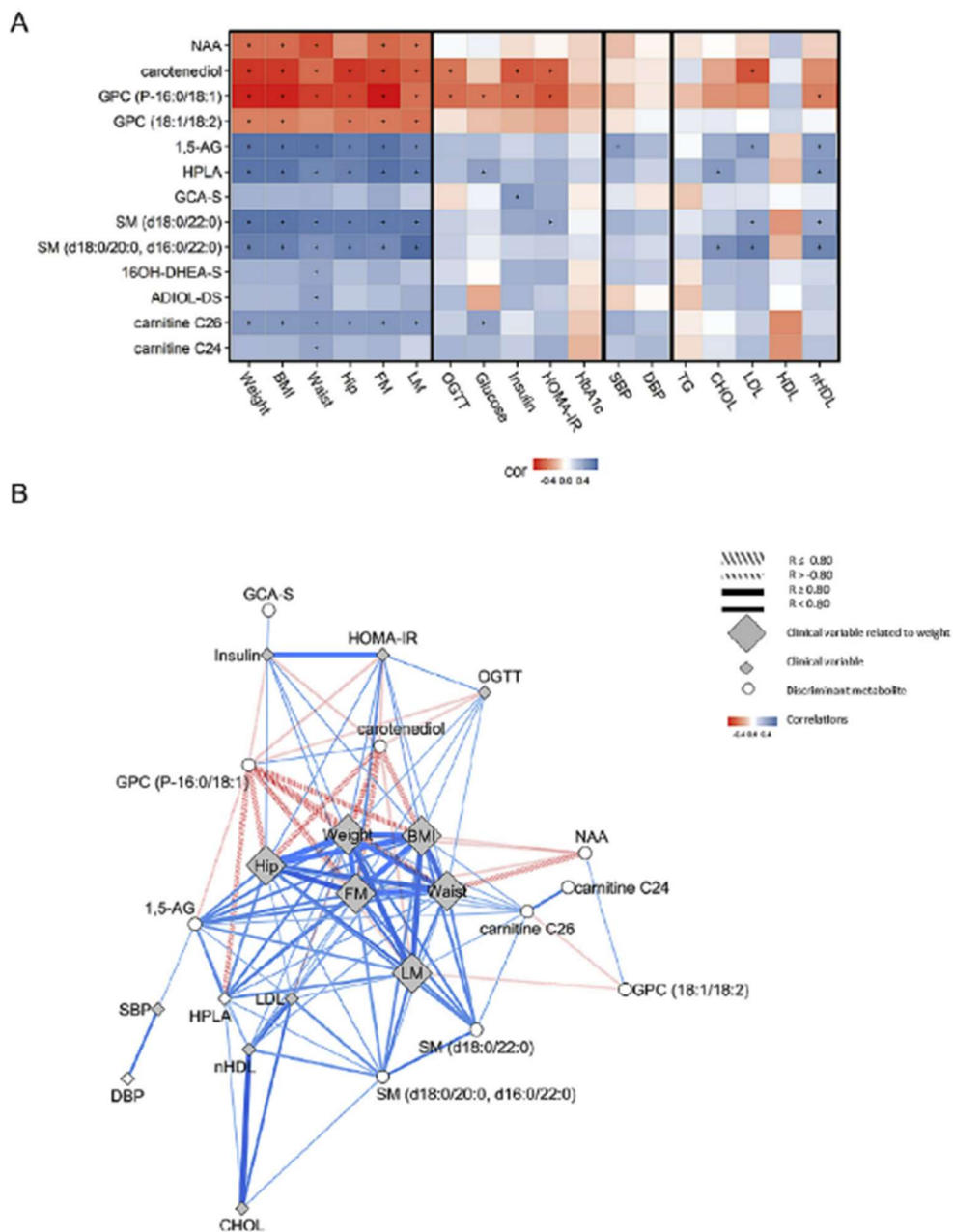
487 **Fig. 2.** Differences between weight loss groups in metabolites selected by repeated double-cross
 488 validated random forest model. p values were obtained by permutation test (n = 1000) of the
 489 differences of the changes between groups. 1,5-AG, 1,5-anhydroglucitol; 16OH-DHEA-S, 16a-
 490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

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



494

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



497

498 **Fig. 4.** Correlations of changes between metabolites and clinical parameters during the intervention
 499 program: (A) Metabolite-clinical correlations; (B) Metabolite-metabolite/clinical significant
 500 correlations. Associations determined by Spearman correlations adjusting p values by FDR, with
 501 significant threshold set at $p < 0.1$. Negative correlations are colored in red and positive correlations
 502 are colored in blue. 1,5-AG, 1,5-anhydroglucitol; 16-OH-DHEA-S, 16 α -hydroxy DHEA 3-sulfate;
 503 ADIOL- DS, androstenediol (3 β ,17 β) disulfate (1); BMI, body mass index; carnitine C26,
 504 cerotylcarnitine (C26); carnitine C24, lignoceroylcarnitine (C24); CHOL, total cholesterol; DBP,
 505 diastolic blood pressure; FM, fat mass; GCA-S, glycochenate sulfate; GPC (18:1/18:2), 1-oleoyl-
 506 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);
 507 HbA1c, glycated hemoglobin A1c; HDL, high-density lipoproteins cholesterol; Hip, hip
 508 circumference; HOMA-IR, insulin resistance calculated by homeostatic model assessment; HPLA,
 509 3-(4- hydroxyphenyl)lactate; LDL, low-density lipoproteins cholesterol; LM, lean mass; NAA, N-
 510 acetylaspartate; OGTT, oral glucose tolerance test; non-HDL, non-high-density lipoproteins
 511 cholesterol; SBP, systolic blood pressure; SM sphingomyelin; TG, triglycerides; Waist, waist
 512 circumference. (For interpretation of the references to color in this figure legend, the reader is
 513 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 2Anthropometric 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 3Baseline 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.