

1     **Significant metabolic improvement by a water extract**  
2             **of olives: animal and human evidence**

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4     Nikolaos Peroulis<sup>1</sup>, Vasilis P. Androutsopoulos<sup>1</sup>, George Notas<sup>1,2</sup>, Stella Koinaki<sup>3</sup>, Elsa  
5     Giakoumaki<sup>4</sup>, Apostolos Spyros<sup>5</sup>, Efstathia Manolopoulou<sup>5</sup>, Sophia Kargaki<sup>6</sup>, Maria  
6     Tzardi<sup>7</sup>, Eleni Moustou<sup>7</sup>, Euripides G. Stephanou<sup>6</sup>, Efstathia Bakogeorgou<sup>1</sup>, Niki  
7     Malliaraki<sup>8</sup>, Maria Niniraki<sup>2</sup>, Christos Lionis<sup>3</sup>, Elias Castanas<sup>1,2</sup>, Marilena Kampa<sup>1,2\*</sup>

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9     <sup>1</sup> Laboratory of Experimental Endocrinology, University of Crete, Heraklion, Greece,

10    <sup>2</sup> Laboratory of Experimental Endocrinology and Clinical Immunology, University  
11    Hospital of Heraklion, Heraklion, Greece

12    <sup>3</sup> Clinic of Social and Family Medicine, School of Medicine, University of Crete, Greece

13    <sup>4</sup> Creta Farm S.A. Rethymnon, Crete, Greece

14    <sup>5</sup> NMR Laboratory, Department of Chemistry, University of Crete, Heraklion, Greece

15    <sup>6</sup> Environmental Chemical Processes Laboratory, Department of Chemistry, University  
16    of Crete, Heraklion, Greece

17    <sup>7</sup> Department of Pathology, University of Crete, Heraklion, Greece

18    <sup>8</sup> Laboratory of Clinical Chemistry, University Hospital of Heraklion, Heraklion, Greece

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20    *\*Corresponding Author*

21    *All correspondence should be addressed to Dr Marilena Kampa, Laboratory of*  
22    *Experimental Endocrinology, University of Crete, P.O. Box 2208, Heraklion, 71003*  
23    *Greece, e-mail: [kampam@uoc.gr](mailto:kampam@uoc.gr)*

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29 **ABSTRACT**

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31 Dyslipidemia and impaired glucose metabolism, are main health issues of growing  
32 prevalence and significant high Health Care cost, requiring novel prevention and/or  
33 therapeutic approaches. Epidemiological and animal studies revealed olive oil as an  
34 important dietary constituent for normolipidemia. However, no studies have  
35 specifically investigated the polyphenol rich water extract of olives (OLWPE),  
36 generated during olive oil production. Here, we explore OLPWE in animals and human  
37 metabolic parameters. High fat-fed rats developed a metabolic dysfunction, which  
38 was significantly impaired when treated with OLWPE, with decreased LDL and insulin  
39 levels and increased HDL. Moreover, they increased total plasma antioxidant capacity,  
40 while several phenolic compounds were detected in their blood. These findings were  
41 also verified in humans that consumed OLWPE daily for four weeks in a food matrix.  
42 Our data clearly show that OLWPE can improve glucose and lipid profile, indicating its  
43 possible use in the design of functional food and/or therapeutic interventions.

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## 45 INTRODUCTION

46 Impaired glucose tolerance and lipid metabolism are the most common metabolic  
47 dysfunctions in humans and they have been closely associated with obesity, now  
48 recognized as a chronic disease of alarming incidence (close to 40% of adults in the  
49 world are overweight or obese) <sup>1</sup>. Obesity complications can further result to a  
50 number of life-threatening pathological conditions. In fact it is the various metabolic  
51 disorders (such as dyslipidemia and impaired glucose tolerance) are usually seen in  
52 central type obesity <sup>2</sup> together with increased blood pressure, that characterize a pro-  
53 inflammatory state <sup>3,4</sup>, leading to an increased likelihood of insulin resistance/type 2  
54 diabetes, atherosclerosis/cardiovascular disease <sup>5</sup>. This, together with a resulting pre-  
55 thrombotic state <sup>6</sup> may result in premature death. This, obesity induced cascade of  
56 events characterizes a pathophysiological state, commonly referred as “metabolic  
57 inflammation”. This is a low-grade inflammation triggered by adipose tissue  
58 hypertrophy and hyperplasia and subsequent hypoxia. As a result, there is an altered  
59 lipid metabolism and an increased production of several hormones, chemokines and  
60 cytokines and coagulation factors that lead to hyperinsulinemia,  $\beta$  pancreatic cell  
61 dysfunction, type II diabetes, increased sodium uptake, vasoconstriction and  
62 hypertension, increased lipoprotein synthesis, gluconeogenesis and dyslipidemia,  
63 endothelial dysfunction, atherosclerosis and clotting disorders and ultimately to  
64 coronary heart disease. The collective cluster of (central) obesity, dyslipidemia,  
65 impaired glucose tolerance/insulin resistance and hypertension is commonly referred  
66 as the “metabolic syndrome” <sup>7</sup>

67 Obesity, due to its high global prevalence and comorbidities, has become an  
68 international health care priority, with the major aim being early diagnosis of  
69 metabolic dysfunction and improvement of body weight and adverse metabolic  
70 disturbances (mainly lipids and glucose) by dietary modifications and pharmaceutical  
71 interventions. The cost of the latter is extremely high <sup>8,9</sup>and therefore alternative  
72 approaches, which may improve the above elements, are of great importance, both  
73 for the health of the patients and for a possible reduction of the pharmaceutical  
74 expenditure.

75 A great variety of animal and human epidemiological studies, report beneficial effects  
76 of olive oil and / or olive -olive oil polyphenol extracts <sup>10-12</sup> on glucose and lipid  
77 metabolism. However, during olive harvesting and olive oil production, a water phase  
78 is also produced, commonly discarded. This phase is rich in olive (poly)phenols, which  
79 distribute between the olive and water phase as a result of time of olive paste  
80 malaxation and temperature. Here, we studied the effect of a microencapsulated  
81 olives water phenolic extract (OLWPE) in a rat model of diet induced obesity and  
82 extended our study (as a proof of principle) in a healthy human population that  
83 consumed this extract in the context of a food matrix. Our findings clearly show that  
84 OLWPE can ameliorate main metabolic parameters, such as fasting glucose levels and  
85 lipid profile, indicating its possible dietary and/or therapeutic use.

## 86 RESULTS

### 87 Polyphenolic characterization of OLOWPE

88 The phenolic content of concentrated OLOWPE was initially estimated as 10mg/ml  
89 Trolox equivalents, while NMR analysis (Figure 1A), revealed that the extract, as  
90 expected, contains phenolic compounds along with other larger amounts of small  
91 molecular weight chemicals, such as ethanol, lactic, succinic and acetic acid and  
92 carbohydrates. Analysis of the phenolic area of the spectrum revealed that the main  
93 phenolic compound present is the phenylethanoid tyrosol, along with small amounts  
94 of ligstroside and possibly elenolic acid, which is a common phenylethanoid hydrolysis  
95 product. Further analysis of the extract by the more sensitive LC-ESI-MS/MS method  
96 showed also the presence of the phenylethanoids oleuropein and verbascoside, the  
97 flavanols catechol, catechin, and epicatechin, the flavones apigenin, apigenin-7-o-  
98 glucoside and luteolin, the flavonols quercetin and rutin and a number of phenolic  
99 acids such as caffeic, ferrulic, gallic, 3-hydroxy-4-methoxy-cinamic, homovanillic,  
100 hippuric, coumaric, siringic, p-hydroxy-benzoic acid, protocatechuic acid  
101 (Supplemental Table 1).

102 The above extract was stabilized by micro-encapsulation and used in the subsequent  
103 long-term metabolic studies, described below.

### 104 Animal study

#### 105 OLWPE bioavailability and absence of toxicity in an animal model

106 Initially, in order to examine the bioavailability of the phenolic compounds in the  
107 extract, rats were given a single dose (corresponding to the dose D3 of the long-term  
108 study, see below and Material and Methods for further details) of OLOWPE by gavage,  
109 blood was withdrawn at different time points (1-24h) and serum was analyzed by LC-  
110 ESI-MS/MS. As soon as one hour after treatment, a number of phenolic compounds  
111 have been detected in animal serum (Figure 1B and C) including epicatechin,  
112 quercetin, caffeic, gallic, coumaric, homovanillic, and p-hydroxy-benzoic acid. This  
113 early appearance of phenolics in the blood suggests an early gastric absorption. For

114 caffeic, gallic, and coumaric acid, increased levels were also detected after 18 and/or  
115 24h, indicating significant intestinal absorption, as well as, a possible increase as a  
116 result of (poly)phenol metabolism. As expected, oleuropein, was not detected since,  
117 due to its high molecular weight, it does not cross the intestinal barrier. Interestingly,  
118 its primary metabolite, hydroxytyrosol, was also not equally detected. Comparing the  
119 different AUC values, the compound with the greater bioavailability is ferrulic acid,  
120 followed by p-hydroxy-benzoic and homovanillic acid. The latter being a metabolite of  
121 hydroxytyrosol possibly explains its absence from the serum of treated animals.

122 Bioavailability data were also obtained during a long-term animal study, in which rats  
123 were fed with three different doses ( $D1 < D2 < D3$ ) of microencapsulated OLWPE  
124 extract, for a period of 16 weeks. As shown in Figure 1D, increased levels of rutin,  
125 caffeic and p-coumaric acid, were detected in animal serum. These compounds were  
126 also present in rats fed only high fat food (HF), while their levels increased dose  
127 dependently when OLWPE was included in their diet. Moreover, in the highest extract  
128 dose (D3), hydroxytyrosol was detected, possibly as a product of long-term continuous  
129 oleuropein metabolism. Further evidence supporting the bioavailability of OLWPE  
130 were obtained by measuring the plasma total antioxidant capacity (TAC) of the 16  
131 weeks-fed animals. TAC levels were dose-dependently increased in rats fed with  
132 different OLWPE extract; dose D3 exhibited a statistically significant increase  
133 compared to the high fat diet only group (Figure 1E).

134 Finally, at the end of the long-term animal study, rat livers and kidneys were examined  
135 in order to rule out any signs of toxicity that could be attributed to the polyphenolic  
136 extract. For this reason, organs were removed, formalin-fixed, paraffin embedded and  
137 sectioned for haemotoxylin-eosin staining. In Figure 2 such sections are presented for  
138 all study groups along with the levels of certain biochemical markers (GOT, GPT  
139 creatinine and urea,) of liver and kidney toxicity for all study groups. No signs of  
140 toxicity were identified, while a slight fatty liver infiltrates, as a consequence of high  
141 fat diet, were not modified by OLWPE.

142

143 **Effect of diets on rat weight**

144 As it is shown in Figure 3A the final weight, as well as total weight gain, was elevated  
145 in rats receiving HF food alone or in combination with any of the tested OLWPE doses  
146 compared to the control rats. This indicates that HF diet induced obesity and OLWPE  
147 did not have any effect on body weight *per se*.

148 **OLWPE significantly lowers circulating lipids and insulin levels**

149 A sixteen-week high fat diet not only increased body weight but also induced  
150 additional features compatible with induction of metabolic syndrome: increased  
151 triglycerides, insulin and LDL and lower HDL (Figure 3B and D) were observed, while  
152 total cholesterol levels did not exhibit significant differences. Enrichment of HF diet  
153 with the different doses of OLWPE, significantly modified HDL and LDL levels (Figure  
154 4). HDL levels were significantly elevated in rats receiving the highest (D3) dose, while  
155 LDL levels of all experimental groups receiving polyphenolic extract were significantly  
156 lower when compared to the HF only group. Accordingly, the HDL/LDL ratio (Figure  
157 4B) was elevated in rats receiving doses 1 and 2, compared to rats of the high fat  
158 group. Additionally, insulin levels that were elevated in the HF only group compared  
159 to the control group, were decreased back to normal levels in rats receiving all three  
160 doses of the polyphenolic extract (Figure 4C). Finally, leptin that was significantly  
161 increased in HF diet rats was not modified by OLWPE (Figure 4D). This finding is in  
162 accordance with the lack of differences in the body weight between the HF only fed  
163 group and the groups with OLWPE in their diet. Similarly, no changes were observed  
164 in the levels of different proinflammatory cytokines like IL6 and TNF $\alpha$  (data not  
165 shown).

166 **OLWPE decreases the levels of CD4<sup>+</sup>CD25<sup>+</sup> T cells**

167 Apart from the OLWPE-induced change of the aforementioned biochemical  
168 parameters, peripheral blood cell characteristics and immunophenotype were also  
169 examined. The major cell population numbers (red blood cells, lymphocytes,  
170 granulocytes and platelets) were not affected (Supplemental Figure 1). However, in  
171 an immunophenotype analysis (Figure 4E), CD4<sup>+</sup> cells population was increased in an

172 OLWPE dose dependent manner, while the CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells were dose-  
173 dependently decreased by OLWPE and attained significant importance in the HF+D3  
174 group, compared to the HF diet only group. All other immune populations did not  
175 present any significant differences among study groups.

## 176 **Human study**

177 The above presented results from the animal study suggest that chronic consumption  
178 of OLWPE polyphenols, can improve the lipid profile of animals and reduce glucose  
179 levels and decrease insulin requirements. However, animal data are not easily  
180 extrapolated in humans, due mainly to a significantly different metabolism between  
181 the two species. Therefore, in order to explore whether the same effect can be  
182 obtained in humans, we have performed a proof of concept study, by administering  
183 microencapsulated OLWPE polyphenols (in a dose equivalent to the D2 dose used in  
184 the animal study, as the total polyphenol content included in the daily dose of olive  
185 oil approved by EFSA) in apparently healthy individuals. However, anthropometric  
186 data (Supplemental Table 2) showed that 19 out of the 35 participants were  
187 overweighted/obese (BMI >26) and 6 had a systolic blood pressure >130 mm Hg. The  
188 baseline biochemical analysis of our group revealed that 14 individuals had a fasting  
189 blood glucose >100 mg/dl, indicative of a pre-diabetic state, 26 presented a total  
190 cholesterol >200 mg/dl, 21 presented LDL cholesterol >130 mg/dl and 6 presented  
191 triglyceride levels >100 mg/dl. However, their HDL levels were astonishingly high  
192 (mean±SD 66.4±10.7 mg/dl), compatible with a high consumption of vegetables and  
193 olive oil, typical of a Cretan diet, followed by all participants.

## 194 **NMR-based plasma metabolomics**

195 At a first approach to identify changes in participants' metabolism, when OLWPE is  
196 included in the diet of humans, serum samples from each participant before and after  
197 a four week OLWPE consumption were analyzed by NMR. Figure 5A depicts the OPLS-  
198 DA (Orthogonal Projection to Latent Structures – Discriminant Analysis) score plots  
199 before (ct1) and after (ct2) consumption of OLWPE, as obtained from the NMR  
200 metabolomic analysis of their serum lipids and water soluble metabolites (see



201 Material and Methods for details). A clear separation of individuals is obtained from  
202 the OPLS-DA models, indicating that OLWPE consumption can be traced at both the  
203 water-soluble metabolite ( $R^2X=0.80$ ,  $Q^2X=0.23$ ) and serum lipid profile ( $R^2X=0.91$ ,  
204  $Q^2X=0.55$ ), with the serum lipids model demonstrating a better discriminatory power  
205 (higher  $Q^2X$ ). In the case of serum lipids, buckets in the spectral region characteristic  
206 of LDL/HDL lipoprotein signal contribute significantly to the classification of  
207 individuals, indicating that OLWPE may affect their lipidemic profile.

### 208 **OLWPE decreases glucose and lipid levels**

209 Based on the above metabolomic results and the beneficial effect of the  
210 microencapsulated OLWPE in animals, the lipidemic and glycemic profile was further  
211 explored. In normo-glycemic or normo-lipidemic individuals, the administration of the  
212 product did not modify blood glucose and lipid levels. However, in the sub-group of  
213 individuals with at least two biochemical or anthropometric elements of cardio-  
214 metabolic risk ( $n=18$ ), we observed (Figure 5B) that, administration of OLWPE in a food  
215 matrix significantly reduced elevated glucose levels ( $p<0.002$ ,  $n=15$ ), while insulin  
216 levels were significantly reduced ( $p<0.03$ ,  $n=6$ , paired analysis). In addition, OLWPE  
217 administration significantly reduced elevated total cholesterol ( $p<0.009$ ,  $n=14$ ),  
218 triglyceride ( $p<0.005$ ,  $n=5$ ) and LDL levels ( $p<0.01$ ,  $n=5$ ), while it decreased  
219 significantly oxLDL ( $p<0.01$ ,  $n=18$ ). oxLDL was also significantly reduced in the  
220 normolipidemic individuals, presumably as a result of the ingestion of polyphenols  
221 (paired t-test,  $p=0.0082$ ,  $n=19$ ) present in the extract.

222 Moreover, in the immunophenotype analysis (Figure 5C) no changes were observed  
223 in the major lymphocyte populations after OLWPE consumption. However, it should  
224 be noted that obese individuals have a slightly increased  $CD4^+CD25^+$  T regulatory cells  
225 as expected from the animal study. Possibly the inability of OLWPE to decrease them  
226 can be attributed to the dose administered that corresponds to dose 2 of the animal  
227 model that equally has no significant effect on the different cell populations.

228 In all participants, no modification of circulating hepatic enzymes (SGOT, SGPT), urea  
229 or creatinine levels was observed (not shown), ensuring that this product does not

230 express any hepatic or renal toxicity, at least for the period of its administration.  
231 Furthermore, no significant modification of body weight was observed, as expected,  
232 in the one-month interval of OLWPE consumption.

## 233 DISCUSSION

234 Polyphenols (more than 8000 identified molecules containing a phenolic scaffold)  
235 constitute a large family of plant-derived compounds. They exhibit powerful  
236 antioxidant properties in parallel with a large number of biological actions, depending  
237 on their absorption and metabolism<sup>13,14</sup> A variable amount of ingested polyphenols  
238 can be found in blood<sup>15</sup>, and can interfere with major cellular processes having a  
239 beneficial impact on cancer reviewed in<sup>13</sup>, vascular function<sup>16,17</sup>, and metabolism<sup>18,19</sup>.

240 The beneficial role of olive oil consumption is now-a-days widely recognized, with the  
241 European Food Safety Authority (EFSA) approving two health claims regarding olive  
242 oil<sup>20</sup>. They suggest its use to replace saturated fats in order to keep normal blood  
243 cholesterol levels and protect blood lipids from oxidative stress with the later effect  
244 to be achieved by olive oil polyphenols contained in a daily intake of 20 g of olive oil.  
245 In numerous studies, diets enriched either in virgin olive oil or following the pattern  
246 of the Mediterranean Diet (which is *per se* rich in olive oil, vegetables and legumes)  
247 have provided further evidence about the beneficial role of plant and olive oil  
248 antioxidant fractions in the prevention of cardiovascular disease<sup>21</sup>, diabetes<sup>11,22,23</sup>  
249 and hyperlipidemia<sup>10</sup>. Moreover, in an animal obesity and diabetes model, a  
250 polyphenol-enriched extract from olive leaves has been shown to reverse the chronic  
251 inflammation and oxidative stress that induces the cardiovascular, hepatic, and  
252 metabolic dysfunction<sup>12</sup>. Olives are a rich source of polyphenols; during their  
253 harvesting and olive oil extraction, contained polyphenols are distributed between the  
254 oil and water phase, depending on the malaxation time and the applied temperature.  
255 Therefore, this water phase is a rich source of olive polyphenols, not yet exploited as  
256 a beneficial constituent of human functional foods/medicinal preparations. At this  
257 point It needs to be stressed that, according to a number of in vitro studies, the  
258 beneficial effect of food extracts is maximized when the total extract is used<sup>24,25</sup>. This  
259 suggests: (1) either a synergistic effect of polyphenol molecules, that cannot be totally  
260 mimicked by the artificial combination of isolated phenolic molecules; or (2) that the  
261 effect of the extract is attributed to minor constituents, present in the total extract,  
262 beyond the lead molecules identified so far. Furthermore, an interesting observation

263 that is in favor of the use of total plant extracts, is that isolated phenolic molecules  
264 with antioxidant properties (including vitamin C) in the context of a food matrix <sup>26</sup>,  
265 may induce pro-oxidant activities, when administered isolated in vitro or in vivo <sup>27,28</sup>.

266 In this light, in our study we used the total polyphenolic water extract from olives  
267 (OLWPE) that contains a number of different phenolic compounds (phenylethanoids,  
268 flavones, flavanols, flavonols and phenolic acids-See Results for details). Our findings  
269 can be summarized as follows: (1) We show that polyphenols and simple phenolic  
270 products are absorbed from different parts of the GI tract, as derived from acute  
271 metabolic studies; (2) The profile of absorbed polyphenols after chronic  
272 administration is different from that of an acute one; (3) Both in obesity-metabolic  
273 syndrome animal model and in humans, chronic administration of OLWPE in a food  
274 matrix results in improvement of glycemic control and lipid metabolic parameters,  
275 with no apparent toxicity. (4) In the animal model. OLWPE normalize the number of  
276 circulating CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells that have been elevated in the metabolically  
277 dysfunctional high fat fed animals.

278 In our experimental conditions, animals fed with the high fat diet were significantly  
279 overweighed compared to those fed with a normal fat diet and exhibited metabolic  
280 dysfunction characterized by increased cholesterol, triglyceride, LDL and insulin levels,  
281 suggesting the establishment of insulin resistance. OLWPE ameliorates a number of  
282 these metabolic parameters such as lipidemic and glycemic profile, with lack of  
283 toxicity. These results are in accordance with a previous study on the effect of OMW  
284 biophenols on alloxan-induced diabetic rats <sup>29</sup>. Especially in the case of increased LDL  
285 and decreased HDL levels, OLWPE seems to exhibit a highly beneficial effect.  
286 Additionally, even though fasting glucose was not significantly reduced, OLWPE  
287 reduced diet-induced hyperinsulinemia. Another interesting finding is that OLWPE can  
288 bring circulating CD4<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells that have been reduced and  
289 elevated respectively by high fat diet, back to normal levels. CD4<sup>+</sup>CD25<sup>+</sup> T regulatory  
290 cells are important anti-inflammatory cells. However, their proportion in obesity-  
291 related metabolic disturbances is still controversial. There are studies reporting a  
292 reduction in obesity <sup>30-32</sup> and others, including our present findings, an increased

293 number of peripheral blood Tregs<sup>33</sup>. In fact, their percentage seems to be  
294 interdependent to their concentration in inflamed tissues. For instance inflamed  
295 obese visceral adipose tissue has been shown to have a reduced proportion of Tregs  
296<sup>34,35</sup> and that could be a possible explanation for their increased number in the blood.

297 The above presented effects of OLWPE are greatly supported by our findings that the  
298 phenolic compounds of the extract are bioavailable (detected in rats' sera) and the  
299 fact that the animals treated with the extract exhibited increased total plasma  
300 antioxidant capacity. Indeed several phenolic compounds present in the extract were  
301 detected in the blood of the animals (epicatechin, quercetin, caffeic, gallic, coumaric,  
302 homovanillic, and p-hydroxy-benzoic acid) as early as one hour after ingestion,  
303 pointing out an additional absorption via stomach that is followed by intestinal  
304 absorption<sup>36</sup>. Although oleuropein was not detected, as expected, samples collected  
305 in longer time points after the OLWPE administration showed significant levels of its  
306 metabolite hydroxytyrosol. However, the aforementioned effects of OLWPE cannot  
307 be solely attributed to the detected molecules, due to certain limitations such as the  
308 doses used and the detection limit of the LC-ESCI-MS/MS method. Nevertheless, our  
309 data present a proof of the bioavailability of the polyphenolic olive extract, as  
310 previously described<sup>37</sup>.

311 Obesity in humans is considered an emerging health problem<sup>38</sup> and in spite of the  
312 number of studies, its prevalence continues to rise. Diet is certainly very important to  
313 obesity incidence, the metabolic dysfunction that usually occurs in obese individuals  
314 and to its negative consequences, such as cancer<sup>39</sup>, aging<sup>40</sup>, cardiovascular disease<sup>41</sup>  
315 and a number of other pathologies<sup>42</sup>. Metabolic dysfunction includes changes in their  
316 lipid and glucose metabolism, characterized by increased LDL cholesterol and low HDL  
317 levels, high glucose and insulin levels that can result to endothelial dysfunction,  
318 atherosclerosis and subsequent heart disease<sup>43</sup>. It is therefore of great importance to  
319 find and exploit substances that will improve the above metabolic parameters. When  
320 healthy young individuals consumed OLWPE daily for a period of 4 weeks,  
321 metabolomic analysis revealed clear differences in their lipid and water soluble  
322 metabolites compared to the period that they did not consumed OLWPE.

323 Furthermore, a significant amelioration of specific metabolic parameters was  
324 observed in those individuals identified with elevated cardio-metabolic risk factors (at  
325 least 2 factors among fasting glucose and insulin, triglycerides, total and LDL  
326 cholesterol, n=18). These findings are in accordance to previous studies that report a  
327 beneficial effect of olive oil and its phenolic constituents on lipid profile <sup>44-49</sup>.  
328 Moreover, oxidized LDL which is known to actively participate in atheromatous plaque  
329 formation and atherosclerosis, was significantly decreased in OMWPE-treated  
330 individuals, supporting the beneficial effect of OLWPE on cardiovascular risk factors.  
331 Additionally, it is of great importance that with the inclusion of OLWPE in the diet  
332 (both in animals and humans) a normal insulin sensitivity was restored. In fact, it is the  
333 first study reporting a direct effect of olive water extract polyphenols on fasting  
334 glucose and insulin levels, while all previous studies were conducted with olive oil see  
335 for example <sup>50</sup>, for a recent publication. Finally, eventhough overweighted/obese individuals  
336 exhibited an increase in peripheral blood CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells (as also  
337 observed in the animal model), OLWPE consumption had no effect mainly due to the  
338 given dose which was also ineffective in animals.

339 Overall, we conclude that OLWPE can exhibit a beneficial health effect, mainly by  
340 modifying circulating lipids and glucose/insulin levels, and most importantly without  
341 the presence of monounsaturated fats of olive oil, on which there are contradictory  
342 data concerning their role on development of insulin resistance, type 2 diabetes  
343 mellitus and impaired vascular integrity and cardiovascular disease <sup>51-54</sup>. Under this  
344 scope we suggest that this microencapsulated polyphenolic extract could possibly be  
345 used in the development of "functional" OLWPE polyphenol-enriched foods or as an  
346 alternative/adjuvant therapeutic approach in hyperlipidemia and impaired  
347 glucose/insulin regulation.

## 348 **METHODS**

### 349 **Isolation and characterization of OLVPE**

350 Olive water total polyphenolic extract (OLWPE) was obtained by using olive mill waste  
351 water, immediately collected during olive oil production through centrifugation,  
352 passing through a multilevel ion-exchange proprietary resin filter (patent no.  
353 GR20030100295 20030708 & WO2005003037) and elution with ethanol (75%);  
354 ethanol was subsequently fully evaporated and the water extract was concentrated  
355 through a rotor evaporator. The total content of polyphenols was estimated by the  
356 Folin-Ciocalteu method<sup>55</sup> that has been modified in order to use small volumes.  
357 Briefly, 20  $\mu\text{l}$  sample was mixed with 80  $\mu\text{l}$  of distilled water, 400  $\mu\text{l}$   $\text{Na}_2\text{CO}_3$  (10%  
358  $\text{Na}_2\text{CO}_3$  in 0.85 N NaOH), and 500  $\mu\text{l}$  Folin-Ciocalteu reagent (10%). The mixture was  
359 allowed to stand for 1 hour in the dark and absorbance was measured at 750 nm. The  
360 total phenolic profile was expressed as Trolox (a water-soluble analog of Vitamin E)  
361 equivalents.

362 The specific composition of the polyphenolic content was obtained by using NMR  
363 spectroscopy and Mass Spectrometry. NMR spectroscopy experiments were  
364 conducted on a Bruker Avance III NMR spectrometer, operating at 500 MHz for the  
365 proton nucleus. OLVPE extracts' NMR analysis was performed by mixing 100  $\mu\text{l}$  of the  
366 sample with 400  $\mu\text{l}$  MeOD. The mixture was vigorously shaken and then placed in a 5  
367 mm NMR tube, where 1D (zg30, zgpr) and 2D dimensional (gCOSY, gHSQC, gHMBC)  
368 NMR spectra were acquired.  $^1\text{H}$  NMR spectra were acquired using the standard one-  
369 dimensional NOESY pulse sequence with water presaturation. Quantitative analysis  
370 was performed by the ChenomX software.

371 Mass spectrometric analysis was carried out on a ThermoFinnigan Liquid  
372 Chromatography/triple quadrupole mass spectrometer on Electrospray Ionization  
373 (LC-ESI-MS/MS). The experimental conditions for the mass spectrometric analysis  
374 were the following: negative ionization mode; capillary voltage 4000V; argon pressure  
375 1mTorr. For quantification purposes data were collected in the selected ion  
376 monitoring (SIM) mode. The applicability and reliability of this analytical approach was  
377 confirmed by method validation and successful analysis of several samples with  
378 different matrices. Extraction of polyphenols from samples was performed using solid-

379 phase extraction (SPE) Strata-X, 30mg/1mL (Phenomenex), a vacuum manifold, and a  
380 vacuum source.

### 381 **Plasma samples polyphenolic content analysis**

382 Plasma samples were enzymatically hydrolyzed with  $\beta$ -glucuronidase/sulfatase from  
383 *Helix pomatia* ( $\geq 100,000$  U/mL) at 37 °C for 45 min before polyphenol extraction;  
384 analysis was performed by SPE and LC–ESI-MS/MS respectively, as described above.

### 385 **Extract microencapsulation**

386 OLWPE extract was microencapsulated using Maltodextrins and Spray Drying (Mean  
387 particle size 10 $\mu$ m) by XEDEV bvba (Zelzate, Belgium) in order to protect polyphenols  
388 from oxidation and heat, and simultaneously mask their unpleasant bitter taste.

389

### 390 **Animal Study**

#### 391 ***Short-term study***

392 Male Sprague-Dawley rats (16 weeks old, weighting 400-500 gr), purchased from  
393 Harlan Laboratories were used (n=4). In each animal, a single dose of the extract  
394 (containing 3.42mg total polyphenols, corresponding to Dosage 3, see below) was  
395 given by gavage, directly to the stomach and the animals were single caged, had  
396 unlimited access to food and water and were kept under normal laboratory  
397 conditions. They were closely monitored for 24h and blood sample was collected at  
398 different time points (1, 3, 6, 18 and 24h), up to 24 hours. The specific polyphenolic  
399 content of their plasma at different time points was determined by LC-ESI-MS/MS  
400 under the experimental conditions described above. For each phenolic compound that  
401 has been detected the following pharmacokinetic parameters have been calculated:  
402 i) maximum concentration (C<sub>max</sub>), ii) time required to achieve maximum  
403 concentration (T<sub>max</sub>), iii) area under the curve (AUC), iv) half-life (t<sub>1/2</sub>), and v)  
404 elimination rate constant (K<sub>e</sub>), according to<sup>56</sup> and using PK Functions for Microsoft  
405 Excel by Joel I. Usansky (<http://www.boomer.org>).

#### 406 ***Long-term study***

407 **Diets:** Normal food (2018S) and high fat (HF) food (TD.06414), containing 60% Kcal  
408 from fat were purchased from Teklad, Harlan Laboratories (Supplemental Table 3).



409 Both diets were acquired in their paste form, so that microencapsulated OLWPE could  
410 be better incorporated. OLWPE was given in three dosages: 0.375 mg of total  
411 polyphenols or 0.85 gr of microcapsules per Kg of food (Dosage 1, D1), 3.75 mg of total  
412 polyphenols or 8.5 gr of microcapsules per Kg of food (Dosage 2, D2) and 37.5 mg of  
413 total polyphenols or 85 gr of microcapsules per Kg of food (Dosage 3, D3). Dosage 2  
414 corresponds to the total polyphenol content of 20g olive oil, being the daily dose of  
415 olive oil approved by EFSA that when used to replace saturated fat contributes to the  
416 maintenance of normal blood cholesterol levels and to the protection of blood lipids  
417 from oxidation <sup>20</sup>.

418 ***Animals and experimental design:*** Male Sprague-Dawley rats (8 weeks old, weighting  
419 230-270 gr), were purchased from Harlan Laboratories and used in our experiments.  
420 Animals were caged in groups of 3-4 rats, had unlimited access to food and water and  
421 were kept under normal laboratory conditions. They were randomly assigned in 5  
422 study groups (n=8 animals per group): 1) the Control group (normal diet), 2) the high  
423 fat (HF) diet group, 3) the high fat + Dosage 1 (HF +D1) group, 4) the high fat + Dosage  
424 2 (HF +D2) group and 5) the high fat + Dosage 3 (HF +D3) group. Rats were kept on  
425 these diets for 4 months *ad libitum*. During this period, animals were closely  
426 monitored, their weight was measured weekly. At the start and at the end of the  
427 study, animals were fasted for 12-14h and afterwards blood samples were collected,  
428 for complete blood cell counting, immunophenotyping and biochemical analysis.  
429 When animals were sacrificed at the end of the study, selected tissues (kidney, liver,  
430 lungs, fat and heart) were also kept for histological analysis.

431 Animal studies were approved by the School of Medicine, University of Crete  
432 Committee for animal welfare (Protocol no. 6174/7-5-14) and all experiments were  
433 performed in accordance with relevant guidelines and regulations.

#### 434 ***Blood sample analysis***

435 *Complete blood cell count* was performed at the University Hospital of Heraklion,  
436 Laboratory of Haematology, in an Abbott Emerald Hematology (Abbott, CA, USA)  
437 Analyzer according to standard operating procedures.

438 Immunophenotyping was performed as follows: 100µl of whole blood were incubated  
439 with the different fluorescently labelled antibodies for 30 min, followed by red blood  
440 cell lysis with the addition of 2 ml BD FACS Lysing solution (for a least 10 min) and  
441 afterwards each sample was analyzed in a flow cytometer (Attune® Acoustic Flow  
442 cytometer, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) using  
443 lymphocyte population of at least 5000 cells. FITC mouse Anti-Rat CD4 (561834), PE  
444 mouse Anti-Rat CD45 (554878), PE mouse Anti-Rat CD8a (559976), PE mouse Anti-Rat  
445 CD25 (554866), FITC mouse Anti-Rat CD32 (550272) and FITC mouse Anti-Rat CD3  
446 (561801) were from BD Pharmingen® (BD Biosciences, San Jose, CA USA). FITC mouse  
447 Anti-Rat CD19 (MA5-16536) was from Thermo Fisher Scientific (Waltham, MA USA).

448 Biochemical and Metabolic parameters: All biochemical and metabolic parameters  
449 (glucose, triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol, hsCRP, urea,  
450 creatinine, γGT, SGOT and SGPT) were measured according to standard operating  
451 procedures using Olympus AU2700 Analyzer at the Laboratory of Biochemistry of the  
452 University Hospital of Heraklion.

453 Hormones and pro-inflammatory cytokines: Insulin, leptin, TNFα, and IL6, were  
454 measured in duplicate using a multiplex kit (MILLIPLEX® MAP Rat Adipokine Magnetic  
455 Bead Panel RADPKMAG-80K-04; Millipore Corp., St. Charles, Missouri, USA), in a  
456 Luminex 100 apparatus.

457 Total Antioxidant Capacity (TAC): TAC was measured colorimetrically using the crocin  
458 bleaching assay as described in <sup>46</sup>.

459

## 460 **Histology**

461 Toxicity was assessed using haematoxylin-eosin staining in formalin-fixed paraffin-  
462 embedded sections of selected tissues (liver, kidney) for all study groups.

## 463 **Human study**

### 464 ***Participants and study design***

465 Thirty-five volunteers, without any diagnosed disease, participated in this study. The  
466 study was implemented in the following phases: participant recruitment using a

467 number of exclusion criteria (Supplemental Table 4), baseline data  
468 collection, test phase I (4 weeks), washout period (2 weeks) and a test phase II (4  
469 weeks). Participants were divided into two groups. Group A consumed the meat  
470 product containing the microencapsulated OLWPE in test phase I and the same meat  
471 product without the microencapsulated OLWPE in test phase II and group B in the  
472 reverse order. Blood sample collection and weight measurements were taken at the  
473 start of the study (baseline values, t=0) at the end of phase I (t=1) and at the end of  
474 phase II (t=2)

475 All participants gave written informed consent to participate in the study. The study  
476 was approved by the Ethics Committee of the Heraklion University Hospital  
477 (Protocol no 10714) and was performed in accordance with relevant guidelines and  
regulations.

#### 478 **Diet**

479 All participants in each test phase were on a free diet (a 3 day food record at t0, which  
480 represents the participants' usual diet before study, and at t1 and t2 was obtained. All  
481 participants adhered to Mediterranean diet as estimated by Mediterranean Diet Score  
482 <sup>57</sup>. One portion (30g) of a meat product with or without the microencapsulated OLWPE  
483 (containing 7mg Trolox equivalents of total polyphenols, that is the average amount  
484 that can be found in 20g of olive oil and corresponds to D2 of the animal study) was  
485 provided in each participant per day.

#### 486 **Data collection**

487 Different social - demographic data, such as date of birth, gender, citizenship, marital  
488 status, place of residence, profession and contact information were collected along  
489 with a number of Anthropometric measurements, including weight, height, waist and  
490 hip circumference and body mass index (BMI) was calculated. Additionally, blood  
491 pressure and pulse rate were monitored and several health habits, like alcohol  
492 consumption, smoking, individual's medical history and the use of any medication  
493 were recorded. All patients were followed, once a week with telephone interviews  
494 and a complete physical examination at the beginning and the end of the  
495 corresponding period of intervention. At the end of each intervention period a  
496 complete biochemical and hematology workup was performed.

497 ***Blood sample analysis***

498 *Metabolomic analysis*

499 The metabolomic analysis was performed according to published protocols by  
500 Beckonert et al.<sup>58</sup> and Dona et al.<sup>59</sup> Briefly, aliquots of human plasma (200 µl) were  
501 added in Eppendorf tubes to 400 µl of 0.9% saline solution, vortexed for 30 seconds,  
502 then centrifuged at 12,000g for 5 min at 4 °C and the sample (600 µl) was transferred  
503 into 5mm NMR glass tubes<sup>58</sup>.

504 A Carr-Purcell-Meiboom-Gill spin echo sequence with presaturation was used for  
505 acquiring <sup>1</sup>H NMR spectra and obtaining the low MW metabolite profile of plasma. A  
506 diffusion edited sequence (ledbpgppr2s1d) with bipolar gradients and LED scheme  
507 was used to suppress low MW compounds and obtain the <sup>1</sup>H NMR spectrum of  
508 lipoproteins.<sup>59</sup> Low MW metabolites were quantified in the CPMG NMR spectra using  
509 the ChenomX suite (ChenomX, SA). Both CPMG and diffusion-edited LED spectra were  
510 bucketed and the data were used directly for metabolomics analysis, which was  
511 performed using the Simca 13.02 software package by Umetrics.

512 Complete blood cell count, the levels of different biochemical and metabolic  
513 parameters (glucose, insulin, triglycerides, total cholesterol, LDL cholesterol, HDL  
514 cholesterol, CRP, urea, creatinine, γGT, SGOT and SGPT) and Total Antioxidant  
515 Capacity (TAC) were obtained as described above.

516 Immunophenotyping was performed as described above using the following anti-  
517 human antibodies from BD (BD Biosciences, San Jose, CA USA): anti-CD45  
518 PERCP(554878), anti CD3FITC (555332), anti-CD4 PE(555347), anti-CD8 PE (555635),  
519 anti-CD25 FITC (555432), anti-CD19 FITC (555412), anti-CD20 PE(555623), anti-  
520 CD16PE (555407), anti CD56 FITC (562794).

521 Oxidized Low Density Lipoprotein (OxLDL) was assayed using an ELISA kit (Cloud-Clone  
522 Corp. Houston, TX, USA), according to the manufacturer's instructions.

523

524 **Statistical analysis**

525 Statistical analysis was performed using SPSS, V21 (IBM Corporation, NY USA), Origin  
526 V8 (OriginLab Corporation, Northampton, USA) and Prism V6, (GraphPad Software, Inc  
527 La Jolla Inc).

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724

## 725 **AUTHOR CONTRIBUTIONS**

726 Conceived and designed the experiments: MK, EC, CL,  
727 Performed the experiments and analyzed the data: NK, VPA, EM, SK, EG, SK, MT, EM,  
728 NM, MN, EB, EGS and GN  
729 Participated in its design and coordination and helped to draft the manuscript: GN,  
730 EC, CL, AS, EGS  
731 Wrote the paper: MK, EC  
732 All authors read and approved the final manuscript.

733

## 734 **COMPETING FINANCIAL INTERESTS**

735 Authors would like to disclose that EC is stated as inventor in patent no.  
736 GR20030100295, 20030708 & WO2005003037. MK, CL, AS and EC are stated as  
737 inventors in patent GR1008734/2016, and patent application PCT/EP2015/077814.

738

## 739 **FIGURE LEGENDS**

740 **Figure 1. A.** A characteristic  $^1\text{H}$  NMR spectrum of OLWPE in MeOD solvent and  
741 magnetic field 500MHz that shows its major constituents. **B.** The concentration

742 (ppb:µg/L) of the different phenolic compounds detected in the plasma of rats by LC-  
743 ESC-MS/MS at different time points( 1, 3, 6, 12, 18 or 24 h) after administration of  
744 OLWPE by gavage. Abbreviations: GA: Gallic acid, EPCTCN: Epicatechin, p-11-BNZC: p-  
745 11-Benzoic acid, CA: Caffeic acid, HVMNLC: Homovanillic acid p-CMRC: p-Coumaric  
746 acid, FA: Ferulic acid, QRCTN: Quercetin **C.** Basic pharmacokinetic parameters of the  
747 phenolic compounds detected in OLWPE. Cmax: Maximum blood concentration,  
748 Tmax: Time required in order to achieve maximum blood concentration, AUC: Area  
749 under the curve “concentration vs time”,  $t_{1/2}$ : half-life, Ke: Elimination rate constant.  
750 Initial Dose: the amount of each phenolic compound in OLWPE administered. % in  
751 plasma/24h: the percentage of each compound in OLWPE found in blood within 24h  
752 (% AUC/initial dose). Abbreviations: GA: Gallic acid, EPCTCN: Epicatechin, p-11-BNZC:  
753 p-11-Benzoic acid, CA: Caffeic acid, HVMNLC: Homovanillic acid p-CMRC: p-Coumaric  
754 acid, FA: Ferulic acid, QRCTN: Quercetin **D.** The concentration (ppb:µg/L) of the  
755 different phenolic compounds detected in the plasma of rats by LC-ESC-MS/MS after  
756 a 16-week consumption of the 3 different doses (D1-D3) of microencapsulated OLWPE  
757 extract in their food. HF: High fat diet. The parallel line to the x-axis represents the  
758 detection limit of the method for each compound. **E.** Total plasma antioxidant capacity  
759 (TAC) of animals fed either with normal diet (control) or a high fat diet (HF) with or  
760 without three different doses (D1, D2, D3) of microencapsulated OLWPE. Data are  
761 represented as mean  $\pm$  SD.

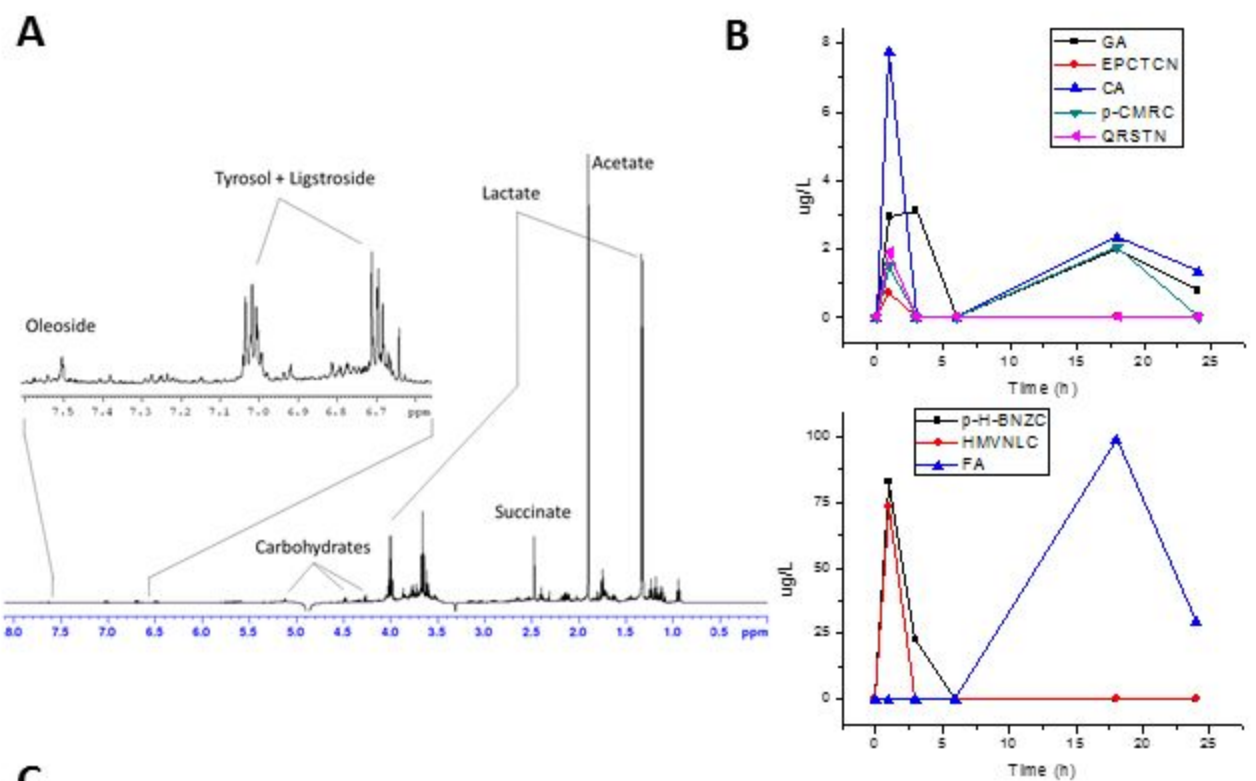
762 **Figure 2. A.** Changes in the concentration of the hepatic enzymes SGOT and SGPT in  
763 animals fed either with normal diet (control) or a high fat diet (HF) with or without  
764 three different doses (D1, D2, D3) of microencapsulated OLWPE for 16 weeks. Data are  
765 represented as mean  $\pm$  SD. **B.** Representative microphotographs of hematoxylin-eosin  
766 stained liver sections of animals from the different study groups. Blue arrows indicate  
767 fat infiltration of the liver. **C.** Changes in the concentration of the urea and creatinine  
768 in animals fed either with normal diet (control) or a high fat diet (HF) with or without  
769 three different doses (D1, D2, D3) of microencapsulated OLWPE for 16 weeks. Data  
770 are represented as mean $\pm$ SD. **D.** Representative microphotographs of hematoxylin-

771 eosin stained kidneys sections of animals from the different study groups.  
772 Bars=100 $\mu$ M.

773 **Figure 3. A.** Changes in the weight of the rats fed either with normal diet (control) or  
774 high fat diet (HF) with or without three different doses (D1, D2, D3) of  
775 microencapsulated OLWPE within the period of 16 weeks. **B and C.** Changes of  
776 different metabolic parameters with time, in animals fed with high fat diet (HF)  
777 compared to normal diet (control). Data are represented as mean  $\pm$  SD.

778 **Figure 4. A.** Changes in the lipidemic profile of animals fed either with a high fat diet  
779 (HF) with or without three different doses (D1, D2, D3) of microencapsulated OLWPE  
780 for 16 weeks. Values of the animals fed with a normal diet (control) are also presented.  
781 **B.** Changes of the HDL to LDL ratio at the different study groups. **C. and D.** Changes in  
782 glucose, insulin and leptin in animals fed either with a high fat diet (HF) with or without  
783 three different doses (D1, D2, D3) of microencapsulated OLWPE for 16 weeks. **E.**  
784 Immunophenotype: Changes in the different lymphocyte populations in animals fed  
785 either with a high fat diet (HF) with or without three different doses (D1, D2, D3) of  
786 microencapsulated OLWPE for 16 weeks. Values of the animals fed with a normal diet  
787 (control) are also presented. Data are represented as mean  $\pm$  SD.

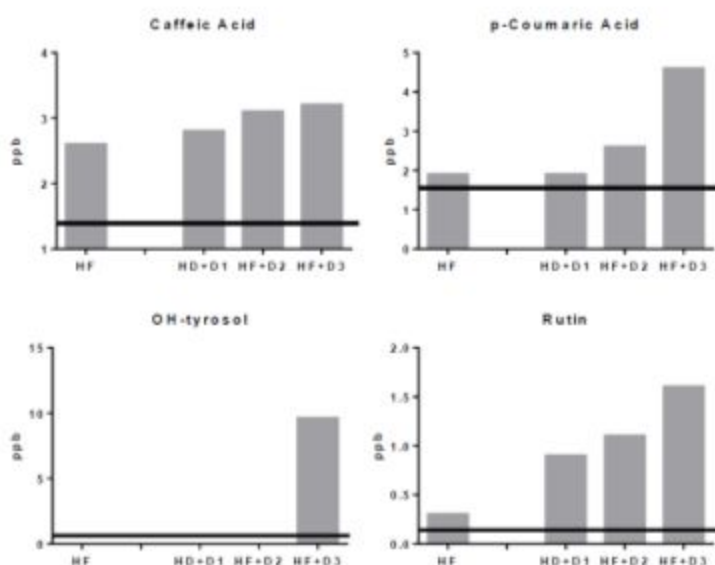
788 **Figure 5. A.** OPLS-DA models of  $^1\text{H}$  NMR spectroscopy obtained serum lipid and water  
789 soluble metabolite profiles of individuals (n=17) after consumption of a plain meat  
790 product (green) and one supplemented with OLWPE microencapsulated polyphenols  
791 (blue). **B.** The lipidemic and glycemic profile of individuals with at least two  
792 biochemical or anthropometric elements of cardio-metabolic risk (n=18) when  
793 consumed the meat product with the microencapsulated extract (OLWPE+) compared  
794 to their profile when the meat product was without the microencapsulated extract  
795 (OLWPE-). **C.** Immunophenotype: Changes in the different lymphocyte populations of  
796 individuals when consumed the meat product with the microencapsulated extract  
797 (OLWPE+) compared to their immunophenotype when the meat product was without  
798 the microencapsulated extract (OLWPE-). Individuals with BMI>26 (over  
799 weighted/obese) are presented as a separate group from the ones with BMI<26  
800 (Lean). Data are represented as mean  $\pm$  SD.



**C**

Parameter	Unit	GA	EPCTCN	p-H-BNZC	CA	HMVNLC	p-CMRC	FA	QRSTN
Cmax	µg/L	3.12	0.72	77.52	7.74	72.94	2.05	98.67	1.92
Tmax	Hours	3.00	1.00	1.00	1.00	1.00	18.00	18.00	1.00
ElimRateConst (Ke)	h-1	0.27	0.31	0.46	0.48	0.64	0.36	0.20	0.38
HalfLife	hours	2.57	2.27	1.52	1.46	1.09	1.94	3.40	1.85
AUC_0-24	µg/L//24h	32.76	1.31	173.10	36.83	109.64	20.84	975.27	3.11
Initial Dose	µg	51.30	86.20	12608.80	1799.30	150453.10	9846.10	97.90	3.80
% in plasma/24h		63.87	1.51	1.37	2.05	0.07	0.21	996.19	81.71

**D**



**E**

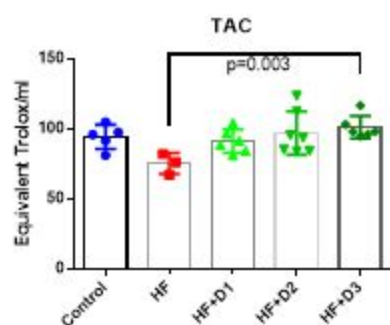


Figure 1

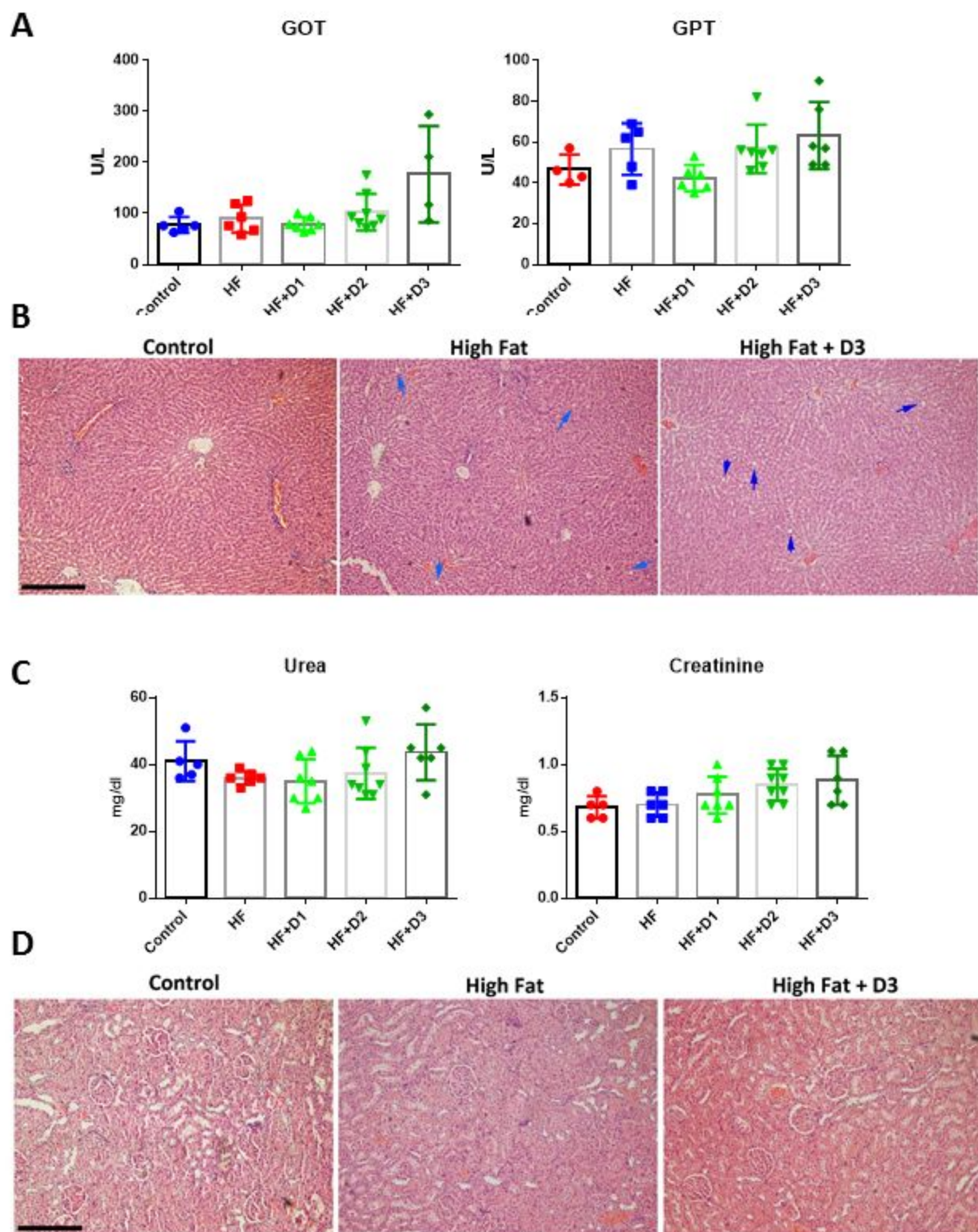


Figure 2

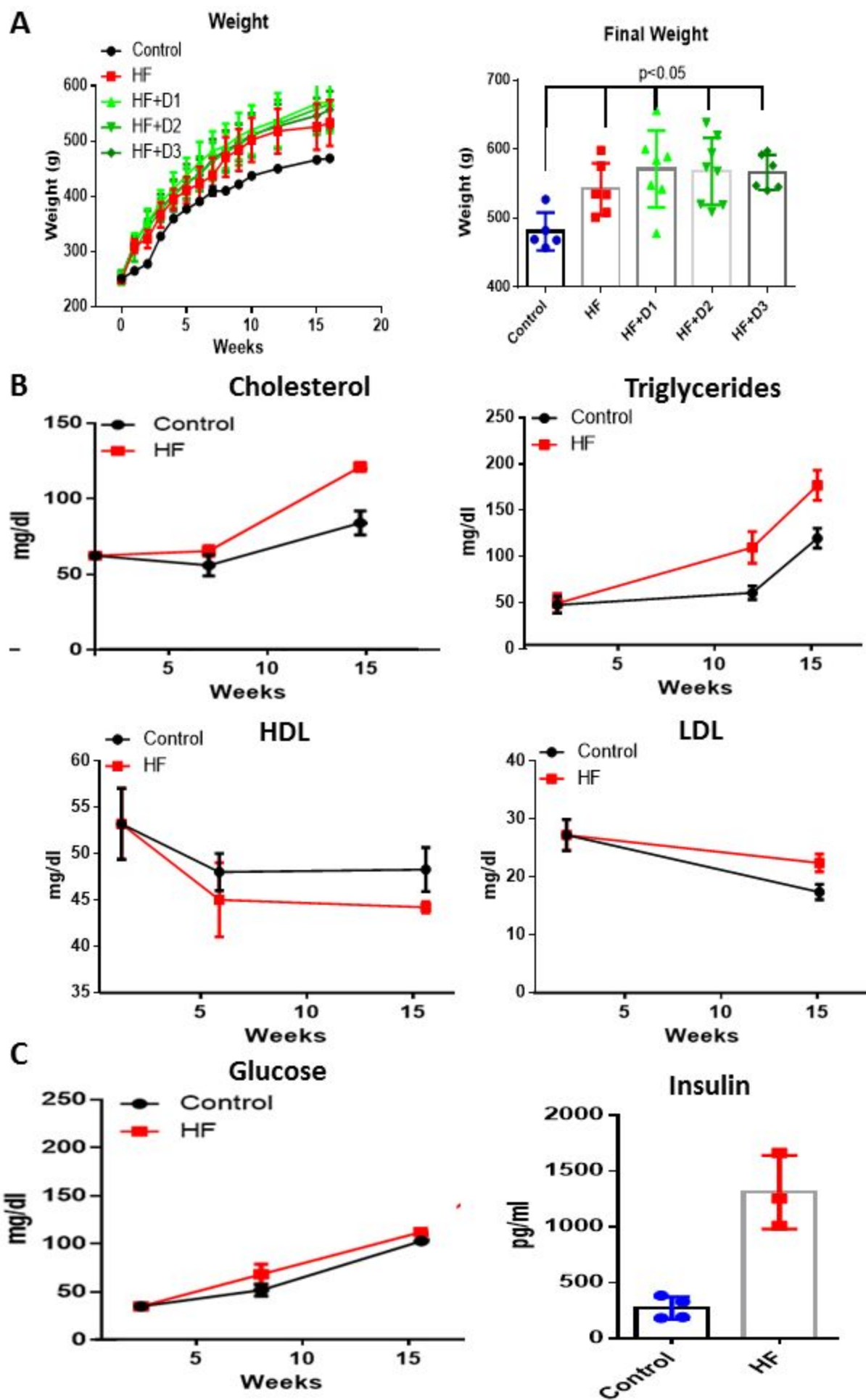


Figure 3

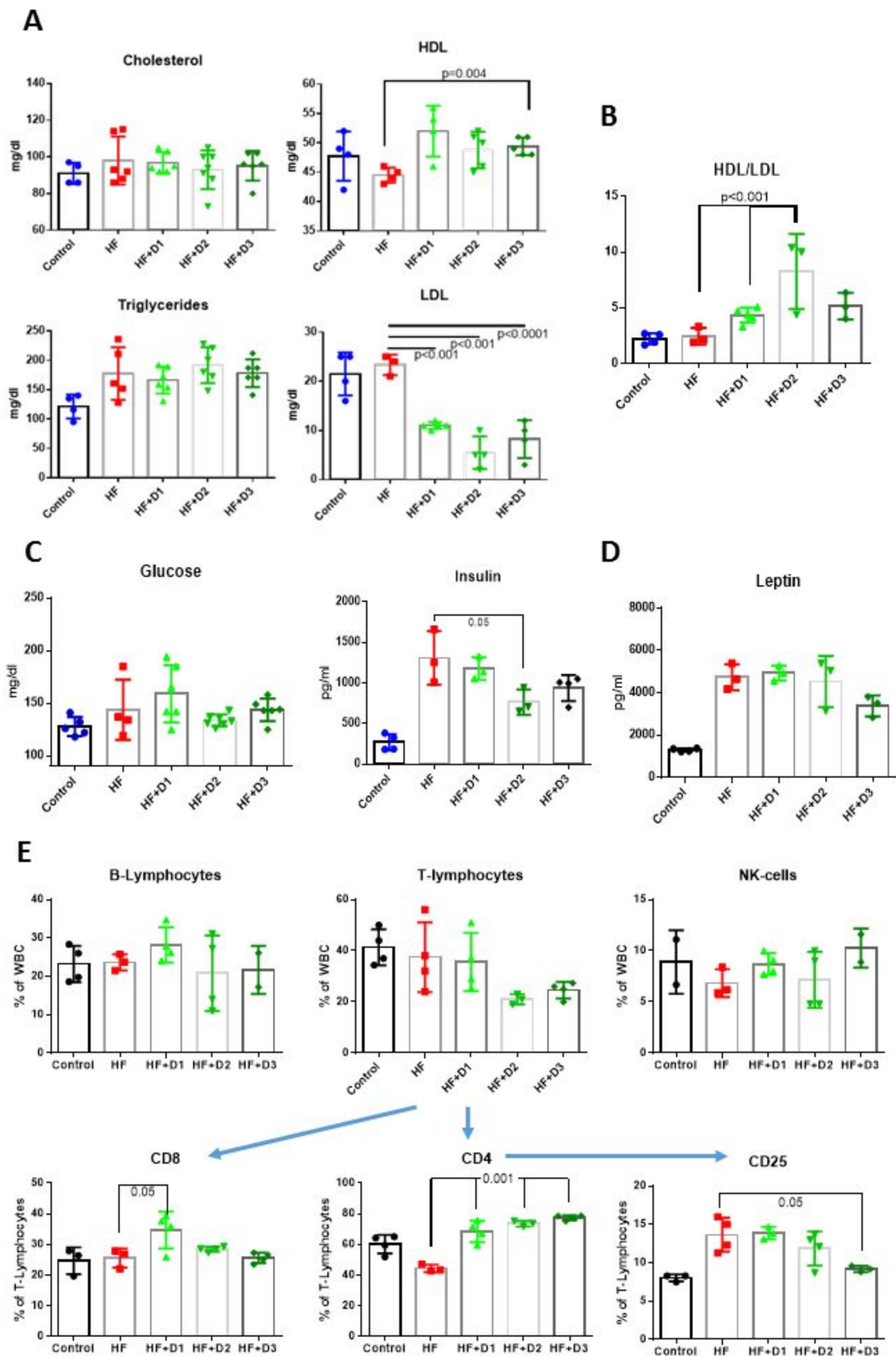


Figure 4



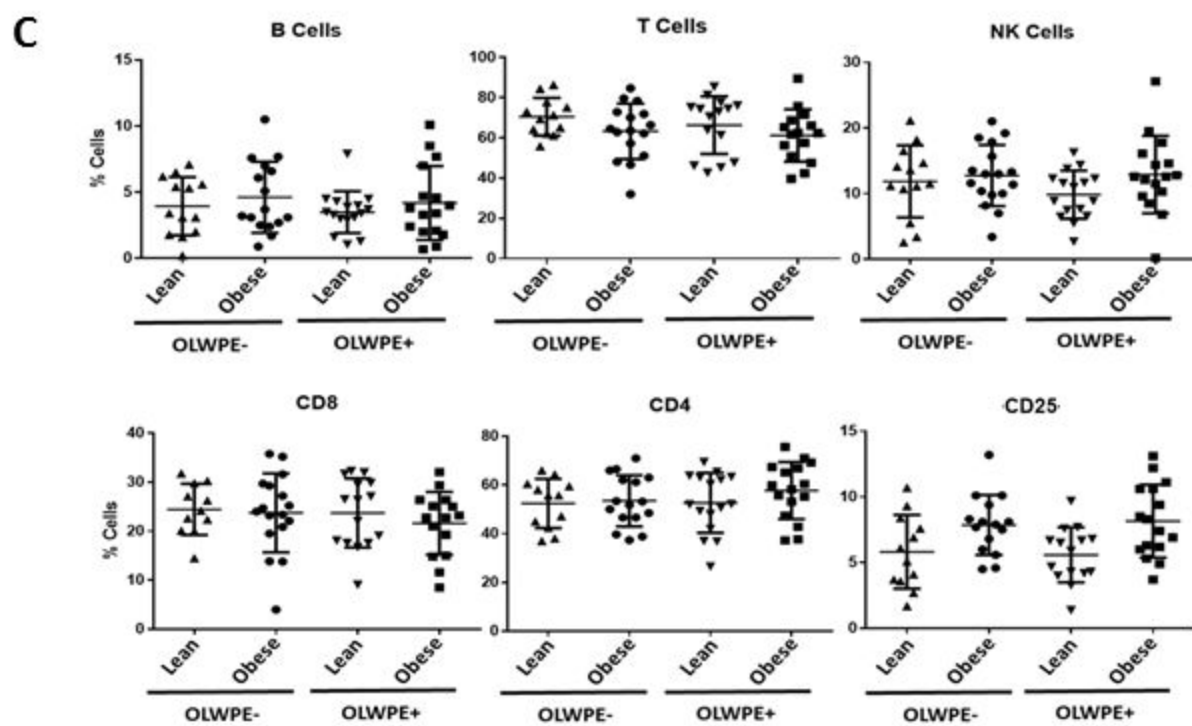
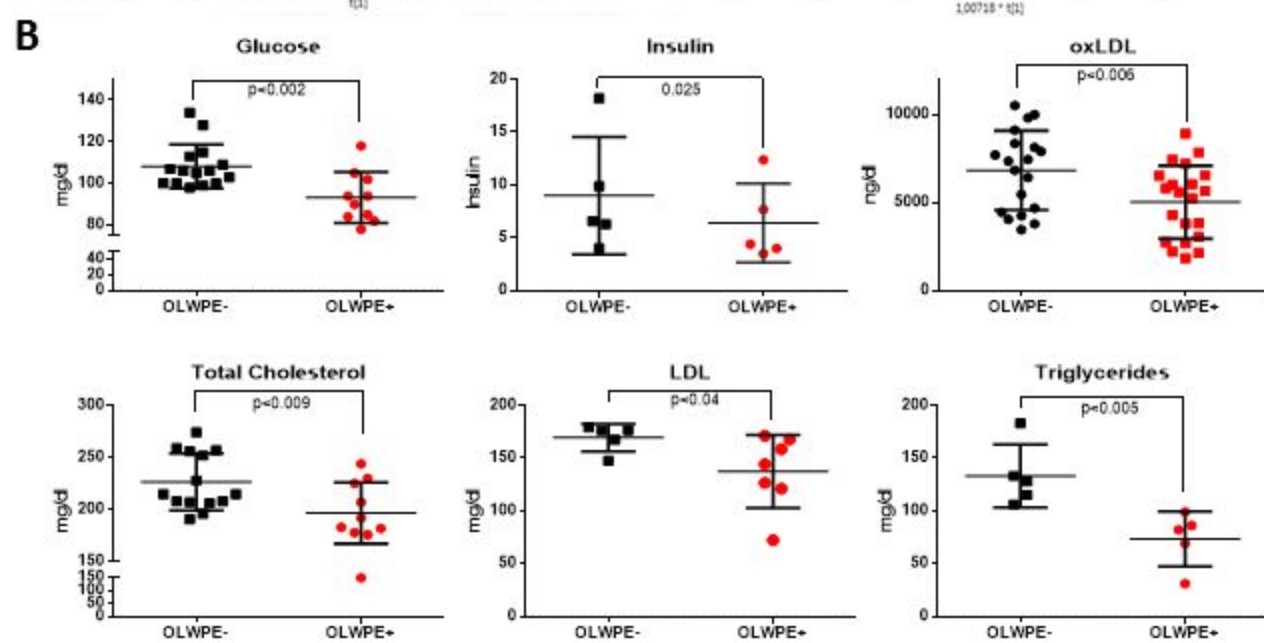
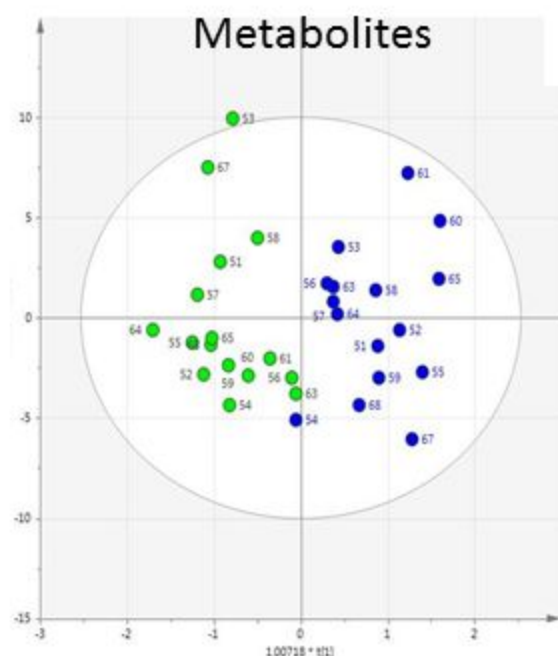
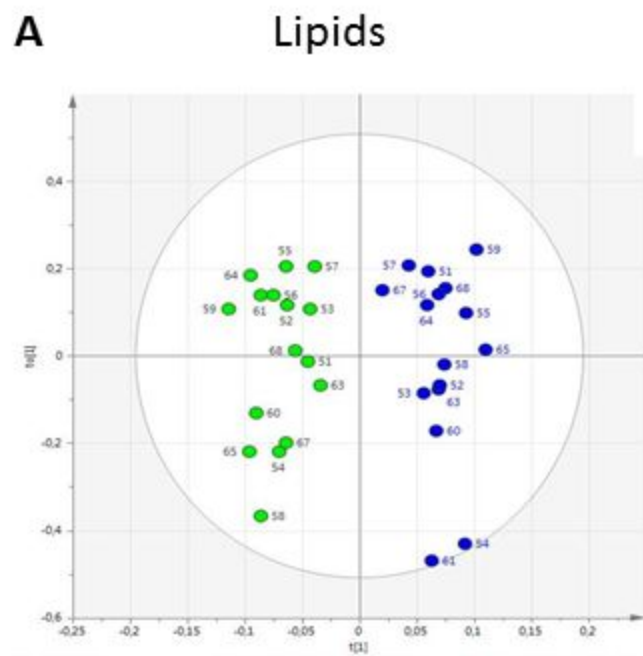


Figure 5