

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

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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.

44

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) ¹. 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 ² together with increased blood pressure, that characterize a pro-
53 inflammatory state ^{3,4}, leading to an increased likelihood of insulin resistance/type 2
54 diabetes, atherosclerosis/cardiovascular disease ⁵. This, together with a resulting pre-
55 thrombotic state ⁶ 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, β 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” ⁷

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 ^{8,9}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 ¹⁰⁻¹² 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 OLVPE**

88 The phenolic content of concentrated OLVPE 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 **OLVPE 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 OLVPE 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 α (data not
165 shown).

166 **OLWPE decreases the levels of CD4⁺CD25⁺ 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⁺ cells population was increased in an

172 OLWPE dose dependent manner, while the CD4⁺CD25⁺ 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 glycemetic 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^{13,14} A variable amount of ingested polyphenols
238 can be found in blood¹⁵, and can interfere with major cellular processes having a
239 beneficial impact on cancer reviewed in¹³, vascular function^{16,17}, and metabolism^{18,19}.

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 oil²⁰. 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 disease²¹, diabetes^{11,22,23}
249 and hyperlipidemia¹⁰. 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 dysfunction¹². 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^{24,25}. 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 ²⁶,
265 may induce pro-oxidant activities, when administered isolated in vitro or in vivo ^{27,28}.

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⁺CD25⁺ 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 ²⁹. 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⁺ and CD4⁺CD25⁺ T regulatory cells that have been reduced and
289 elevated respectively by high fat diet, back to normal levels. CD4⁺CD25⁺ 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 ³⁰⁻³² and others, including our present findings, an increased

293 number of peripheral blood Tregs³³. 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^{34,35} 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 absorption³⁶. 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 described³⁷.

311 Obesity in humans is considered an emerging health problem³⁸ 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 cancer³⁹, aging⁴⁰, cardiovascular disease⁴¹
315 and a number of other pathologies⁴². 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 disease⁴³. 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 ⁴⁴⁻⁴⁹.
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 ⁵⁰, for a recent publication. Finally, eventhough overweighted/obese individuals
336 exhibited an increase in peripheral blood CD4⁺CD25⁺ 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 ⁵¹⁻⁵⁴. 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 method⁵⁵ that has been modified in order to use small volumes.
357 Briefly, 20 μl sample was mixed with 80 μl of distilled water, 400 μl Na_2CO_3 (10%
358 Na_2CO_3 in 0.85 N NaOH), and 500 μl 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 μl of the
366 sample with 400 μl 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. ^1H 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 β -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 μ 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 (Cmax), ii) time required to achieve maximum
403 concentration (Tmax), iii) area under the curve (AUC), iv) half-life ($t_{1/2}$), and v)
404 elimination rate constant (Ke), according to⁵⁶ 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 ²⁰.

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 ⁴⁶.

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 ⁵⁷. 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.⁵⁸ and Dona et al.⁵⁹ 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 tubes⁵⁸.

504 A Carr-Purcell-Meiboom-Gill spin echo sequence with presaturation was used for
505 acquiring ¹H 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 ¹H NMR spectrum of
508 lipoproteins.⁵⁹ 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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718

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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 ^1H 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.

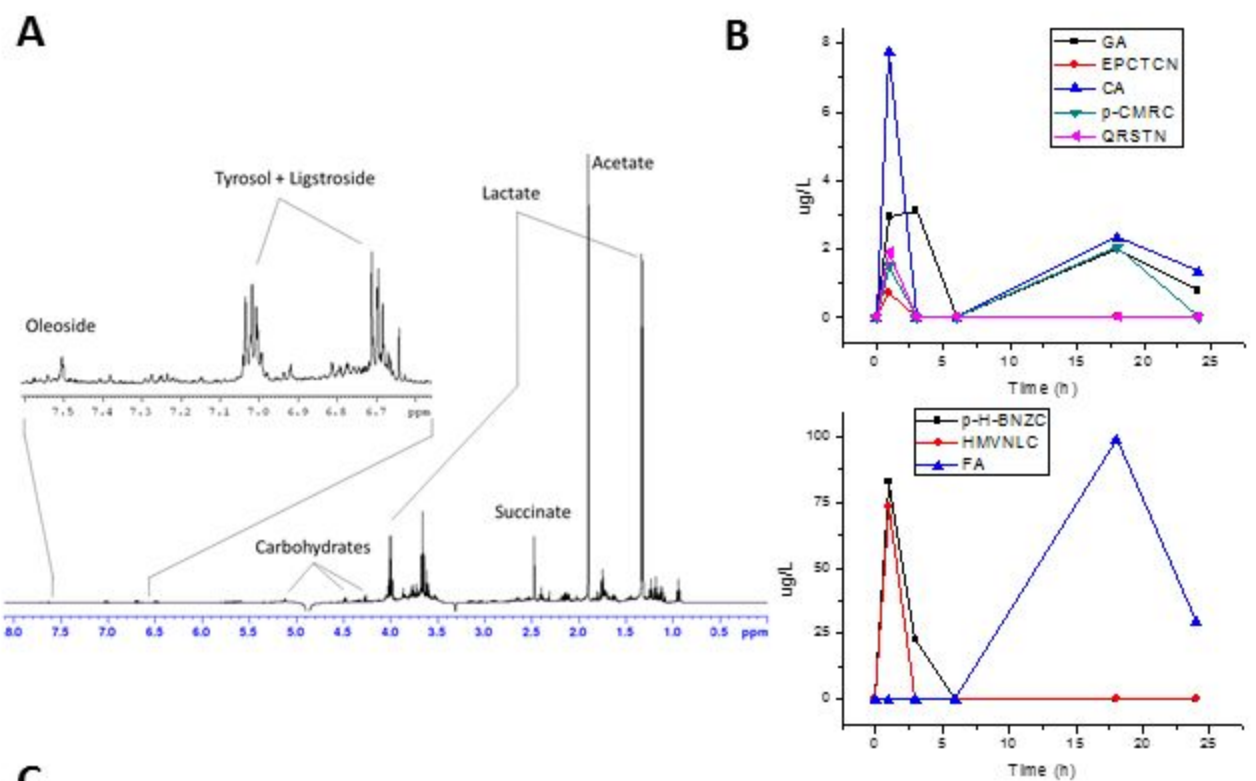
762 **Figure 2. A.** Changes in the concentration of the hepatic enzymes SGOT and SGPT in
763 animals fed either with normal diet (control) or a high fat diet (HF) with or without
764 three different doses (D1, D2, D3) of microencapsulated OLWPE for 16 weeks. Data are
765 represented as mean \pm SD. **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 μ 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.

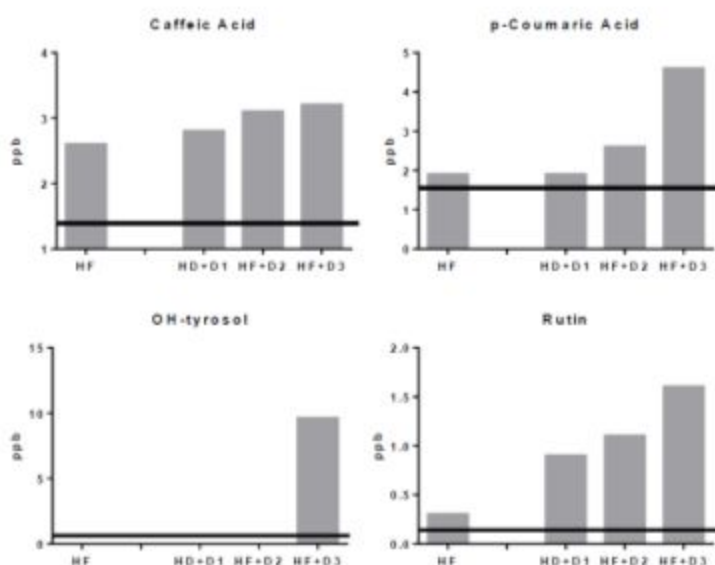
788 **Figure 5. A.** OPLS-DA models of ^1H NMR spectroscopy obtained serum lipid and water
789 soluble metabolite profiles of individuals (n=17) after consumption of a plain meat
790 product (green) and one supplemented with OLWPE microencapsulated polyphenols
791 (blue). **B.** The lipidemic and glycemic 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

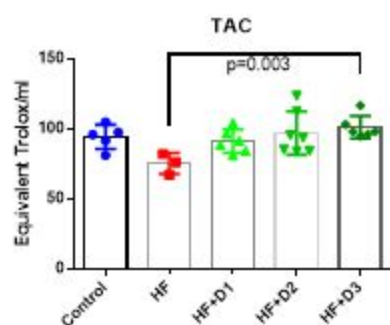


Figure 1

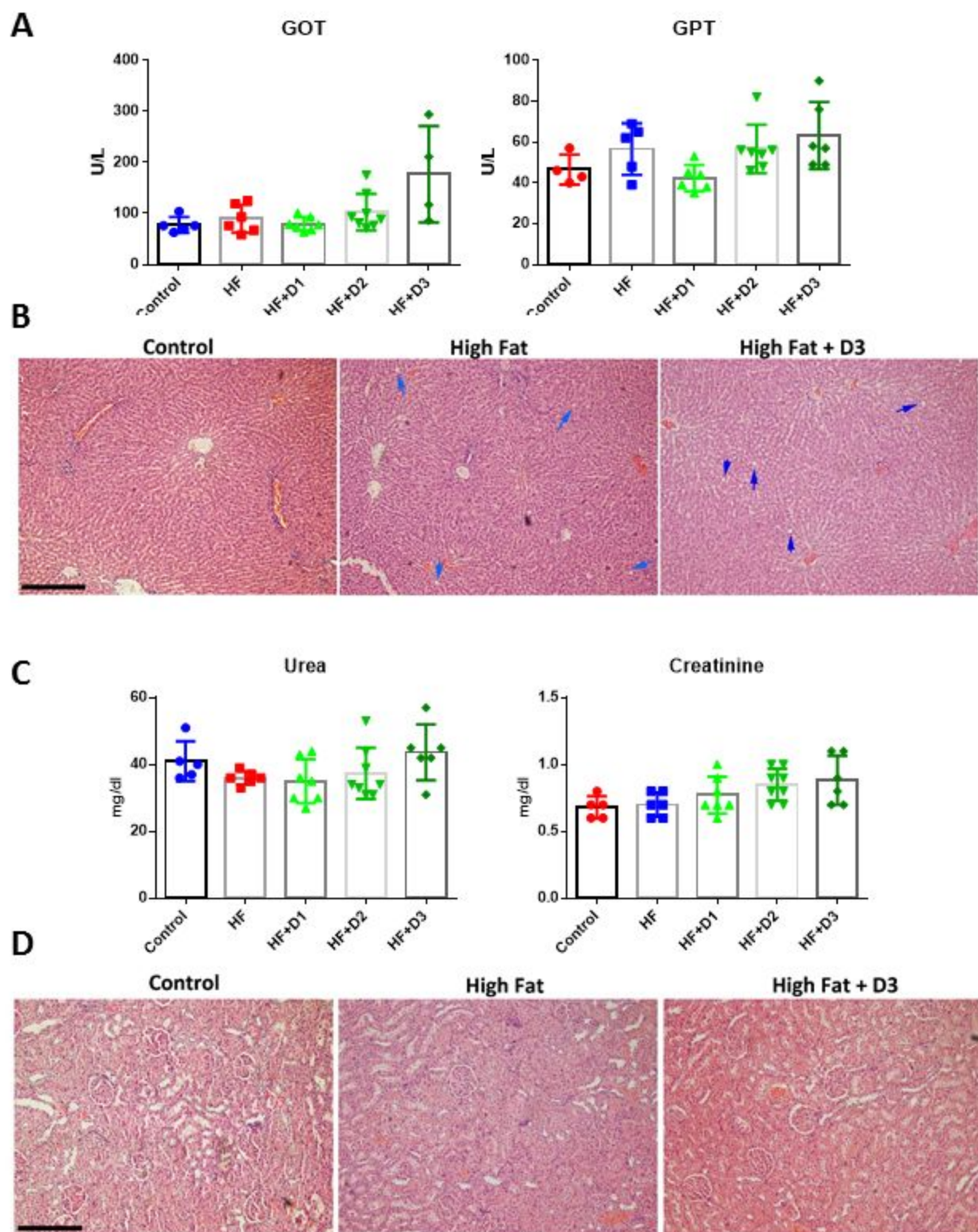


Figure 2

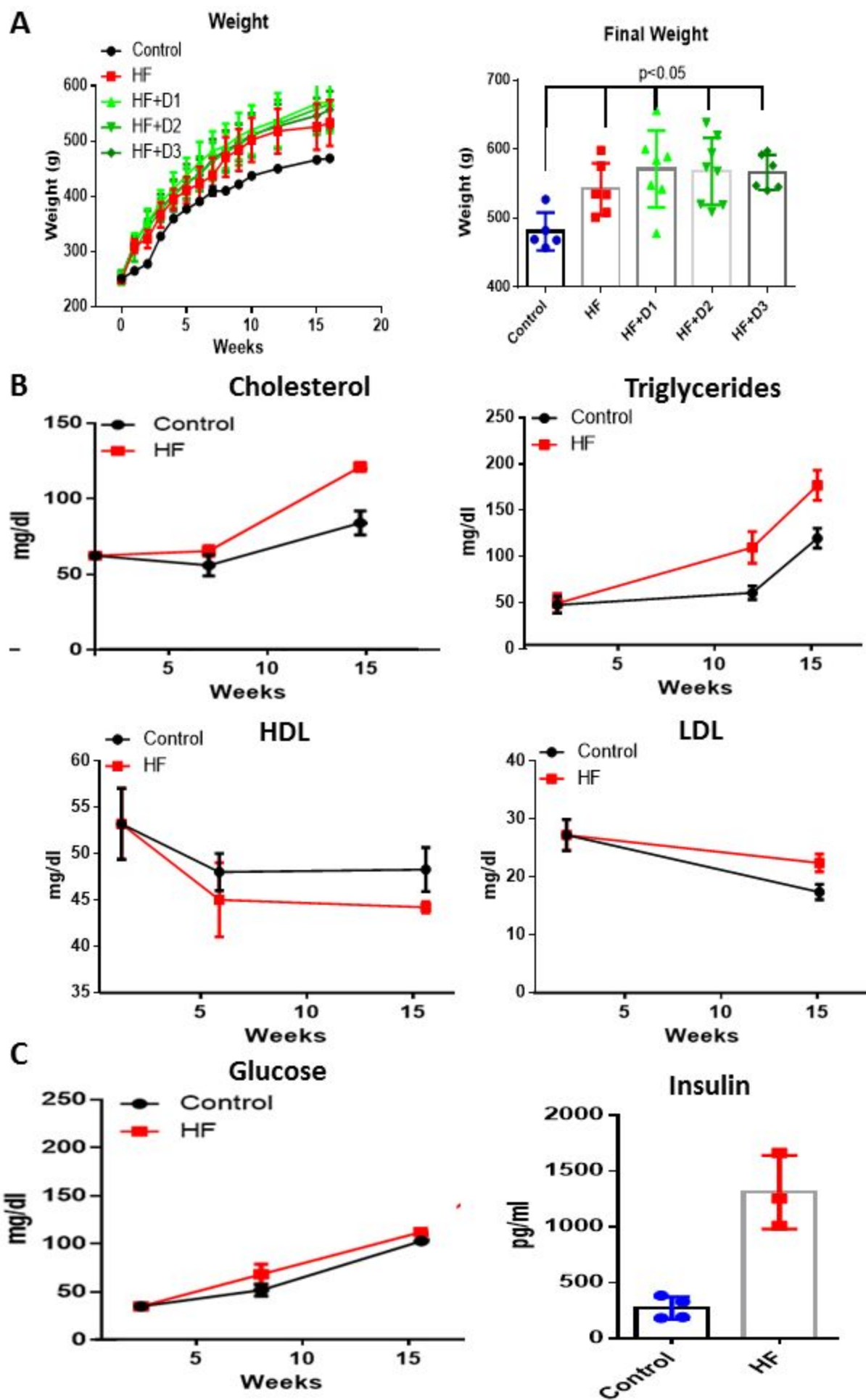


Figure 3

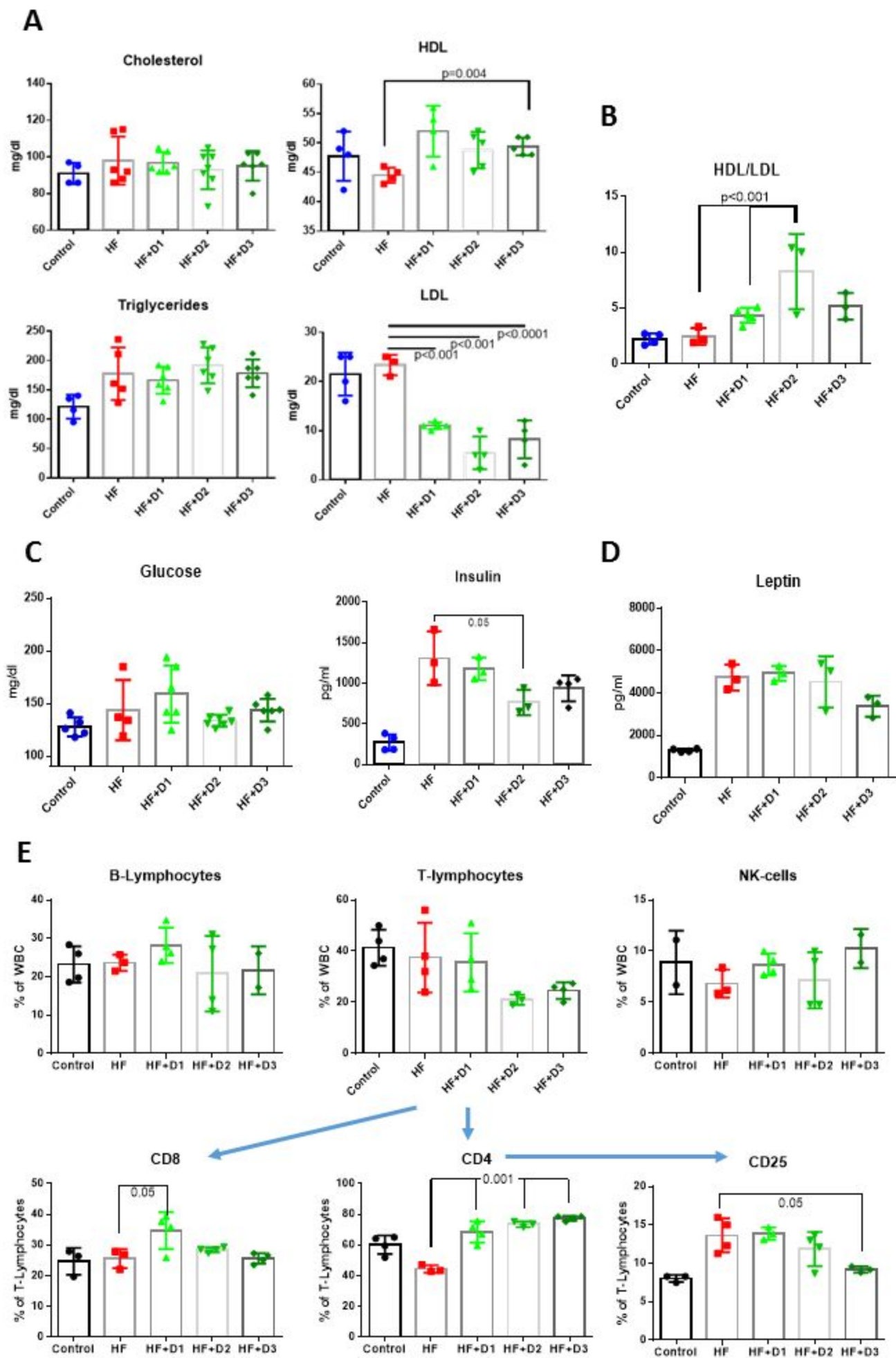


Figure 4

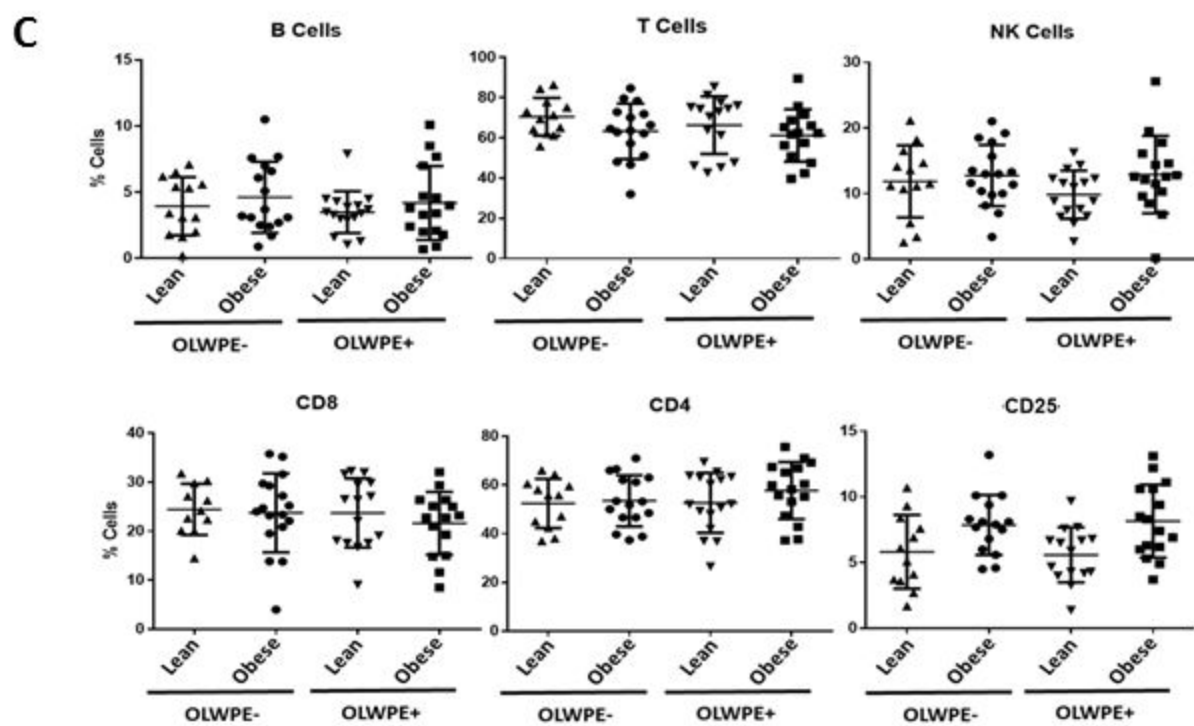
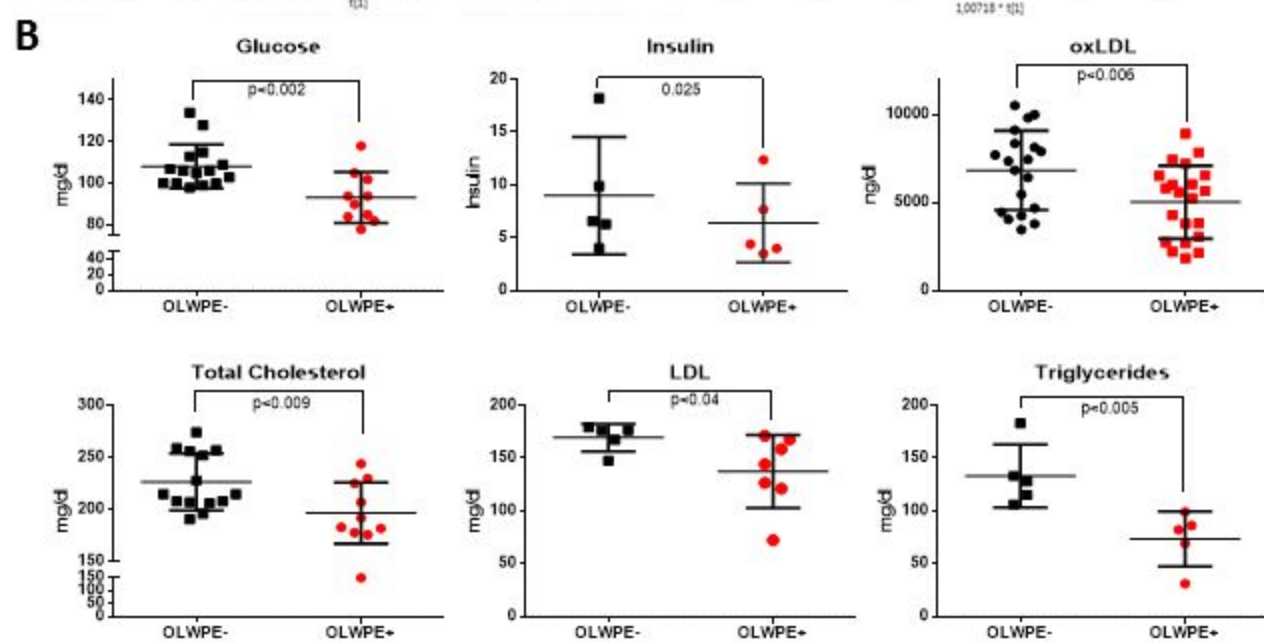
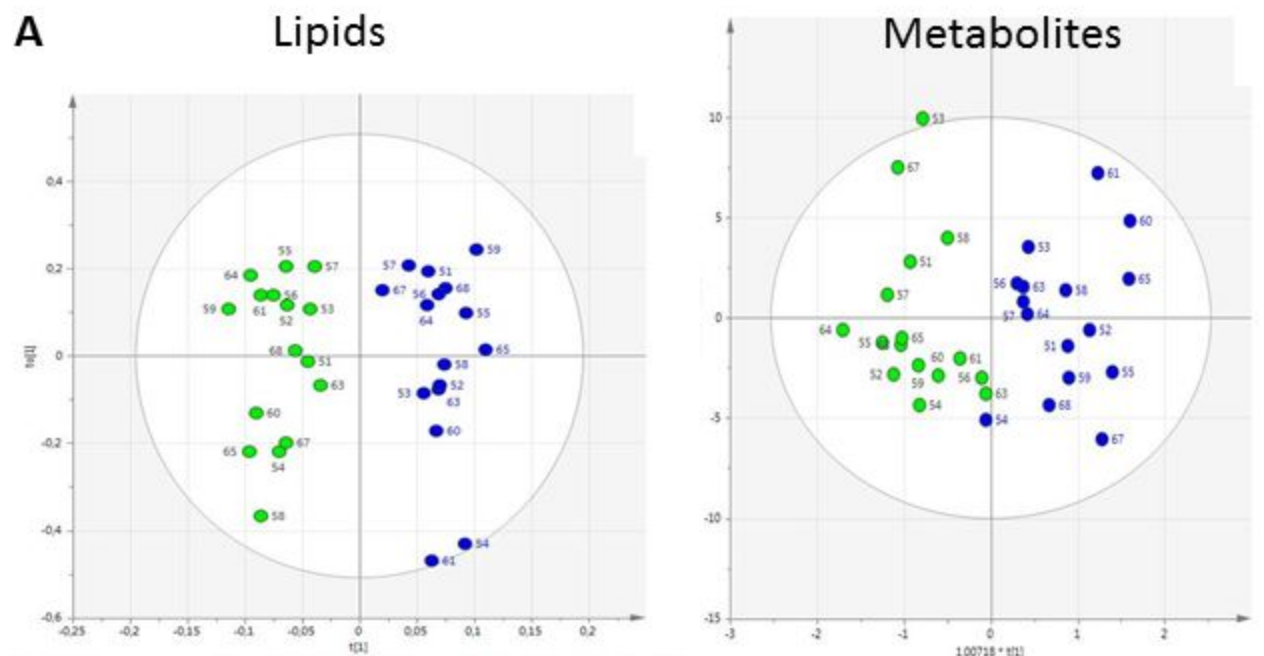


Figure 5