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

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ABSTRACT

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

INTRODUCTION

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

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

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

RESULTS

Polyphenolic characterization of OLWPE

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

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

Animal study

OLWPE bioavailability and absence of toxicity in an animal model

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

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

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

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

Effect of diets on rat weight

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

OLWPE significantly lowers circulating lipids and insulin levels

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

OLWPE decreases the levels of $CD4^+CD25^+$ T cells

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

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

Human study

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

NMR-based plasma metabolomics

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

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

OLWPE decreases glucose and lipid levels

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

METHODS

Isolation and characterization of OLWPE

Olive water total polyphenolic extract (OLWPE) was obtained by using olive mill waste water, immediately collected during olive oil production through centrifugation, passing through a multilevel ion-exchange proprietary resin filter (patent no. GR20030100295 20030708 & WO2005003037) and elution with ethanol (75%); ethanol was subsequently fully evaporated and the water extract was concentrated through a rotor evaporator. The total content of polyphenols was estimated by the Folin-Ciocalteu method [55] that has been modified in order to use small volumes. Briefly, 20 µl sample was mixed with 80 µl of distilled water, 400 µl Na_2CO_3 (10% Na_2CO_3 in 0.85 N NaOH), and 500 µl Folin-Ciocalteu reagent (10%). The mixture was allowed to stand for 1 hour in the dark and absorbance was measured at 750 nm. The total phenolic profile was expressed as Trolox (a water-soluble analog of Vitamin E) equivalents.

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

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

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

Plasma samples polyphenolic content analysis

Plasma samples were enzymatically hydrolyzed with β-glucuronidase/sulfatase from Helix pomatia (≥100,000 U/mL) at 37 ^{o}C for 45 min before polyphenol extraction; analysis was performed by SPE and LC–ESI-MS/MS respectively, as described above.

Extract microencapsulation

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

Animal Study

Short-term study

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

Long-term study

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

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

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

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

Blood sample analysis

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

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

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

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

Total Antioxidant Capacity (TAC): TAC was measured colorimetrically using the crocin bleaching assay as described in [46].

Histology

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

Human study

Participants and study design

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

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

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

Diet

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

Data collection

Different social - demographic data, such as date of birth, gender, citizenship, marital status, place of residence, profession and contact information were collected along with a number of Anthropometric measurements, including weight, height, waist and hip circumference and body mass index (BMI) was calculated. Additionally, blood pressure and pulse rate were monitored and several health habits, like alcohol consumption, smoking, individual's medical history and the use of any medication were recorded. All patients were followed, once a week with telephone interviews and a complete physical examination at the beginning and the end of the corresponding period of intervention. At the end of each intervention period a 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 tubes [58].

504 A Carr-Purcell-Meiboom-Gill spin echo sequence with presaturation was used for
505 acquiring ^{1}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 ^{1}H 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

Statistical analysis

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

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MK, EC, CL,

Performed the experiments and analyzed the data: NK, VPA, EM, SK, EG, SK, MT, EM, NM, MN, EB, EGS and GN

Participated in its design and coordination and helped to draft the manuscript: GN, EC, CL, AS, EGS

Wrote the paper: MK, EC

All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS

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

FIGURE LEGENDS

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

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

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

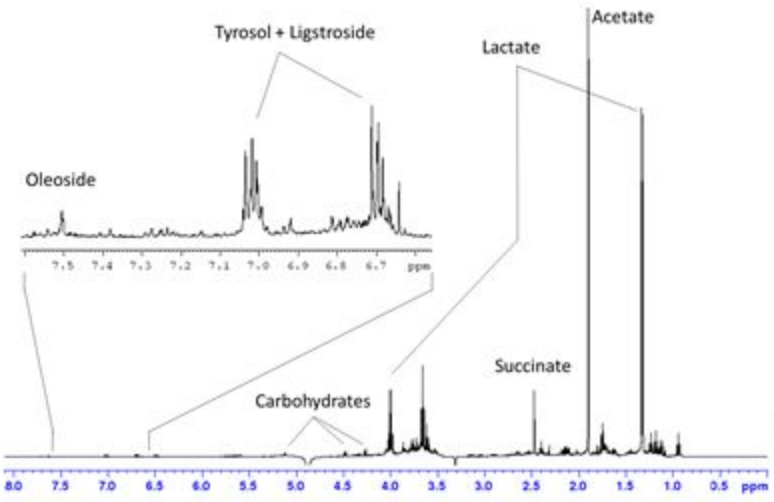
771 eosin stained kidneys sections of animals from the different study groups.
772 Bars=100μM.

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 ± 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 ± SD.

788 **Figure 5. A.** OPLS-DA models of ^{1}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 ± SD.

A



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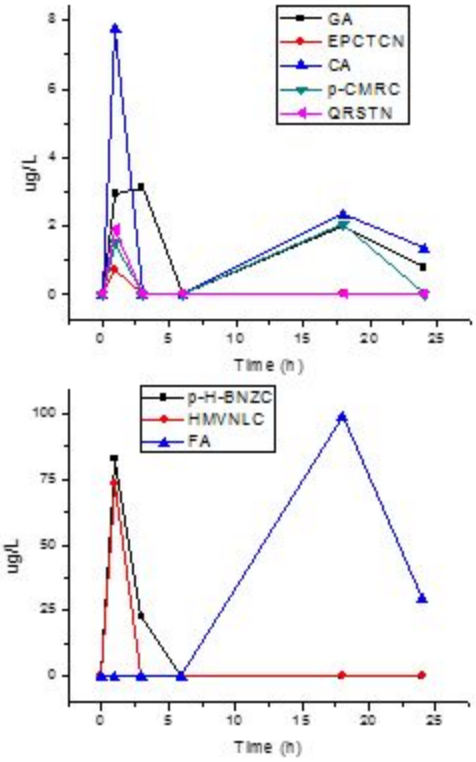
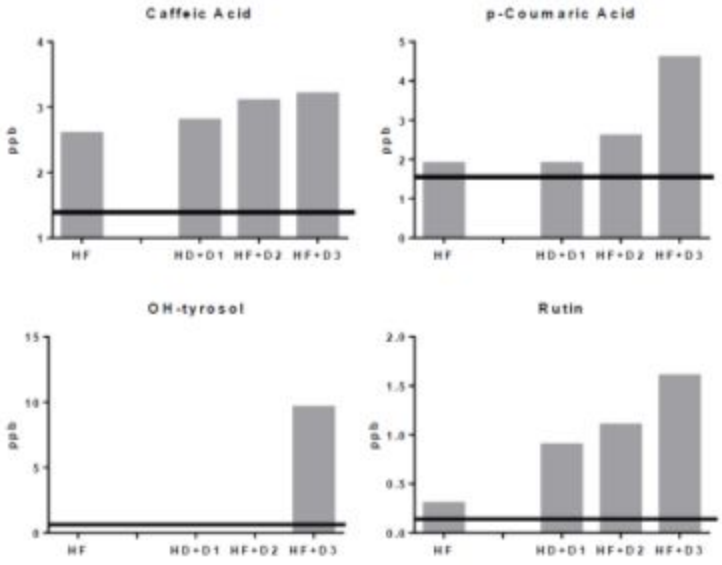


C

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



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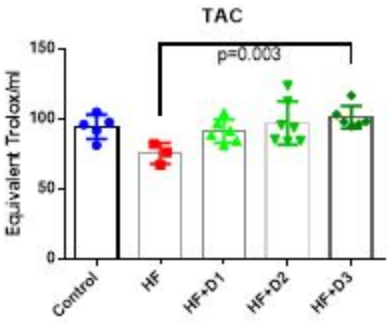

Figure 1

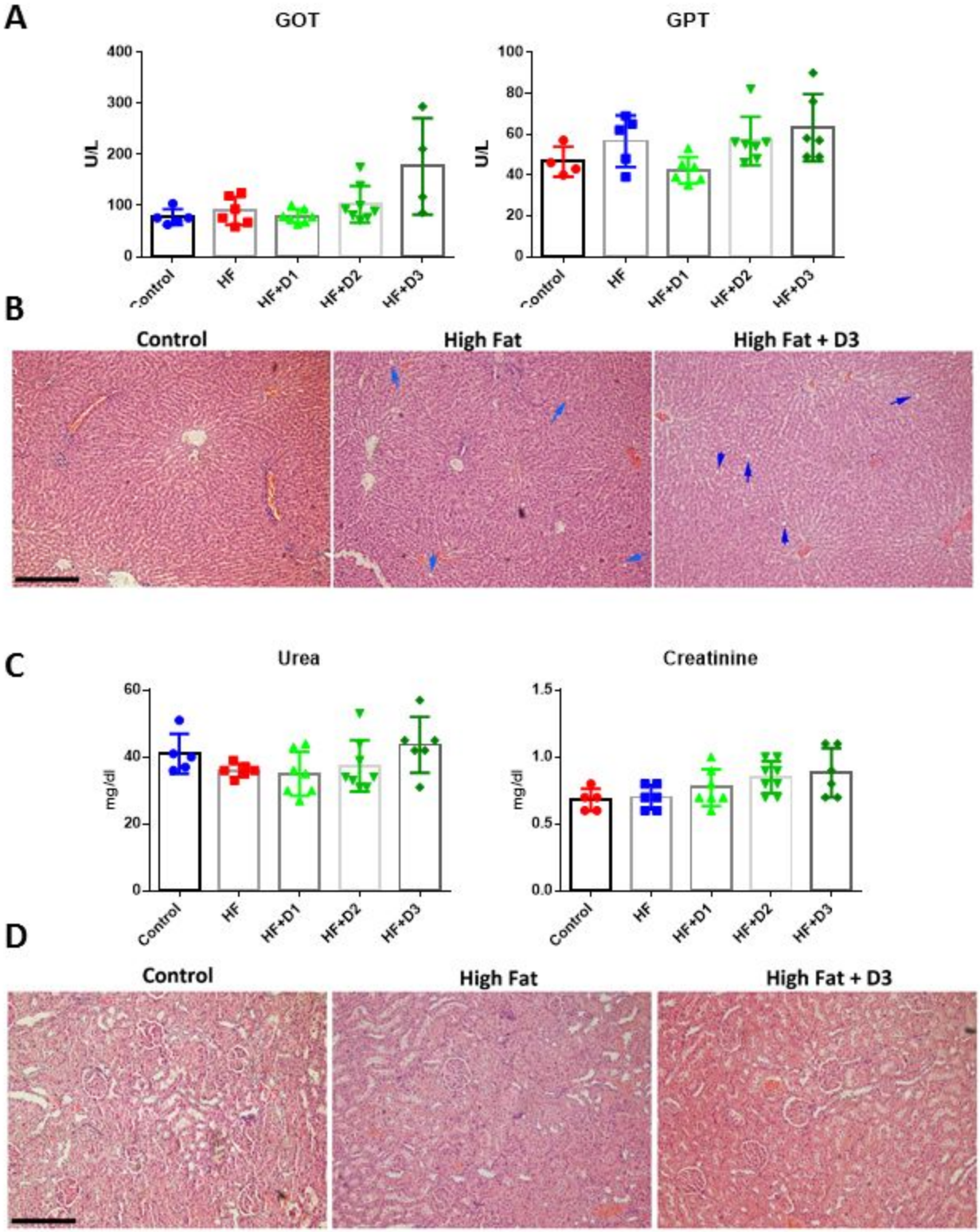
A
GOT
GPT
U/L
400
300
200
100
0
100
80
60
40
20
0
Control
HF
HF+D1
HF+D2
HF+D3
B
Control
High Fat
High Fat + D3
C
Urea
Creatinine
mg/dl
60
40
20
0
1.5
1.0
0.5
0.0
D
Control
High Fat
High Fat + D3

Figure 2

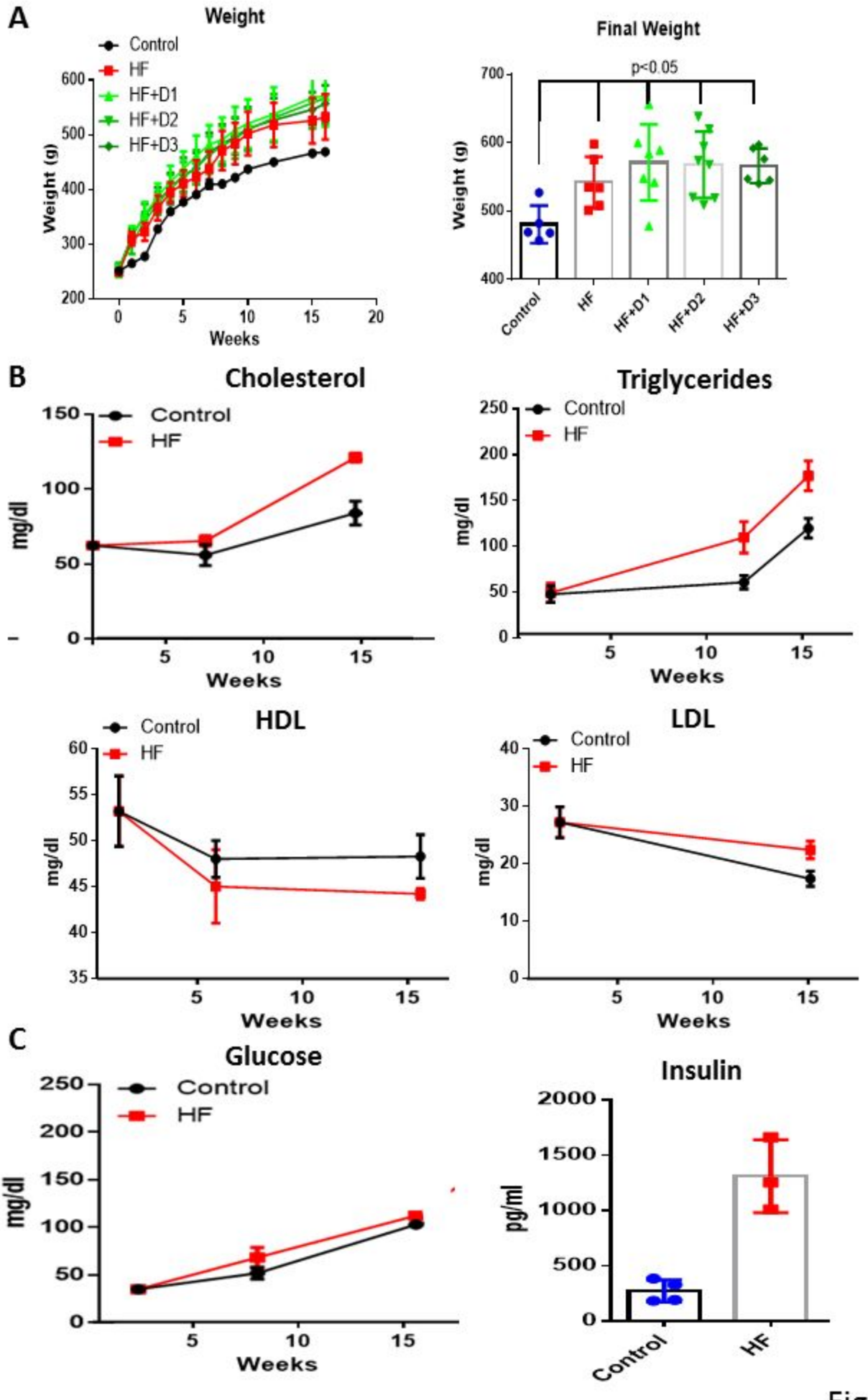
A
Weight
Control
HF
HF+D1
HF+D2
HF+D3
Weight (g)
Weeks
Final Weight
p<0.05
Weight (g)
Control
HF
HF+D1
HF+D2
HF+D3
B
Cholesterol
Control
HF
mg/dl
Weeks
Triglycerides
Control
HF
mg/dl
Weeks
HDL
Control
HF
mg/dl
Weeks
LDL
Control
HF
mg/dl
Weeks
C
Glucose
Control
HF
mg/dl
Weeks
Insulin
pg/ml
Control
HF

Figure 3

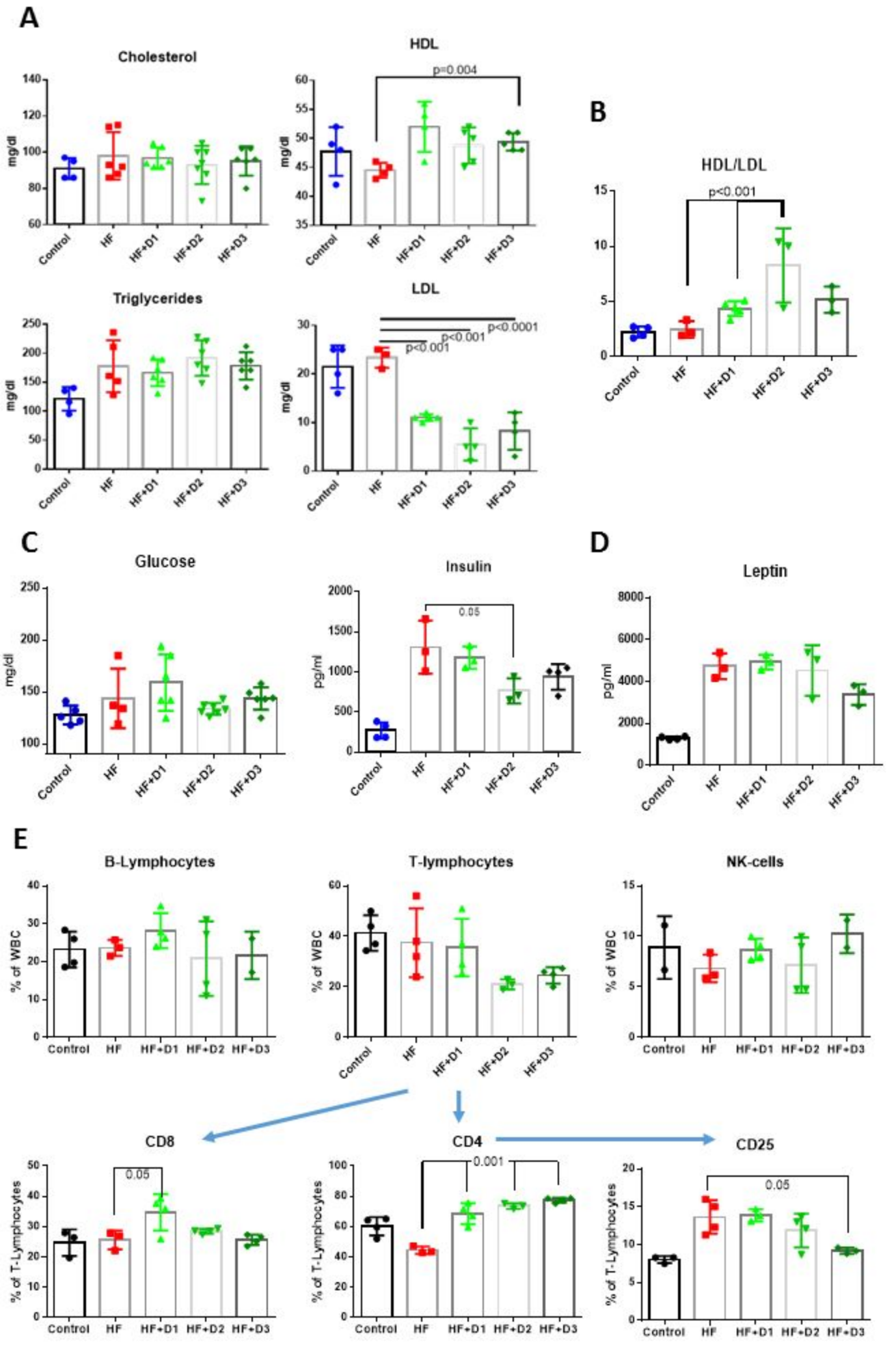
A
Cholesterol
mg/dl
HDL
p=0.004
Triglycerides
LDL
p<0.0001
p<0.001
p<0.001
B
HDL/LDL
p<0.001
C
Glucose
Insulin
0.05
D
Leptin
pg/ml
E
B-Lymphocytes
T-lymphocytes
NK-cells
% of WBC
CD8
CD4
CD25
0.05
0.001
0.05
% of T-Lymphocytes
Control
HF
HF+D1
HF+D2
HF+D3

Figure 4

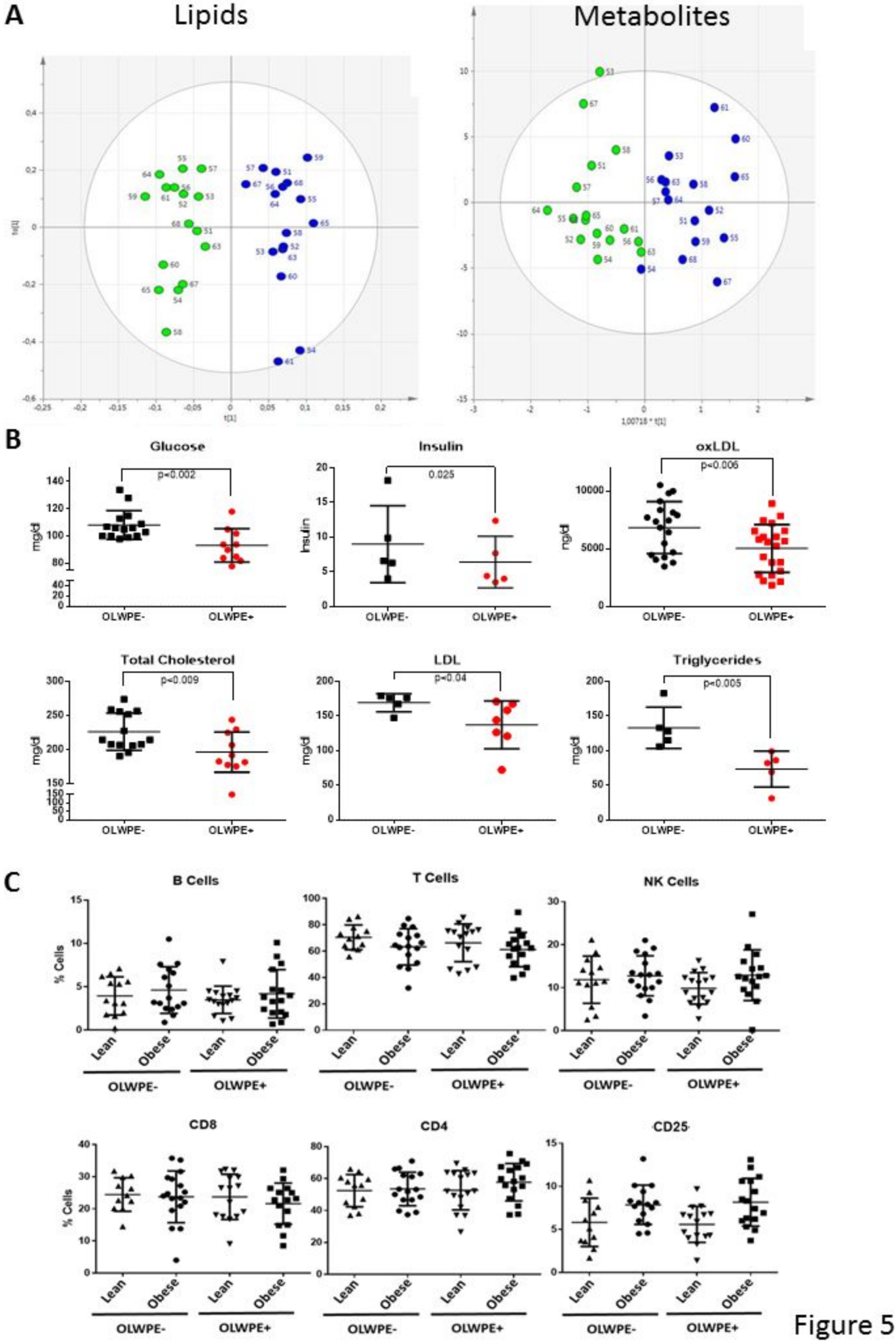
A
Lipids
Metabolites
B
Glucose
p=0.002
mg/d
OLWPE-
OLWPE+
Insulin
0.025
Insulin
oxLDL
p=0.006
ng/d
Total Cholesterol
p=0.009
LDL
p=0.04
Triglycerides
p=0.005
C
B Cells
T Cells
NK Cells
% Cells
Lean
Obese
CD8
CD4
CD25

Figure 5