RESEARCH ARTICLE

Alperujo extract, hydroxytyrosol, and 3,4-dihydroxyphenylglycol are bioavailable and have antioxidant properties in vitamin E-deficient rats—a proteomics and network analysis approach

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Scope: Olive products are rich in phenolic compounds, which are natural antioxidants in vitro. We tested the in vivo effects of alperujo, an olive production by-product, as well as hydroxytyrosol and 3,4-dihydroxyphenylglycol (DHPG) isolated from alperujo, on indices and pathways of oxidative and metabolic stress in a vitamin E-deficient rat model.

Methods and results: Rats were fed a vitamin E-deficient diet for 10 weeks, followed by this diet supplemented with either 100 mg/kg diet dα-tocopherol, alperujo extract, hydroxytyrosol, or 10 mg/kg diet DHPG, for a further 2 weeks. We detected alperujo phenolics in tissues and blood, indicating they are bioavailable. Alperujo extract partially ameliorated elevated plasma levels of thiobarbituric acid reactive substances and also lowered plasma cholesterol levels, whereas hydroxytyrosol increased plasma triglyceride levels. Proteomics and subsequent network analysis revealed that hepatic mitochondrial aldehyde dehydrogenase (ALDH2), of which protein and activity levels were regulated by dα-tocopherol and olive phenolics, represents a novel central regulatory protein hub affected by the dietary interventions.

Conclusion: The in vivo free radical scavenging properties of olive phenolics appear relatively modest in our model. But alternative mechanisms, including regulation of ALDH2, may represent relevant antioxidant mechanisms by which dietary olive phenolics could have beneficial impact on cardiovascular health.

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1 Introduction

The beneficial effects of a Mediterranean diet rich in olive oil and table olives have been widely reported [1–4]. Olive products are rich in natural antioxidants that may inhibit oxidative stress during the development of major diseases such as coronary heart disease [5, 6], cancer [7] or neurodegenerative diseases [8, 9]. The antioxidant properties of olive products have been attributed to the presence of phenolic compounds [10, 11], which act by scavenging free radicals and chelating metallic ions to lower hydroxyl radical formation [1]. They may also activate the transcription factor nuclear factor erythroid-2-related factor-2 (Nrf2), thereby

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Abbreviations: ALDH2, mitochondrial aldehyde dehydrogenase; **DHPG**, 3,4-dihydroxyphenylglycol; **4-HNE**, 4-hydroxynonenal; **KEGG**, Kyoto Encyclopedia of Genes and Genomes; **Nrf2**, nuclear factor erythroid-2-related factor-2; **TBARS**, thiobarbituric acid reactive substances

regulating the induction of over 200 genes encoding antioxidant enzymes and other proteins that control redox status in the cell [12]. After the extraction of oil from the olive, many phenolic compounds remain in the by-product alperujo, extracts of which contain a similar phenolic profile to that of the original fruit [13]. One of the most extensively studied phenolics in olives is hydroxytyrosol [13–15], an o-diphenol whose marked antioxidant activity in vitro can be ascribed to the electron donating ability of hydroxyl groups in the ortho position and subsequent formation of stable intramolecular hydrogen bonds with the phenoxylic radical [16]. However, recently, we isolated another phenol, 3,4-dihydroxyphenylglycol (DHPG), from alperujo derived from *Olea europaea* fruits [17, 18] with potentially even greater antioxidant activity in vitro [19].

In vitro antioxidant activity of compounds such as phenolics may not reflect activity in vivo. Factors such as poor absorption through the intestinal wall and rapid metabolism and excretion may limit their biological relevance. Consequently, in order to assess efficacy in vivo, we have determined the effects of dietary consumption of alperujo extract, hydroxytyrosol, and DHPG on indices and pathways of oxidative and metabolic stress in an oxidatively stressed rat model. The physiological effects of the phenolics could however be mediated through multiple biochemical and molecular pathways. Therefore, we also applied proteomics to hepatic samples from the rats to further our understanding of mechanisms by which olive oil phenolics affect hepatic metabolic pathways and oxidative stress, and to extend the availability of relevant biomarkers to properly assess their effects.

2 Materials and methods

2.1 Phenolic compounds and extracts

All extracts were produced at the Instituto de la Grasa, Seville, Spain. Alperujo, a waste product of the olive oil production process, was obtained by crushing olives to a fine paste, which was subsequently pumped into a two-phase decanter generating a liquid phase (oil) and a semisolid waste (alperujo) [20]. Alperujo extract was produced by organic solvent extraction from alperujo pre-treated under hydrothermal conditions, as described by us previously [21]. Briefly, alperujo samples were centrifuged and the liquid fraction was extracted with ethyl acetate and dried under vacuum at 35°C [21]. Hydroxytyrosol was produced from alperujo extract by a patented industrial system [22]. DHPG purification from alperujo extract was performed by a new system that has been patented [23]. The amounts of hydroxytyrosol and DHPG in the alperujo extract were determined by HPLC on a Hewlett-Packard Series 1100 liquid chromatograph system with an UV-visible detector and a Rheodyne injection valve (20-μL loop) as described by us recently [24].

2.2 Animals and diets

The study protocol was approved by the Ethical Review Committee of Animal Studies at the Rowett Institute of Nutrition and Health, and was conducted in compliance with the Animals (Scientific Procedures) Act, 1986. Sixty weanling male rats of the Rowett Hooded Lister strain were randomly allocated to six intervention groups of ten animals each. Five intervention groups were offered, ad libitum, a semisynthetic diet containing less than 0.5 mg/kg vitamin E for 10 weeks. Rats were then offered this diet supplemented with either alperujo extract, hydroxytyrosol and dα-tocopherol (vitamin E) at a concentration of 100 mg/kg diet, or DHPG at a concentration of 10 mg/kg diet, or no additional supplement, for a further 2 weeks. These diets provided 0.0242 mg hydroxytyrosol and 0.00242 DHPG per kcal diet, which is comparable with a daily intake of 48.4 mg hydroxytyrosol and 4.84 mg DHPG per 2000 kcal in humans. The phenolics were dissolved in water, and dα-tocopherol in chloroform, and mixed to an even distribution in the diet. One intervention group was maintained on a vitamin E-adequate diet (100 mg dα-tocopherol/kg) throughout the trial (Supporting Information Fig. S1). Diets were stored at –40°C until use.

After 12 weeks of intervention, rats were anesthetized with isofluorane and blood samples were collected by cardiac puncture into heparinized tubes (Becton Dickinson, Oxford, UK). Plasma was separated by centrifugation for 15 min at 1750 × *g*, 4°C, divided into aliquots and stored at –80°C before analysis. Red cells were isolated and washed twice prior to being re-suspended to the original volume in PBS. Tissues samples of liver, heart, kidney, muscle, testes, white adipose tissue (WAT), and brain were perfused in situ with chilled isotonic KCl (0.154 M) via the hepatic portal vein and snap frozen in liquid nitrogen before storage at –80°C until analysis.

2.3 Analytical chemistry

Plasma vitamin E was determined by HPLC with fluorescence and visible detection [25]. The determination of cholesterol, triglycerides, and glucose in plasma was performed on a multiparametric analyzer Konelab 30 (Thermo Electron Corporation, Loughborough, UK) using commercial reagents (Thermo Scientific, Loughborough, UK) according to the manufacturer's instructions.

2.4 Indices of oxidative damage, muscle damage, and redox status

Plasma levels of thiobarbituric acid reactive substances (TBARS) were determined by HPLC with fluorimetric detection [26] using tetramethoxypropane (Aldrich, Gilligham, Dorset, UK) as a standard. The results were expressed as malondialdehyde (MDA) equivalents or TBARS. The susceptibility of washed erythrocytes to hydrogen peroxide-induced peroxidation was used as a measure of functional

antioxidant status [27]. Plasma hemoglobin concentration was determined using the sysmex hematology analyzer (KX-21N, Sysmex, Norderstedt, Germany). Plasma creatine kinase (CK) and pyruvate kinase (PK) activities were measured as indices of muscle damage. CK activity was determined by the Enzychrom™ Creatine Kinase Assay kit (Bioassay Systems, Cambridge, UK, ECPK-100), and PK activity was determined by the method of Beisenherz et al. [28].

2.5 Levels of phenols in plasma and tissues

Hydroxytyrosol, DHPG, tyrosol, and homovanillic acid were extracted from plasma using solid-phase extraction and from tissues using homogenization with zirconia beads and centrifugation. Levels of hydroxytyrosol, DHPG, and other phenols were analyzed by an HPLC with UV-Vis detection method using catechol as an internal standard [29], and expressed as ng/mL plasma or ng/g of tissue.

2.6 Hepatic fat

Hepatic fat was extracted by the Folch lipid extraction method [30]. Hepatic levels of triglycerides were determined in the hepatic fat fraction by an enzymatic assay (Konelab, Thermo Electron, Loughborough, UK).

2.7 Hepatic proteomics

Proteomics of hepatic tissue was performed as described by us previously [6, 31–34]. Briefly, cytosolic protein homogenates from each animal liver were separated by two-dimensional (2-D) gel electrophoresis using a BioRad (Hemel Hempstead, UK) immobilized pH gradient (IPG) 17 cm strips, pH 5–8, for the separation of the proteins in the first dimension. SDS-PAGE was performed on 18 × 18 cm acrylamide gradient (8–16%) gels for the separation of proteins in the second dimension. Samples were run in batches of 12 that were randomized for intervention group to prevent running order bias. Flamingo (Biorad) fluorescent-stained gels were scanned using a Bio-Rad FX scanner, set at medium intensity with a resolution of 100 μm, and analyzed using SameSpots (Nonlinear Dynamics Newcastle upon Tyne, UK). Spot density values from the gels were then exported from the SameSpots software into R 2.11.1. (R Foundation for Statistical Computing, Vienna, Austria) to perform statistical analysis as described below. Spots with densities that significantly differed between treatments were excised from the SDS-PAGE gels using a robotic BioRad spot cutter. The proteins represented by these spots were trypsinized using a protocol of the MassPrep Station (Micromass, Hemel Hempstead, UK). Samples from 2-D PAGE gels were analyzed using a nano LC system (LC Packings, Camberly, Surrey, UK) consisting of an "Ultimate" nano LC system, with a column flow rate of 0.3 μL/min, a "Famos" autosampler set to an injection volume of 10 μL, and a "Switchos" microcolumn switching device. The nanocolumn was a C18 PepMap 100, 15 cm × 75 μm id, 3 μm, 100 Å (LC Packings). HPLC-grade solvents comprised 2% acetonitrile and 0.1% formic acid (A), and 80% ACN, and 0.08% formic acid (B). The gradient started at 5% B, increasing to 50% B over 30 min, then ramping to 80% B over a further 2 min. The MS was performed using a Q-Trap (Applied Biosystems/MDS Sciex, Warrington, Cheshire, UK) triple quadrupole fitted with a nanospray ion source, where Q3 was operated as a linear ion trap. The nanospray needle voltage was set at 2800 V. The collision energy was compound dependent (set to a maximum of 80 eV). The total ion current data were submitted for database searching using the MASCOT search engine (Matrix Science, London, UK) using the MSDB database with the following search criteria—allowance of zero or one missed cleavages; peptide mass tolerance of ±1.5 Da; fragment mass tolerance of ±1.5 Da, trypsin as digestion enzyme; carbamidomethyl fixed modification of cysteine; methionine oxidation as a variable modification and charged states as 2+ and 3+.

2.8 Measurement of mitochondrial aldehyde dehydrogenase (ALDH2) activity

ALDH2 activity was assessed according to the method described by Tank et al. [35], including some modifications suggested by Moon et al. [36]. Briefly, a reaction mixture containing 100 mM Na-phosphate buffer (pH 7.4), 1 mM EDTA, 1 mM NAD, and a homogenate of cytosolic proteins from the liver (~1 mg protein/assay). Ten millimolars of pyrazole was added to inhibit alcohol dehydrogenase activity. The reaction mixture was incubated for 2 min at room temperature, after which the enzyme reaction was initiated by adding 10 μM acetaldehyde. The change in absorbance was monitored for 2 min to calculate the rate of NADH production. Specific activity of aldehyde dehydrogenase was calculated using the extinction coefficient of reduced NAD of 6.22 × 106 cm^2 at 340 nm (Merck Index, Whitehouse Station, NJ, USA), and 1 unit represents a reduction of 1 nmol NAD+/min/mg protein at room temperature.

2.9 Statistical analysis

Data are presented as mean ± standard deviation (SD). Log-transformed physiological data were analyzed by analysis of variance with a term for treatment group. Log-transformed protein spot densities, originating from normalized data as calculated by the SameSpots software, were analyzed by analysis of variance with terms for gel batch and treatment group. Post-hoc comparisons were based on the least significant difference. Principal component analysis was carried out on the correlation matrix of the log-transformed physiological data and spot densities, and scatter plots of scores of the first

few components were examined. Tests were calculated in R (R foundation for Statistical Computing). Spots significant at $p < 0.05$ were selected for identification.

2.10 Network analysis

To visualize the regulated proteins in the context of their biological function, a network was generated using pathways from the Kyoto Encyclopedia of Genes Genomes (KEGG) [37], Wikipathways [38], and Reactome [39]. The network is a bipartite graph containing a set of nodes representing regulated proteins and a set of nodes representing pathways. An edge is added between a protein and pathway node when the protein plays a role in that pathway. To map the measured proteins with the protein and gene identifiers in the pathways, both were translated to UniProt identifiers using the BridgeDb library [40]. The constructed network was visualized using Cytoscape (version 2.8.1) [41], and the log2 ratios for the protein expression of the three compounds were visualized using barcharts in the protein nodes. In a few cases where more than one protein isoform was identified per UniProt identifier, a distinct bar is shown in the barchart for each isoform. The network visualization was used to objectively identify regulatory protein hubs (defined as a protein with a high number of connected pathways) and main pathways (defined as pathways with a high number of connected proteins) regulated by the intervention diets.

3 Results

3.1 Effects on measures of oxidative stress

Compared with animals maintained on the vitamin E-deficient ration, plasma concentrations of vitamin E were significantly higher ($p < 0.001$) in the rats consuming the vitamin E-sufficient ration and also in those repleted with vitamin E for 2 weeks (Fig. 1). Plasma levels of TBARS were significantly decreased by vitamin E repletion ($p < 0.01$) and also, albeit to a lesser extent, by alperujo extract ($p < 0.01$), compared with the vitamin E-deficient group. Levels of TBARS in the erythrocyte membrane, as a measure of erythrocyte membrane lipid peroxidation, were significantly lowered only by vitamin E ($p < 0.001$) compared with the vitamin E-deficient group (Fig. 1). Similarly, plasma creatinine and PK activity were significantly lowered only by vitamin E ($p < 0.001$) compared with the vitamin E-deficient group (Fig. 1).

3.2 Effects on metabolic health

The final bodyweights of the rats was similar between the intervention groups and thus unaffected by the dietary inclusion of alperujo extract, hydroxytyrosol, or DHPG in the vitamin E-deficient rats (Fig. 2). Levels of plasma glucose tended to be lowered by hydroxytyrosol compared with the vitamin E-deficient group, but this effect did not reach statistical significance ($p = 0.078$) (Fig. 2). Both vitamin E and alperujo extract significantly lowered levels of plasma cholesterol ($p < 0.01$), whereas hydroxytyrosol increased levels of plasma triglycerides ($p = 0.01$) compared with the vitamin E-deficient group (Fig. 2). Interestingly, hepatic fat was significantly lower ($p < 0.05$) in the vitamin E-sufficient animals compared with vitamin E-deficient animals, even when vitamin E was added back into the diet ($p < 0.05$) (Fig. 2). Levels of hepatic triglycerides were similar in all intervention groups (Fig. 2).

3.3 Levels of phenolic compounds in plasma and tissues

In general, levels of hydroxytyrosol were highest in plasma, but high levels of hydroxytyrsol were also detected in WAT and in testes, especially in the groups supplemented with hydroxytyrosol and DHPG (Table 1). Levels of DHPG were present in plasma, kidney, brain, muscle, and WAT, but tissue levels were mostly detectable when the diet was depleted in vitamin E. Levels of DHPG were highest in animals where the vitamin E-deficient diet had been supplemented with DHPG (Table 1). Levels of tyrosol, a metabolite of hydroxytyrosol, were markedly elevated in WAT in all intervention groups, but also in plasma and testes in the groups where the vitamin E-deficient diet was supplemented with alperujo extract or hydroxytyrsol (Table 1). Levels of homovanillic acid were highest in the brain in all intervention groups, but this metabolite of hydroxytyrosol was also present in plasma of all intervention groups. Furthermore, low levels of homovanillic acid had been accumulating in liver, heart, muscle, and WAT in some intervention groups (Table 1).

3.4 Proteomics results

An image of a typical gel image is shown in Supporting Information Fig. S2. Levels of 42 unique single hepatic protein were regulated in our study by the various interventions. Most of these proteins were involved in oxidative phosphorylation, glycolysis/gluconeogenesis, fatty acid metabolism, amino acid metabolism, bile acid biosynthesis, and energy metabolism (Table 2). Measures of confidence for protein identification and characterization by LC- MS/MS analysis of hepatic cytosolic proteins that were significantly regulated in the various intervention groups can be found in Supporting Information Table S1.

3.5 Multivariate data analysis

Principal component analysis was used to examine the effects of the various treatments on the physiological outcomes and hepatic protein levels in the complete dataset.

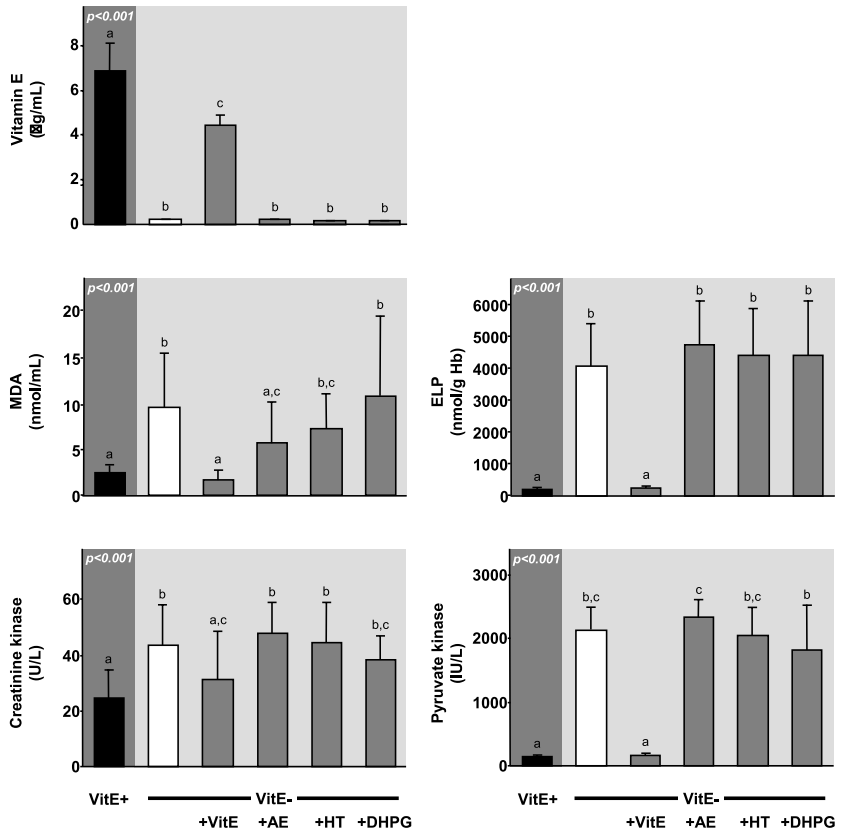

Figure 1. Indices of oxidative damage, muscle damage, and redox status. Effects of alperujo extract, hydroxytyrosol or dα-tocopherol (100 mg/kg diet), or DHPG (10 mg/kg diet), compared with no dietary repletion in vitamin E-deficient rats, or compared with rats maintained on a vitamin E-adequate diet, on plasma levels of vitamin E and thiobarbituric acid reactive substances (TBARS), levels of TBARS in washed erythrocytes upon hydrogen peroxide-induced peroxidation (ELP: erythrocyte lipid peroxidation), and plasma creatine kinase (CK) and pyruvate kinase (PK) activities. Data are presented as means ± SEM ($n = 10$ per group). Figures provide overall *p*-values, and different letters indicate significant differences between intervention groups following post hoc comparisons.

This approach visualizes the extent to which different treatments have similar, or very different, effects. Principal component analysis revealed a clear separation of the vitamin E-sufficient and depleted groups on the first principal component when considering the physiological data. This effect was mainly explained by plasma vitamin E levels, as well as levels of TBARS in plasma and erythrocyte membranes, and plasma levels of CK and PK activity. A similar but notably less clear separation between the vitamin E-sufficient and depleted groups could be observed both on the first and second principal component analysis when considering the proteomics data. This effect was mainly explained by regulation of the proteins heat shock cognate 71 kDa protein (SSP 444), fructose-1,6-bisphosphatase 1 (SSP761), isocitrate dehydrogenase [NAD] subunit alpha (SSP 877), protein disulfide-isomerase A6 (SSP699), selenium-binding protein 1 (SSP 634), and 3-oxo-5-beta-steroid-4-dehydrogenase (SSP 908), as evidenced by the highest positive or negative loadings (Fig. 3).

3.6 Network analysis

In order to assess the impact of changes in protein levels on the regulation of major hepatic pathways, we explored the proteomics results with Cytoscape (Fig. 4). Cytoscape [42] is an open source software platform to visualize a complex network of regulated proteins assessed by 2-D gel electrophoresis and their pathway annotations. Thirty-six of the 42 regulated and identified proteins were part of 54 different pathways annotated in KEGG, 41 different pathways in Wikipathways, and 21 different pathways in Reactome (Fig. 4). The central regulatory protein hub affected by dietary interventions was ALDH2 (SSPs 597 and 654, UniProt accession number P11884, Supporting Information Table S1) with the corresponding gene being involved in 16 biochemical pathways. Other important regulatory protein hubs were phosphoglucomutase (SSP 516, UniProt accession number Q499Q4, Supporting Information Table S1), proteasome activator complex subunit 2 (SSP 1051, UniProt accession number Q63798, Supporting Information Table S1), proteasome (prosome, macropain) activator subunit 1 (SSPs 1068 and 1074, UniProt accession number Q6P9V7, Supporting Information Table S1), and heat shock protein HSP 90-beta (SSP 366, UniProt accession number P34058, Supporting Information Table S1), all with the corresponding gene being involved in nine biochemical pathways.

3.7 Hepatic activity of aldehyde dehydrogenase

Based on the proteomics findings and subsequent network analysis, we analyzed activity levels of aldehyde dehydrogenase in frozen liver, as this protein represented a main regulatory protein hub. We took into account that the mitochondrial protein ALDH2, which we found regulated in the proteomics analysis, would have leaked into the cytosolic fraction of the

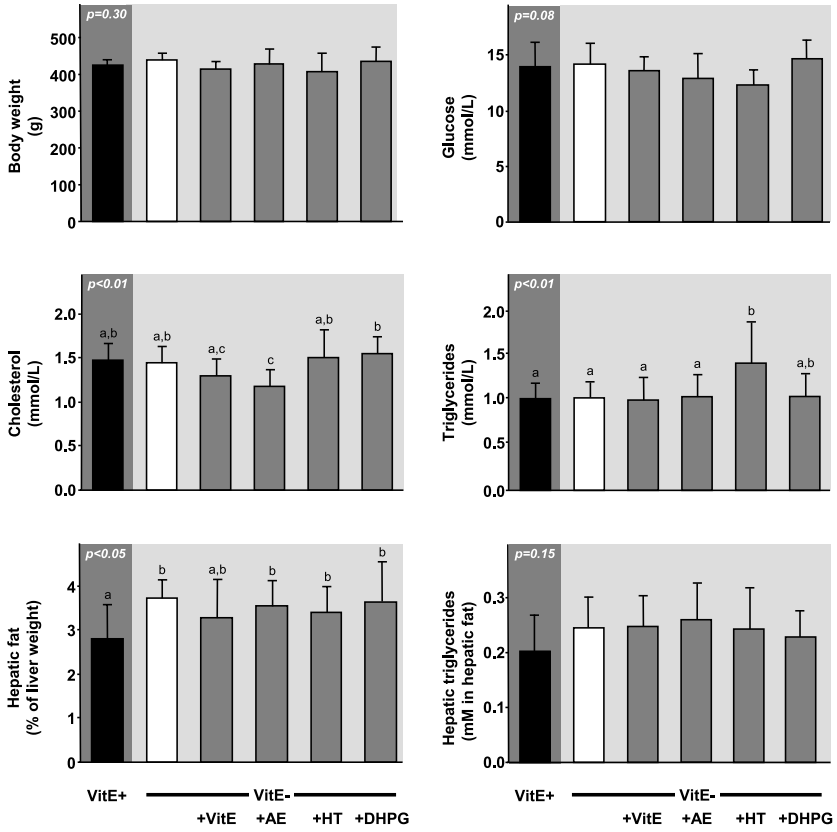

Figure 2. Metabolic indices. Effects of alperujo extract, hydroxytyrosol or dα-tocopherol (100 mg/kg diet), or DHPG (10 mg/kg diet), compared with no dietary repletion in vitamin E-deficient rats, or compared with rats maintained on a vitamin E-adequate diet, on body weight, plasma levels of glucose, cholesterol and triglycerides, and hepatic fat and triglyceride levels. Data are presented as means ± SEM ($n = 10$ per group). Figures provide overall *p*-values, and different letters indicate significant differences between intervention groups following post hoc comparisons.

liver cells. We also took into account that measurement of aldehyde dehydrogenase would represent a range of aldehyde dehydrogenase isoforms that could have been present in the liver. Because of the limited number of hepatic samples from the vitamin E-sufficient group available for measurement of aldehyde dehydrogenase activity, we specifically focused on the results obtained in rats fed the vitamin E-deficient diet by comparing the effects of vitamin E, alperujo extract, hydroxytyrosol, and DHPG as compared with no supplement. Hepatic aldehyde dehydrogenase activity was significantly decreased upon intervention with both vitamin E and DHPG in the vitamin E-deficient animals. This observation was in line with a decrease in protein levels of two ALDH2 isoforms by vitamin E and the olive phenolics (Fig. 5).

4 Discussion

In our vitamin E-deficient rat model, alperujo extract partially ameliorated elevated plasma levels of TBARS, but not to the same extent as dα-tocopherol. Alperujo extract also lowered plasma cholesterol, whereas hydroxytyrosol increased plasma triglyceride levels. Network analysis of hepatic proteomics results revealed that aldehyde dehydrogenase, which was down-regulated by dα-tocopherol as well as by olive phenolics, represented a central regulatory protein hub affected by dietary interventions.

4.1 Antioxidant effects

Previously, phenolic extracts and purified hydroxytyrosol from olive mill waste showed clear antioxidant effects in alloxan-induced diabetic rats by increasing renal levels of superoxide dismutase (SOD), hepatic and kidney catalase, and glutathione peroxidase activities and decreasing plasma TBARS [43]. Maintaining our rats on diets deficient in vitamin E resulted in an increase in markers of oxidative stress and tissue damage. This was ameliorated by repletion with dα-tocopherol for 2 weeks. Analogous responses were not observed following repletion with similar dietary concentrations of hydroxytyrosol or DHPG (Fig. 1). The implication is that despite potent antioxidant activity in vitro, these olive-derived phenolics are ineffective chain-breaking antioxidants in vivo, at least in this oxidatively stressed rodent model. This relative ineffectiveness cannot be ascribed solely to poor uptake and absorption from the diet as they and their metabolites were detected in tissues and blood. Limited access to relevant cellular sites such as the lipid components of cell membranes may limit in vivo efficacy. Repletion with alpurejo extract did, however, partially ameliorate elevated markers of plasma TBARS induced by vitamin E deficiency (Fig. 1). This is not due to confounding effects by residual vitamin E as none was detected in the extract but may reflect activity of other as yet unidentified compounds. It is important to note that the measurement of MDA as a marker of lipid peroxidation remains

Table 1. Concentrations of phenolic compounds in plasma and tissues rat samples

Plasma/tissues	Phenolic compound	Vitamin E-adequate diet	Vitamin E-deficient diet				
			Control	+VitE	+AE	+HT	+DHPG
Plasma (ng/mL plasma)	Hydroxytyrosol	83.9 ± 3.8	90.3 ± 2.7	109.7 ± 2.2	77.4 ± 1.4	148.4 ± 4.3	129.0 ± 5.4
	DHPG	5.6 ± 0.7	6.1 ± 1.2	9.3 ± 0.9	15.4 ± 1.2	3.5 ± 0.4	9.3 ± 0.3
	Tyrosol	n.d.	n.d.	n.d.	375.4 ± 2.1	19.7 ± 0.8	n.d.
	Homovanillic acid	3.8 ± 0.3	57.4 ± 3.0	4.1 ± 0.4	163.9 ± 1.0	204.9 ± 1.5	139.3 ± 1.1
Liver (ng/g tissue)	Hydroxytyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DHPG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Tyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Homovanillic acid	n.d.	n.d.	n.d.	n.d.	20.5 ± 5.7	114.8 ± 19.3
Kidney (ng/g tissue)	Hydroxytyrosol	n.d.	n.d	10.5 ± 1.0	4.0 ± 0.3	16.1 ± 1.1	40.3 ± 3.0
	DHPG	n.d.	2.0 ± 0.2	3.5 ± 0.1	10.4 ± 0.7	5.0 ± 0.1	42.8 ± 0.9
	Tyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Homovanillic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Heart (ng/g tissue)	Hydroxytyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DHPG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Tyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Homovanillic acid	1.9 ± 0.2	n.d.	69.7 ± 1.9	n.d.	16.4 ± 2.0	49.2 ± 1.7
Brain (ng/g tissue)	Hydroxytyrosol	n.d.	n.d.	n.d.	9.7± 0.5	12.1 ± 0.8	n.d.
	DHPG	n.d.	4.3 ± 0.1	10.9 ± 0.6	4.3 ± 0.2	4.3 ± 0.3	8.7 ± 0.1
	Tyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Homovanillic acid	78.8 ± 0.9	1926.2 ± 1.2	2377.0 ± 2.6	1311.5 ± 3.5	1024.6 ± 2.3	819.7 ± 1.2
Muscle (ng/g tissue)	Hydroxytyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	DHPG	10.9 ± 0.7	60.9 ± 0.9	10.9 ± 0.5	7.2 ± 0.5	13.0 ± 0.4	2.2 ± 0.2
	Tyrosol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Homovanillic acid	3.3 ± 0.7	110.7 ± 1.4	110.5 ± 3.6	n.d.	28.7 ± 1.4	n.d.
White adipose tissue (ng/g tissue)	Hydroxytyrosol	n.d.	n.d.	n.d.	112.9 ± 2.2	72.6 ± 1.7	129.0 ± 3.1
	DHPG	n.d.	n.d.	6.5 ± 0.9	9.8 ± 0.8	n.d.	n.d.
	Tyrosol	819.7 ± 7.6	983.6 ± 10.1	2049.2 ± 12.4	819.8 ± 4.1	942.6 ± 5.6	696.7 ± 9.8
	Homovanillic acid	n.d.	n.d.	n.d.	n.d.	n.d.	32.8
Testes (ng/g tissue)	Hydroxytyrosol	n.d.	n.d.	10.5 ± 0.8	8.1 ± 1.0	96.8 ± 2.0	80.6 ± 1.4
	DHPG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Tyrosol	n.d.	n.d.	n.d.	565.6 ± 14.0	500.0 ± 17.1	n.d.
	Homovanillic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Data are expressed as means ± SD. Analyses were performed in duplicate in tissues from four randomly chosen animals per intervention group. VitE = vitamin E, AE = alperjuo extract, HT = hydroxytyrosol, DHPG = 3,4-dihydroxyphenylglycol.

Table 2. Identification of hepatic proteins which were significantly regulated by alperujo extract, hydroxytyrosol, or DHPG compared with no dietary repletion in vitamin E-deficient rats

	UniProt		KEGG		Fold-change		
SSP	Accession	Identified protein name	Accession	Pathways	Alperujo extract	Hydroxytyrosol	DHPG
Glycolysis/gluconeogenesis							
761	P19112	Fructose-1,6-bisphosphatase 1	rno:24362	rno00010 glycolysis/gluconeogenesis rno00030 pentose phosphate pathway rno00051 fructose and mannose metabolism rno01100 metabolic pathways rno04910 insulin signaling pathway	1.47	1.48	2.03
815	P19112	Fructose-1,6-bisphosphatase 1	rno:24362	rno00010 glycolysis/gluconeogenesis rno00030 pentose phosphate pathway rno00051 fructose and mannose metabolism rno01100 metabolic pathways rno04910 insulin signaling pathway	1.03	1.30	1.13
449	P08461	Pyruvate dehydrogenase complex component E2	rno:81654	rno00010 glycolysis/gluconeogenesis rno00020 citrate cycle (TCA cycle) rno00620 pyruvate metabolism rno01100 metabolic pathways	0.91	1.26	1.32
1000	P49432	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	rno:289950	rno00010 glycolysis/gluconeogenesis rno00020 citrate cycle (TCA cycle) rno00290 valine, leucine, and isoleucine biosynthesis rno00620 pyruvate metabolism rno00650 butanoate metabolism rno01100 metabolic pathways	1.08	1.17	1.00
516	Q499Q4	Phosphoglucomutase 1	rno:24645	rno00010 glycolysis/gluconeogenesis rno00030 pentose phosphate pathway rno00052 galactose metabolism rno00230 purine metabolism rno00500 starch and sucrose metabolism rno00520 amino sugar and nucleotide sugar metabolism rno01100 metabolic pathways	0.99	1.03	1.03
597	P11884	Aldehyde dehydrogenase, mitochondrial	rno:29539	rno00010 glycolysis/gluconeogenesis rno00040 pentose and glucuronate interconversions rno00053 ascorbate and aldarate metabolism rno00071 fatty acid metabolism rno00280 valine, leucine, and isoleucine degradation rno00310 lysine degradation rno00330 arginine and proline metabolism rno00340 histidine metabolism	0.80	0.83	0.78

Table 2. Continued

	UniProt		KEGG		Fold-change		
SSP	Accession	Identified protein name	Accession	Pathways	Alperujo extract	Hydroxytyrosol	DHPG
				rno00380 tryptophan metabolism rno00410 beta-alanine metabolism rno00561 glycerolipid metabolism rno00620 pyruvate metabolism rno00640 propanoate metabolism rno01100 metabolic pathways			
654	P11884	Aldehyde dehydrogenase, mitochondrial	rno:29539	rno00010 glycolysis/ gluconeogenesis rno00040 pentose and glucuronate interconversions rno00053 ascorbate and aldarate metabolism rno00071 fatty acid metabolism rno00280 valine, leucine, and isoleucine degradation rno00310 lysine degradation rno00330 arginine and proline metabolism rno00340 histidine metabolism rno00380 tryptophan metabolism rno00410 beta-alanine metabolism rno00561 glycerolipid metabolism rno00620 pyruvate metabolism rno00640 propanoate metabolism rno01100 metabolic pathways	0.98	0.81	0.82
Lipid and bile acid metabolism							
760	P15650	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	rno:25287	rno00071 fatty acid metabolism rno01100 metabolic pathways rno03320 PPAR signaling pathway	0.95	1.09	0.79
818	P15650	Long-chain specific acyl-CoA dehydrogenase, mitochondrial	rno:25287	rno00071 fatty acid metabolism rno01100 metabolic pathways rno03320 PPAR signaling pathway	0.76	0.92	0.89
632	Q63060	Glycerol kinase	rno:79223	rno00561 glycerolipid metabolism rno01100 metabolic pathways rno03320 PPAR signaling pathway	1.05	1.01	1.09
989	O35077	Glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic	rno: 60666	rno00564 glycerophospholipid metabolism	0.76	1.02	1.00
908	P31210	3-oxo-5-beta-steroid 4-dehydrogenase	rno:192242	rno00120 primary bile acid biosynthesis rno00140 steroid hormone biosynthesis rno01100 metabolic pathways	1.15	1.04	1.06
Protein and amino acid metabolism							
696	Q6AYW2	Phenylalanine hydroxylase	rno:24616	rno00360 phenylalanine metabolism rno00400 phenylalanine, tyrosine, tryptophan biosynthesis rno01100 metabolic pathways	0.89	0.88	0.88
904	P35738	2-oxoisovalerate dehydrogenase subunit beta, mitochondrial	rno:29711	rno00280 valine, leucine, and isoleucine degradation rno01100 metabolic pathways	0.98	1.03	0.86

Table 2. Continued

	UniProt		KEGG		Fold-change		
SSP	Accession	Identified protein name	Accession	Pathways	Alperujo extract	Hydroxytyrosol	DHPG
1044	P46953	3-hydroxyanthranilate 3,4-dioxygenase	rno:56823	rno00380 tryptophan metabolism rno01100 metabolic pathways	1.12	1.26	1.12
Protein processing							
366	34058	Heat shock protein HSP 90-beta	rno:301252	rno04141 protein processing in endoplasmic reticulum rno04612 antigen processing and presentation rno04621 NOD-like receptor signaling pathway rno04914 progesterone-mediated oocyte maturation rno05200 pathways in cancer rno05215 prostate cancer	0.78	0.72	0.62
444	P63017	Heat shock cognate 71 kDa protein	mmu:15481	mmu03040 spliceosome mmu04010 MAPK signaling pathway mmu04141 protein processing in endoplasmic reticulum mmu04144 endocytosis mmu04612 antigen processing and presentation mmu05145 toxoplasmosis mmu05162 measles	0.89	1.00	1.20
555	P63039	60 kDa heat shock protein, mitochondrial	rno:63868	rno03018 RNA degradation rno04940 Type I diabetes mellitus	0.96	0.98	0.98
577	Q3MHS9	Chaperonin containing Tcp1, subunit 6A (Zeta 1)	rno:288620		0.97	0.93	0.94
322	P46462	Transitional endoplasmic reticulum ATPase	rno:116643	rno04141 protein processing in endoplasmic reticulum	1.26	1.54	1.27
509	P61980	Heterogeneous nuclear ribonucleoprotein K	rno:117282	rno03040 spliceosome	0.81	0.88	0.94
699	Q63081	Protein disulfide-isomerase A6	rno:286906	rno04141 protein processing in endoplasmic reticulum	0.88	0.93	1.02
1051	Q63798	Proteasome activator complex subunit 2	rno:100362709 rno:29614	rno03050 proteasome rno04612 antigen processing and presentation	0.95	1.03	1.12
1068	Q6P9V7	Proteasome (prosome, macropain) activator subunit 1	rno:29630	rno03050 proteasome rno04612 antigen processing and presentation	0.64	0.67	0.82
1074	Q6P9V7	Proteasome (prosome, macropain) activator subunit 1	rno:29630	rno03050 proteasome rno04612 antigen processing and presentation	1.14	1.30	1.19
Energy metabolism and oxidative stress							
708	P10719	ATP synthase subunit beta, mitochondrial	rno:171374	rno00190 oxidative phosphorylation rno01100 metabolic pathways rno05010 Alzheimer's disease rno05012 Parkinson's disease rno05016 Huntington's disease	0.98	0.81	0.92
877	Q99NA5	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	rno:114096	rno00020 citrate cycle (TCA cycle) rno01100 metabolic pathways	0.94	0.92	0.96

Table 2. Continued

	UniProt		KEGG		Fold-change		
SSP	Accession	Identified protein name	Accession	Pathways	Alperujo extract	Hydroxytyrosol	DHPG
261	P52873	Pyruvate carboxylase, mitochondrial	rno:25104	rno00020 citrate cycle (TCA cycle) rno00620 pyruvate metabolism rno01100 metabolic pathways	1.21	2.13	0.83
1035	P13803	Electron transfer flavoprotein subunit alpha, mitochondrial	rno:300726		1.12	1.22	0.89
1297	P07632	Superoxide dismutase [Cu-Zn]	rno:24786	rno04146 peroxisome rno05014 amyotrophic lateral sclerosis (ALS) rno05016 Huntington's disease rno05020 Prion diseases	1.19	1.28	1.21
Miscellaneous							
456	P02770	Serum albumin	rno:24186		0.86	1.39	1.21
586	Q3MIF4	Xylulose kinase	rno:316067	rno00040 pentose and glucuronate interconversions rno01100 metabolic pathways	1.20	1.22	1.31
634	Q8VIF7	Selenium-binding protein 1	rno:140927		0.90	1.04	1.01
774	Q5EBA9	Suclg2 protein			1.05	1.00	0.89
879	P05369	Farnesyl pyrophosphate synthase	rno:83791	rno00900 terpenoid backbone biosynthesis rno01100 metabolic pathways	0.58	0.51	0.78
928	P06214	Delta-aminolevulinic acid dehydratase	rno:25374	rno00860 porphyrin and chlorophyll metabolism rno01100 metabolic pathways	0.93	1.16	1.10
969	O35331	Pyridoxal kinase	rno:83578	rno00750 vitamin B6 metabolism rno01100 metabolic pathways	0.82	0.83	1.21
986	P23457	3-alpha-hydroxysteroid dehydrogenase	rno:191574		0.89	1.02	1.08
1065	P52844	Estrogen sulfotransferase, isoform 1			0.76	0.84	0.93
1077	P27605	Hypoxanthine-guanine phosphoribosyltransferase	rno:24465	rno00230 purine metabolism rno00983 drug metabolism—other enzymes rno01100 metabolic pathways	0.80	0.95	0.95
1111	Q6LDG5	Hypoxanthine-guanine phosporibosyltransferase			0.78	1.39	0.86

All spots were significantly regulated by alperujo extract, hydroxytyrosol, or DHPG, compared with no dietary repletion, in vitamin E-deficient rats, assessed by analysis of variance as described in Materials and methods.

controversial. This controversy is mainly caused, however, by the various methods that determine MDA based on colorimetry. These methods are frequently criticized for its lack of specificity and its artifact formation [44]. However, we measured MDA using HPLC that is analytically superior to the colorimetric methods. The validity of the measure within this model system is further emphasized by the decrease noted once nutritional antioxidants are reintroduced in to the diet.

Poor antioxidant ability of hydroxytyrosol and DHPG in vivo does not exclude the possibility of biological activity unrelated to hydrogen donation to reactive free radical species. Indeed, proteomic analysis revealed that levels of SOD had increased upon intervention with dα-tocopherol and all olive phenolic fractions (Table 2 and Supporting Information Table S1). Expression of SOD, as well as other antioxidant enzymes and proteins that control redox status in the cell, is regulated via the redox sensitive transcription factor, Nrf2 interacting with the antioxidant response element. This is a natural defense mechanism initiated by many xenobiotics, including, for example, polyphenols [12].

Network analysis of proteomics data also revealed down-regulation of another antioxidant protein target, mitochondrial ALDH2, by both dα-tocopherol as well as olive phenolics. Down-regulation of hepatic ALDH2 levels was accompanied by a simultaneous decrease in hepatic aldehyde dehydrogenase activity levels (Fig. 5). Expression of many aldehyde

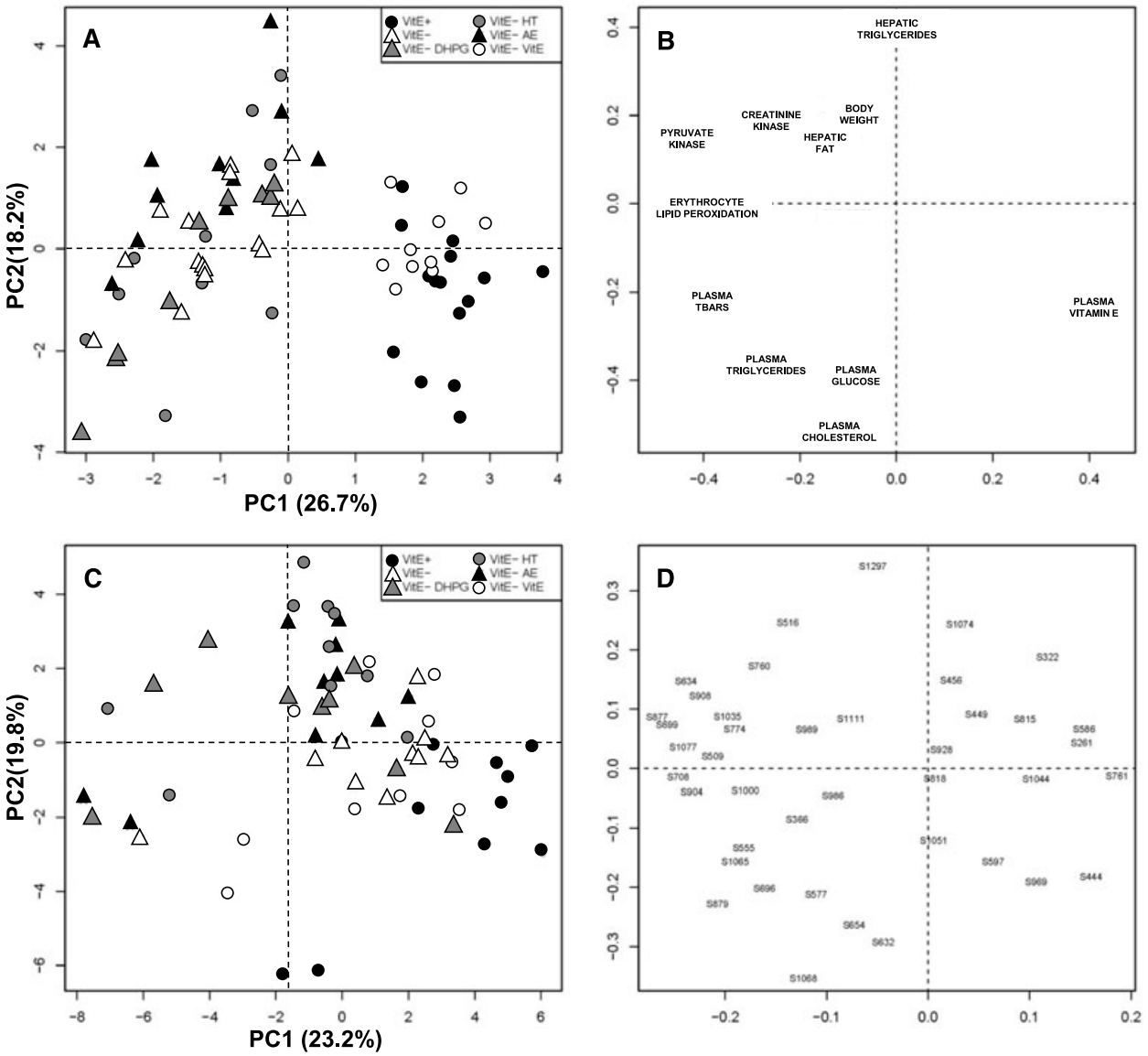

Figure 3. Multivariate data analysis. Scores and loadings plots of a principal component analysis of physiological outcomes (A and B, respectively) and hepatic cytosolic proteins (C and D, respectively) that were significantly regulated between rats receiving different diets: vitamin E-adequate diet (VitE+), vitamin E-deficient diet repleted with dα-tocopherol (VitE-VitE), alperujo extract (VitE-AE), hydroxytyrosol (VitE-HT), DHPG (VitE-DHPG), or no dietary repletion (VitE-). S261 = pyruvate carboxylase, mitochondrial, S322 = transitional endoplasmic reticulum ATPase, S366 = heat shock protein HSP 90-beta, S444 = heat shock cognate 71 kDa protein, S449 = dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial, S456 = serum albumin, S516 = phosphoglucomutase 1, S555 = 60 kDa heat shock protein, mitochondrial, S577 = chaperonin containing Tcp1, subunit 5 (epsilon), S509 = heterogeneous nuclear ribonucleoprotein K, S586 = xylulose kinase, S597 = aldehyde dehydrogenase, mitochondrial, S632 = glycerol kinase/ATP-stimulated glucocorticoid-receptor translocation promoter, S634 = selenium-binding protein 1, S654 = aldehyde dehydrogenase, mitochondrial, S696 = phenylalanine hydroxylase, S699 = protein disulfide-isomerase A6, S708 = ATP synthase subunit beta, mitochondrial, S760 = long-chain specific acyl-CoA dehydrogenase, mitochondrial, S761 = fructose-1,6-bisphosphatase 1, S774 = suclg2 protein, S815 = fructose-1,6-bisphosphatase 1, S818 = long-chain specific acyl-CoA dehydrogenase, mitochondrial, S877 = isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial, S879 = farnesyl pyrophosphate synthase, S904 = 2-oxoisovalerate dehydrogenase subunit beta, mitochondrial, S908 = 3-oxo-5-beta-steroid 4-dehydrogenase, S928 = delta-aminolevulinic acid dehydratase, S969 = pyridoxal kinase, S986 = 3-alpha-hydroxysteroid dehydrogenase, S989 = glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic, S1000 = pyruvate dehydrogenase E1 component subunit beta, mitochondrial, S1035 = electron transfer flavoprotein subunit alpha, mitochondrial, S1044 = 3-hydroxyanthranilate 3,4-dioxygenase, S1051 = proteasome activator complex subunit 2, S1065 = estrogen sulfotransferase, isoform 1, S1068 = proteasome (prosome, macropain) activator subunit 1, S1074 = proteasome (prosome, macropain) activator subunit 1, S1077 = hypoxanthine-guanine phosphoribosyltransferase, S1111 = hypoxanthine-guanine phosporibosyltransferase, S1297 = superoxide dismutase [Cu-Zn].

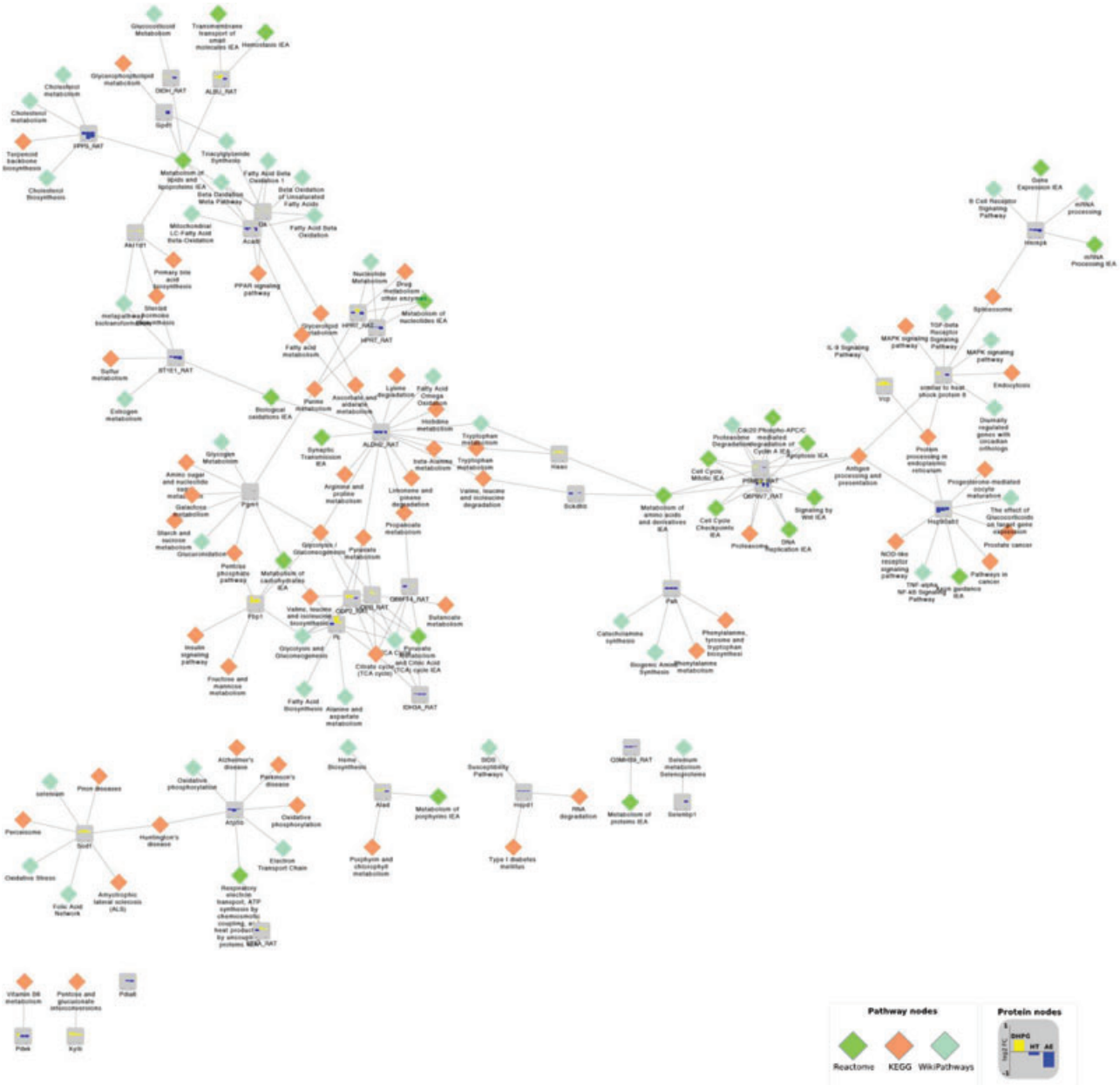

Figure 4. Cytoscape pathway plot of regulated proteins. Protein regulation is illustrated in the bar charts contained in the gray squares. An edge is added between a protein and pathway node. A pathway from KEGG is indicated with an orange rhombus, from Wikipathways with a blue rhombus, and from the Reactome with a green rhombus. Proteins are labeled with their gene identifiers: Acadl = long-chain specific acyl-CoA dehydrogenase, mitochondrial, Akr1c9 = 3-alpha-hydroxysteroid dehydrogenase; Akr1d1 = 3-oxo-5-beta-steroid 4-dehydrogenase, Alad = delta-aminolevulinic acid dehydratase, Alb = serum albumin, Aldh2 = aldehyde dehydrogenase, mitochondrial, Atp5b = ATP synthase subunit beta, mitochondrial, Bckdhb = 2-oxoisovalerate dehydrogenase subunit beta, mitochondrial, Cct6a = chaperonin containing Tcp1, subunit 6A (Zeta 1), Etfa = electron transfer flavoprotein subunit alpha, mitochondrial, Fbp1 = fructose-1,6-bisphosphatase 1, Fdps = farnesyl pyrophosphate synthase, Gk = glycerol kinase, Gpd1 = glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic, Haao = 3-hydroxyanthranilate 3,4-dioxygenase, Hnrnpk = heterogeneous nuclear ribonucleoprotein K, Hprt1 = hypoxanthine-guanine phosphoribosyltransferase, Hsp90ab1 = heat shock protein HSP 90-beta, Hspa8 = heat shock cognate 71 kDa protein, Hspd1 = 60 kDa heat shock protein, mitochondrial, Idh3a = isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial, ODP2_RAT = pyruvate dehydrogenase complex component E2, ODPB_RAT = pyruvate dehydrogenase E1 component subunit beta, mitochondrial, Pah = phenylalanine hydroxylase, Pc = pyruvate carboxylase, mitochondrial, Pdia6 = protein disulfide-isomerase A6, Pdxk = pyridoxal kinase, Pgm1 = phosphoglucomutase 1, Psme2 = proteasome activator complex subunit 2, Psme1 = proteasome (prosome, macropain) activator subunit 1, Selenbp1 = selenium-binding protein 1, Sod1 = superoxide dismutase [Cu-Zn], Suclg2 = Suclg2 protein, Sult1e1 = estrogen sulfotransferase, isoform 1, Vcp = transitional endoplasmic reticulum ATPase, Xylb = xylulose kinase.

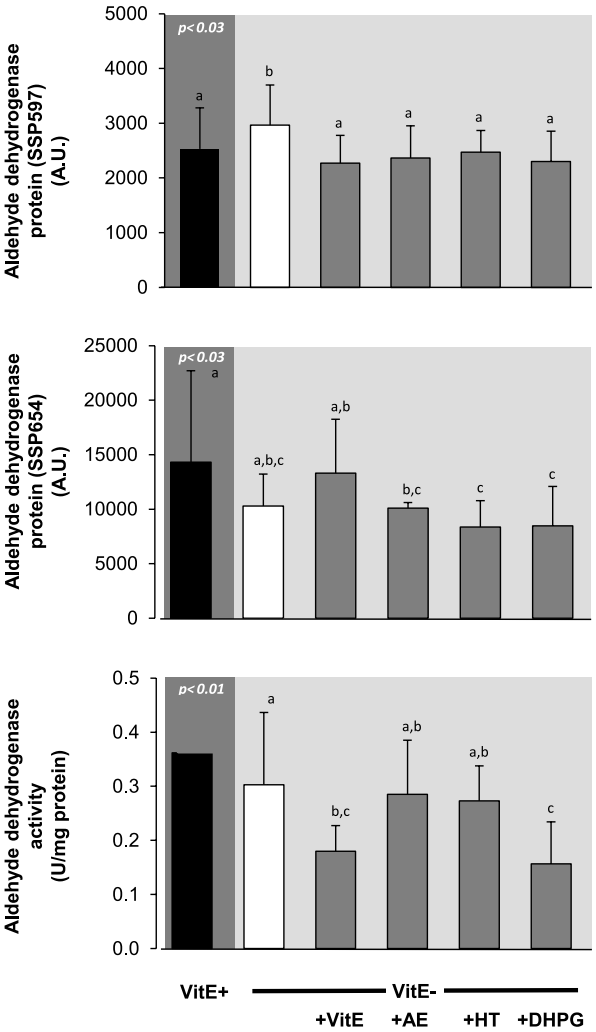

Figure 5. Regulation hepatic aldehyde dehydrogenase protein levels and activity. Effects of alperujo extract, hydroxytyrosol or dα-tocopherol (100 mg/kg diet), or DHPG (10 mg/kg diet), compared with no dietary repletion in vitamin E-deficient rats, or compared with rats maintained on a vitamin E-adequate diet, on the regulation of hepatic protein levels of two aldehyde dehydrogenase isoforms, and hepatic aldehyde dehydrogenase activity levels. Data are presented as means ± SEM ($n = 2$ animals from the group on the vitamin E-adequate diet and $n = 6$ animals from the other groups). Figures provide overall *p*-values, and different letters indicate significant differences between intervention groups following post hoc comparisons.

dehydrogenases is regulated by Nrf2, but it is currently uncertain whether this is also the case for ALDH2 [45]. In our network analysis, ALDH2 was a central regulatory protein hub, which means that this protein, or its downstream products, is more likely to be a good biomarker compared with other proteins and metabolites. In addition, because of the role this protein plays in a relatively large number of annotated pathways, regulation of this protein will automatically have a larger impact on body functions. ALDH2 is expressed abundantly in the liver but also in organs that require high mitochondrial capacity for oxidative ATP generation, such as heart and brain, and has emerged as a key enzyme of cardioprotection [46]. ALDH2 is important in the oxidation of aldehydic substrates, such as, for example, the reactive aldehydes 4-HNE and malonaldehydes, which are lipid peroxidation products and markers of the oxidative stress. Aldehydes induce inactivation of a number of macromolecules including the proteasome, the electron transport chain in the mitochondria, as well as inactivation of ALDH2 itself, leading to mitochondrial impairment and increases in oxidative stress [46]. Olive phenolics regulated, in addition to ALDH2, also proteasome activator complex and proteins involved in the electron transport chain (such as ATP sythase and electron transfer flavoprotein), indicating that they can affect pathways associated with ALDH2. This implies that modulation of ALDH2 activity may well represent a novel antioxidant mechanism for olive phenolics. Further studies are necessary to reveal how exactly regulation of hepatic protein and activity levels of mitochondrial ALDH2 by dα-tocopherol and olive phenolics affects antioxidant mechanisms.

4.2 Metabolic effects

Recent evidence suggests that phenolic acids from olives and olive oil may affect metabolic health. In a randomized multicenter crossover study in 200 subjects, phenolic-rich olive oils increased levels of HDL cholesterol, and lowered the ratio of total HDL cholesterol compared with olive oils low in phenolics [47]. In mice, glucose-stimulated insulin secretion from the pancreas was greater when fed olive oil compared with sunflower oil, an effect that coincided with increased activities of antioxidant enzymes such as catalase and glutathione peroxidase 1 [48]. In alloxan-induced diabetic rats, olive mill waste extracts decreased levels of plasma glucose and serum triglycerides and total cholesterol, but increased serum HDL cholesterol [43]. Furthermore, olive leaf extract rich in oleuropein and hydroxytyrosol prevented the increase in the abdominal fat pads and attenuated the increase in the abdominal circumference, improved oral glucose tolerance, lowered plasma total cholesterol and triglyceride concentrations, normalized hepatic macrovesicular steatosis, portal inflammation, and fibrosis in rats fed a high-carbohydrate and high-fat diet [49]. In our rats on a vitamin E-deficient diet, levels of plasma glucose tended to be lowered by hydroxytyrosol compared with the vitamin E-deficient group, but this effect did not reach statistical significance (Fig. 2). Proteomics analysis indicated up-regulation of fructose 1,6-bisphopshatase especially by DHPG and to a lesser extent by hydroxytyrosol. This may indicate an increased gluconeogenesis, which together with increased ketogenesis and increased fatty acid oxidation, is often observed in an insulin-resistant state. However, we did not observe regulation of other major gluconeogenesis, ketogenesis, or fatty acid oxidation enzymes (Table 1). Alperujo extract did, however, significantly lower

levels of plasma cholesterol, whereas hydroxytyrsol increased levels of plasma triglycerides (Fig. 3), without significantly affecting hepatic fat or hepatic triglyceride levels. Any findings relating to lipoprotein metabolism, however, have to be interpreted with caution as for this outcome, rats are not an optimal model for humans. Indeed, most of the cholesterol in rats would be transported in HDL. Thus, in this study, metabolic pathways were not majorly affected by the phenolics, in contrast to some other studies in rats [43,49]. However, the subtle effects we found on plasma glucose, cholesterol, and triglyceride levels may contribute to the finding that pathways with most regulated proteins mapped in KEGG, Wikipathways, and Reactome were involved in metabolism of lipids and lipoproteins, glycolysis and gluconeogenesis, and the citric acid cycle (Fig. 4). The lack of clear metabolic effects in the vitamin E-deficient animals may be explained by the fact that these animals were not made insulin resistant or diabetic, although levels of hepatic fat were increased compared with vitamin E-sufficient animals (Fig. 2). Another explanation may be that we dosed orally and used lower dietary levels of phenolics compared with other studies.

4.3 Levels of phenolic compounds in plasma and tissues

In humans, some phenolic compounds are relatively well absorbed in a dose-dependent manner [50], but 98% of these are present in plasma or urine in conjugated forms, mainly glucuronide and sulphate metabolites [51]. Relatively little is known about accumulation and availability of phenolic compounds in tissues. Therefore, in this study, we measured levels of the two main dietary intervention compounds, hydroxytyrosol and DPHG, as well as tyrosol and an important metabolite of hydroxytyrosol, homovanillic acid, in plasma and various tissues of the rats, using an HPLC method we recently validated in our laboratory [29]. This analysis provided a comprehensive overview of where these compounds accumulate, and to which level they are bioavailable in plasma and in the various tissues. Hydroxytyrosol appeared mostly available in plasma with relatively little accumulation of the compound in the tissues, apart from WAT and the testes of rats where the diet was supplemented with hydroxytyrosol and DHPG (Table 1). This finding is in agreement with a rat study that showed a fast and extensive uptake of radiolabeled hydroxytyrosol by most organs and tissues, followed by excretion of hydroxytyrosol and its metabolites into urine within 5 h [52]. In addition to being an important component of olive oil, hydroxytyrosol is also a well-known metabolite of dopamine. Therefore, levels in plasma and tissues may result both from dietary ingestion as well as from endogenous production [53]. Homovanillic acid is another metabolite of dopamine metabolism, but also a major metabolite of hydroxytyrosol [53]. Levels of homovanillic acid were highest in the brain, but predominantly in rats that were on a vitamin E-deficient diet (Table 1). Interestingly, levels of DHPG were also found to be increased in brains of animals that were on a vitamin E-deficient diet (Table 1). The oxidative stress caused by vitamin E deficiency could be responsible for the observed increase in levels of DHPG and homovanillic acid in the brain. Indeed, DHPG is formed endogenously from the stress hormone noradrenalin. Basal levels of urinary norepinephrine and epinephrine of aged vitamin E-deficient rats was two- to three-fold higher than those of control rats [54], whereas levels of norepinephrine in the brain and the heart were decreased in these rats, indicating an enhanced release or increased catabolism of norepinephrine [55]. Similarly, an increased turnover of dopamine, the precursor of homovanillic acid, has also been reported in the brain of rats on vitamin E-deficient diet [56]. We observed accumulation of high levels of tyrosol, especially in WAT but also in plasma and in testes of rats fed the alperujo extract and hydroxytyrosol. A study in humans has previously indicated that recovery of dietary tyrosol in urine was a lot lower (20%) compared with the recovery of dietary hydroxytyrosol (between 80 and 100%) from virgin olive oil [57]. Metabolism of tyrosol, compared with hydroxytyrosol, however, appears to be less extensive, at least in rats and in HepG2 cells [58]. Therefore, it could be that tyrosol, unlike hydroxytyrosol, is more likely to accumulate in tissues upon ingestion.

4.4 Conclusion

In this study, we show that olive phenolics have subtle antioxidant properties. These properties were achieved by a moderate dose of phenolics which was around ten times higher than the minimum dose of polyphenols (e.g., hydroxytyrosol and oleuropein complex) in olive (olive fruit, olive mill waste waters, or olive oil, *O. europaea* L. extract and leaf) which, according to a recent Scientific Opinion of the European Food and Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA), is required to substantiate the health claim "contributes to the protection of blood lipids from oxidative damage" (http://www.efsa.europa.eu/en/efsajournal/doc/2033.pdf). However, mechanisms may not necessarily involve hydrogen donation to reactive free radical species, at least in our oxidatively stressed rodent model. Instead, proteomics and network analysis revealed that regulation of ALDH2 and proteasome activators, as well as regulation of SOD, may represent novel and alternative antioxidant pathways by which olive phenolics could impact on cardiovascular health. Indeed, polyphenols are capable of transcriptional gene regulation and modulation of enzyme activities, and should thus be considered as versatile bioactives rather than mere antioxidants [44].

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The authors have no conflict of interest to declare.

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