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Extra Virgin Olive Oils Increase Hepatic Fat Accumulation and Hepatic Antioxidant Protein Levels in *APOE*$^{-/-}$ Mice

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We assessed the effects of Picual and Arbequina olive oil, rich and poor in polyphenols, respectively, on plasma lipid and glucose metabolism, hepatic fat content, and the hepatic proteome in female *Apoe*$^{-/-}$ mice. Both olive oils increased hepatic fat content and adipophilin levels ($p < 0.05$), though Picual olive oil significantly decreased plasma triglycerides ($p < 0.05$). Proteomics identified a range of hepatic antioxidant enzymes that were differentially regulated by both olive oils as compared with palm oil. We found a clear association between olive oil consumption and differential regulation of adipophilin and betaine homocysteine methyl transferase as modulators of hepatic triglyceride metabolism. Therefore, our "systems biology" approach revealed hitherto unrecognized insights into the triglyceride-lowering and anti-atherogenic mechanisms of extra virgin olive oils, wherein the up-regulation of a large array of anti-oxidant enzymes may offer sufficient protection against lesion development and diminish oxidative stress levels instigated by hepatic steatosis.

Keywords: proteomics • antioxidant enzymes • insulin resistance • adipophilin • betaine homocysteine methyltransferase

Introduction

The "Mediterranean Diet" is associated with a lower rate of coronary heart disease (CHD),[1,2] as well as a reduction in all-cause mortality.[3,4] Olive oil is the main source of fat in this type of diet. Low percentages (5−10%) of extra virgin olive oil (EVOO)-enriched diets on a low cholesterol background halted the progression of induced atherosclerosis in rabbits[5] and in female apolipoprotein E knockout (*Apoe*$^{-/-}$) mice,[6] although a higher percentage of dietary EVOO (20%) on a high cholesterol background diet failed to show a difference in atherosclerosis lesion development when compared with a carbohydrate-rich diet.[7]

The Mediterranean diet might protect against coronary heart disease by improving the lipoprotein profile.[8] The Mediterranean diet may also provide additional benefits by acting on other cardiovascular risk factors, including a lowering of blood pressure, and an improvement in insulin sensitivity both in healthy and in type 2 diabetic patients.[9] The beneficial effects of olive oil on both lipoprotein metabolism and potentially insulin resistance suggest that consumption of olive oil affects hepatic lipid and glucose metabolism. To gain understanding in the mechanisms by which olive oil fatty acids, or its minor antioxidant constituents, may affect hepatic metabolic pathways, oxidative stress and eventually atherogenesis, we have applied a systems biology approach. The physiological effects of the individual olive oils could be mediated through multiple biochemical and molecular mechanisms, stressing the need to extend the availability of relevant biomarkers to properly assess their effects. The current study was carried out in *Apoe*$^{-/-}$ mice, a well-characterized and widely used model that spontaneously develops atherosclerosis with features similar to those occurring in humans.[10]

Materials and Methods

The study protocols were approved by the Ethics Committee for Animal Research of the University of Zaragoza, Spain, and were conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" published by the NIH in 1985.

Animals and Diets. The design of this study has been described before.[11] Briefly, *Apoe*$^{-/-}$ animals were bred and kept at the Unidad Mixta de Investigación, Zaragoza, Spain. Animals were housed in sterile filter-top cages under 12 h light/dark

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cycles with *ad libitum* access to food and water. Twenty-four female *Apoe*$^{-/-}$ mice were randomly distributed into three experimental groups matched for baseline plasma cholesterol values. Mice were fed a base diet of standard mouse chow (B & K Universal Ltd., Humberside, UK) supplemented with 0.15% (w/w) cholesterol and either 20% (w/w) Picual EVOO (90 mg polyphenols/kg), 20% (w/w) Arbequina EVOO (25 mg of polyphenols/kg), or 20% (w/w) palm oil. The EVOOs were both from Spanish olive tree cultivars grown on the same field at the same time. The percentage of energy provided by monounsaturated fatty acids was 35% for the Picual olive oil diet, 33% for the Arbequina olive oil diet, and 18% of the palm oil diet. The diets were prepared weekly and stored under N_2 at −20 °C until use. The composition of the diet has been described previously.[11] At the end of the 10-week intervention period, the animals were killed by suffocation with CO_2 after an overnight fast and blood was obtained thereafter by cardiac puncture. The livers were removed, weighed, frozen in liquid nitrogen, and stored at −80 °C until analysis.

Plasma Analysis. Blood was centrifuged at 3000 rpm for 10 min and plasma was collected for the measurement of triglycerides,[11] non-esterified fatty acids (NEFA) (Waku, Madrid, Spain), insulin, and glucose (rat/mouse insulin ELISA kit, Linco Research, Missouri ; Glucose RTU, BioMerieux, Lyon, France) validated with standard controls (Calimat, BioMerieux, Lyon, France), according to the manufacturers' instructions. Fasting plasma insulin and glucose concentrations were used to calculate the biomarker insulin resistance from the homeostasis model assessment for insulin resistance (HOMA) [(glucose$_0$ * insulin$_0$)/22.5].[12] The biomarker insulin sensitivity was calculated with use of the revised quantitative insulin sensitivity check index (QUICKI) [1/(log insulin$_0$ + log glucose$_0$ + log NEFA$_0$)].[13]

Measurement of Hepatic Fat Content and Adipophilin Protein Levels. Paraffin-embedded liver sections (4 μm) were stained with hematoxylin and eosin and observed using a Nikon microscope without prior knowledge of the diet group. Hepatic fat content was evaluated by quantifying the extent of fat droplets in each liver section with Adobe Photoshop 7.0 and expressed as percentage of total liver section.

Hepatic adipophilin in cytosolic protein homogenates was measured by an in-house ELISA using a specific polyclonal antibody (Research Diagnostics) as previously described.[14]

Measurement of Hepatic Glutathione Peroxidase 1 (GPx1), Glutathione S-transferase (GST) and Thioredoxin Reductase (TR) Activity. Hepatic GPx1 and GST activities were measured by the methods described by Arthur et al.,[15] using 1,2 dichloro-4-nitrobenzene as a substrate for the GST activity assay. Hepatic TR activity was measured as described by Rigobello et al.[16] and adapted for measurement in a 96-well plate.

Proteomics. Cytosolic protein homogenates were prepared from each individual animal liver as described previously.[17,18] Proteins were separated by two-dimensional gel electrophoresis, and the gels were analyzed using PDQuest software (BioRad). Spots with densities that significantly differed between treatments were excised from the SDS-PAGE gels using the robotic BioRad spot cutter. These proteins were trypsinized using a protocol of the MassPrep Station (Micromass) and analyzed by MALDI-TOF and electrospray LC mass spectrometric methods as described.[17,18]

Statistical Analysis. Data are presented as means ± SD. Analysis of variance was carried out on plasma parameters and protein spot data, followed by post-hoc unpaired t-tests based on the pooled variance. Data were log-transformed before analysis when not normally distributed. Principal component analysis was performed after centring and unit variance (UV) scaling of the data in SIGMA P+ (Umetrics Ltd, UK). Analysis of correlations was done with Pearson correlation coefficients. The analysis of multiple hypotheses testing for many combinations of variables was done with by determining the false discovery rate or *q*-values[19] within Genstat (VSN intl Ltd., UK). *q*-values estimate the probability that a correlation that is called significant, is false positive. For example, a *q*-value of 0.05 would mean, that we should expect that 5 out of 100 associations that were tested significant, are in fact false positive.

Table 1. Fasting Plasma Triglycerides, Total Cholesterol, HDL Cholesterol, Nonesterified Fatty Acids (NEFA), Glucose and Insulin Concentrations, HOMA and Revised QUICKI, in Female Apolipoprotein E Knockout Mice Fed a High Fat High Cholesterol Diet Supplemented with 20% (w/w) Extra Virgin Picual Olive Oil, 20% (w/w) Extra Virgin Arbequina Olive Oil, and 20% (w/w) Palm Oil (Atherogenic Control Group) for 10 Weeks[a]

	Picual oil (n = 8)	Arbequina oil (n = 8)	palm oil (n = 8)
total triglycerides (mmol/L)	1.71 ± 0.64[b]	2.49 ± 0.79	2.21 ± 0.31
total cholesterol (mmol/L)	40.16 ± 1.89[c]	37.82 ± 3.75[c]	31.13 ± 4.04
HDL cholesterol (mmol/L)	1.42 ± 0.06[b]	1.22 ± 0.12	1.26 ± 0.16
NEFA (mg/dL)	5.00 ± 0.96[b]	4.41 ± 1.29	3.89 ± 0.60
glucose (mmol/L)	17.41 ± 3.58	18.25 ± 4.34*	14.52 ± 1.85
insulin (pmol/L)	101.57 ± 32.02	98.29 ± 22.04	96.61 ± 22.93
HOMA	11.26 ± 3.72	11.91 ± 3.84	8.91 ± 2.26
revised QUICKI	0.22 ± 0.01[c]	0.22 ± 0.01	0.23 ± 0.01

[a] Values represent the mean ± SD. HOMA: homeostasis model for insulin resistance; (revised) QUICKI: quantitative insulin sensitivity check index. [b] Significantly different from the palm oil group: $p < 0.05$. [c] Significantly different from the palm oil group: $p < 0.01$.

Results

Food Intake and Body Weights. Food intake and body weight gain did not differ between the three groups during the 10-week intervention period (data not shown). Fasting plasma triglycerides were 22% lower ($p < 0.05$) upon intervention with Picual EVOO,[11] and NEFA was 30% higher ($p < 0.05$) upon intervention with Picual EVOO, compared with palm oil (Table 1). Plasma glucose concentrations were 26% higher after intervention with Arbequina EVOO compared with palm oil ($p < 0.05$). Intervention with both EVOOs did not change plasma insulin concentrations compared with palm oil intervention, nor did it affect the homeostasis model assessment (HOMA) index of insulin resistance. However, intervention with Picual EVOO did decrease the revised quantitative insulin sensitivity check index of insulin sensitivity (revised QUICKI) ($p < 0.01$) compared with the palm oil group (Table 1).

Liver Weight, Hepatic Fat, and Hepatic Adipophilin. Average liver weight (expressed as percentage of total final body weight) was significantly higher in the Picual EVOO group (5.3 ± 0.4%, $p < 0.001$), but not in the Arbequina EVOO group (4.8 ± 0.6), compared with the palm oil group (4.4 ± 0.4%). Hepatic fat was significantly increased by Picual EVOO ($p < 0.01$) and Arbequina EVOO ($p < 0.05$), as compared with palm oil (Figure 1).

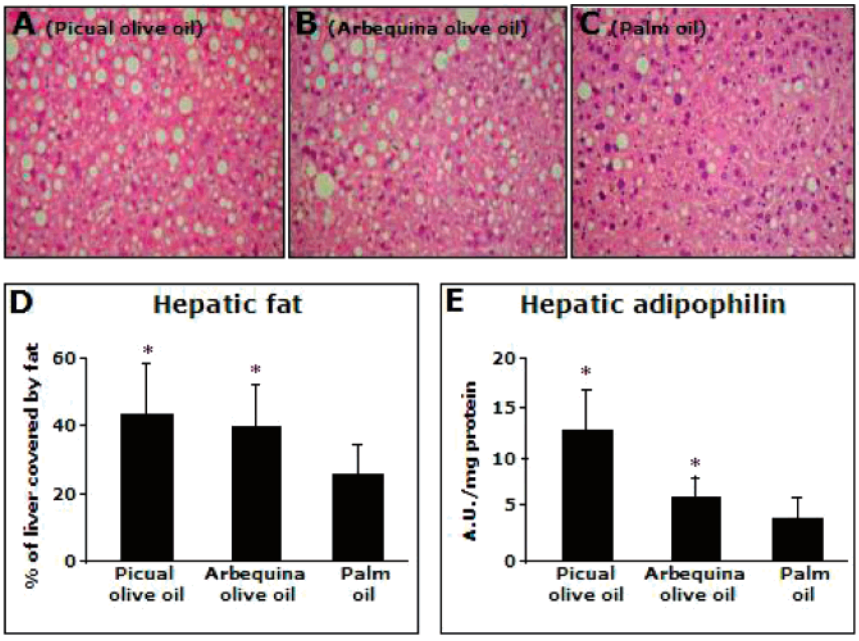

Figure 1. Representative images from a liver from (A) the Picual olive oil group, (B) the Arbequina olive oil group, (C) the palm oil group, stained with hematoxylin and eosin (magnification ×100) as well as the morphometric quantification of the liver fat content in each group (results are expressed as percentage of liver covered by fat in each section) (D) and the amount of hepatic adipophilin protein (E).

Hepatic adipophilin protein levels were significantly higher upon intervention with Picual EVOO ($p < 0.001$) and with Arbequina EVOO ($p < 0.05$), as compared with palm oil (Figure 1).

Hepatic GPx1, GST, and TR Activity. Hepatic GPx1 activity was significantly lower upon intervention with Picual EVOO (0.33 ± 0.04 U/mg protein, mean ± SD) and Arbequina EVOO (0.33 ± 0.03 U/mg protein) compared with palm oil intervention (0.40 ± 0.02) (both $p < 0.01$). Hepatic GST activity was not significantly affected by Picual EVOO (8.70 ± 3.54 μM/g protein) or Arbequina EVOO (6.37 ± 0.82 μM/g protein) as compared with palm oil intervention (6.04 μM/g protein). TR activity was significantly increased by Picual EVOO (1.11 ± 0.23 U/mg protein) ($p < 0.05$), but not by Arbequina EVOO (0.94 ± 0.24 U/mg protein) as compared with palm oil intervention (0.84 ± 0.22 U/mg protein).

Proteomics. Two-dimensional-gel electrophoresis of individual liver cytosolic protein fractions revealed 80 cytosolic proteins of which levels were significantly up- or down-regulated by Picual EVOO and Arbequina EVOO, compared with palm oil. The proteins identified were categorized according to their major biochemical functions to facilitate the elucidation of pattern changes between treatments (Table 2). Significant increases were observed in levels of a range of antioxidant enzymes, enzymes involved in carbohydrate metabolism, and enzymes involved in the methionine cycle or glutathione synthesis, mainly upon intervention with Arbequina EVOO.

Principal Component Analysis. Principal component analysis (PCA) is a mathematical procedure that transforms a number of correlated variables into a smaller number of uncorrelated variables, which may enable the detection of a structure in relationships between them. PCA of the proteomics results as well as physiological outcome parameters allowed us to reduce all initial variables into two principal components. These new uncorrelated factors that were successively extracted revealed that 32% of all variance in the dataset was accounted for by the first principal component (PC1), and an additional 12% was accounted for by the second principal component (PC2) (Figure 2, upper panel). The largest treatment effect on the first principal component (i.e., the largest distance between the spots representing the dietary intervention groups on the X-axis) was between the palm oil control group (red spots) and the Arbequina EVOO intervention group (blue spots). When considering the second principal component (which represents one or more other, independent variables), the Picual EVOO intervention group initiated a specific treatment effect compared with the palm oil control group. The loadings plot revealed that parameters with the highest loadings (i.e., the largest distance from the 0,0 point) which explained most of the treatment effect of the Picual olive oil (green letters) and Arbequina olive oil (blue letters) (Figure 2, lower panel). For Picual olive oil, the proteins that provided the largest contribution to the dietary treatment effects in the principle component analysis were mostly related to hepatic steatosis. For Arbequina olive oil, the proteins that provided the largest contribution to the dietary treatment effects in the principal component analysis were mostly related to oxidative stress, carbohydrate metabolism, and the methionine cycle/glutathione synthesis.

Pairwise Correlation Analysis. In addition to PCA, we performed a pairwise correlation analysis over the different treatments, including the physiological data measured in plasma lipid as well as the data on hepatic protein levels. Such an analysis shows which parameters vary in similar ways throughout the two EVOO treatments, but also highlights which of the parameters are differentially affected by the two EVOO cultivars. Figure 3 shows a network of all pairwise interactions for each of the EVOO diets with a Pearson correlation coefficient > 0.66, p value < 0.0005 and a q-value of 0.0025, using the software tool Cytoscape.[20]

Table 2. Measures of Confidence for Protein Identification and Characterization by MALDI-TOF and MS/MS Analysis of Hepatic Cytosolic Proteins Which Were Significantly Increased or Decreased upon Intervention with Picual Olive Oil or Arbequina Olive Oil, as Compared with Palm Oil

			MS/MS						peptide mass fingerprinting		fold-change[b]	
SSP	protein identification	accession	Mr Exp	Mr Theor	protein score[a]	matched peptides	peptide sequences	peptide charges	matched peptides	sequence coverage	Picual olive oil	Arbequina olive oil
oxidative stress												
0706	Heat shock protein 60 kDa	P63038	59.55	60.95	828	11	LSDGVAVLK	+2				4.4
							VGLQVVAVK	+2				
							VTDALNATR	+2				
							DIGNIISDAMK + Ox(M)	+2				
							NAGVEGSLIVEK	+2				
							VGGTSDVEVNEK	+2				
							TLNDELEIIEGMK + Ox(M)	+2				
							AAVEEGIVLGGGCALLR	+2				
							TLNDELEIIEGMKFDR + Ox(M)	+3				
							KPLVIIAEDVDGEALSTLVLNR	+3				
							LVQDVANNTNEEAGDGTTTATVLAR	+3				
1601	Heat shock protein 60 kDa	P63038	58.59	60.95	1051	14	LSDGVAVLK	+2				0.7
							VGLQVVAVK	+2				
							IGIEIIKR	+2				
							VTDALNATR	+2				
							NAGVEGSLIVEK	+2				
							VGGTSDVEVNEK	+2				
							DIGNIISDAMKK + Ox(M)	+2				
							TVIIEQSWGSPK	+2				
							GYISPYFINTSK	+3				
							TLNDELEIIEGMK + Ox(M)	+2				
							CEFQDAYVLLSEK	+2				
							DPGMGAMGGMGGGMGGGMF + 5 Ox(M)	+2				
							ISSVQSIVPALEIANAHR	+3				
							LVQDVANNTNEEAGDGTTTATVLAR	+3				
2717	HSP 75/TRAP-1 protein	Q9CQN1	75.04	80.21	107				13	22		4.0
0802	Heat shock protein 110 kDa	Q61699	111.37	96.41	584	8	AFSDPFVEAEK	+2			0.4	0.5
							VLATAFDTTLGGR	+2				
							FLEMCDDLLAR + Ox (M)	+2				
							NAVEEYVYEMR + Ox (M)	+2				
							EDIYAVEIVGGATR	+2				
							AGGIETIANEYSDR	+2				
							ELSTTLNADEAVTR	+2				
							NFTTEQVTAMLLSK + Ox (M)	+2				
4002	Thioredoxin peroxidase 2	P35700	23.55	22.18	64				5	24	2.0	2.5
3004	Glutathione peroxidase 1	Q5RJH8	22.43	22.29	339	7	NEEILNSLK	+2				0.8
							AHPLFTFLR	+2				
							NDIAWNFEK	+2				
							FLVGPDGVPVR	+2				
							DYTEMNDLQK + Ox (M)	+2				
							NALPTPSDDPTALMTDPK + Ox (M)	+2				
							NALPTPSDDPTALMTDPK + Ox (M)	+3				
4003	Superoxide dismutase	P08228	16.61	15.81	129	2	LACGVIGIAQ	+2				2.5
							DGVANVSIEDR	+2				
2004	Ferritin light chain 1	P29391	23.9	20.67	74				11	51		1.4

Table 2 (Continued)

			MS/MS						peptide mass fingerprinting		fold-change[b]	
SSP	protein identification	accession	Mr Exp	Mr Theor	protein scorae[a]	matched peptides	peptide sequences	peptide charges	matched peptides	sequence coverage	Picual olive oil	Arbequina olive oil
3105	Antioxidant protein (prdx3 product)	Q8K4K8	26.67	27.69	171	3	ELSLDDFK	+2				1.5
							GTAVVNGEFK	+2				
							HLSVNDLPVGR	+2				
3607	T complex polypeptide 1	P80314	55.26	60.45	66				8	17		0.8
4206	Haoo protein	Q78JT3	30.81	32.80	79				9	42	0.9	0.7
3508a	Aldehyde dehydrogenase	P47738	50.37	56.54	214	3	TIEEVVGR	+2				2.1
							VVGNPFDSR	+2				
							TEQGPQVDETQFK	+2				
3609	Aldehyde dehydrogenase	Q9DBF1	54.94	55.51	74				8	21	0.6	
2205	Alpha-ETF	Q99LC5	35.30	35.01	131				7	34		1.8
lipid metabolism												
1105	Apolipoprotein A−I	Q00623	25.85	30.59	346	5	LSPVAEEFR	+2				0.5
							DFANVYVDAVK	+2				
							VQPYLDEFQK	+2				
							VAPLGAELQESAR	+2				
							VKDFANVYVDAVK	+3				
2108	Apolipoprotein A−I precursor	Q00623	26.17	30.59	80				11	36		0.5
3003	Fatty acid-binding protein	P55050	13.81	14.24	54	1	LTITQDGNK	+2				1.5
8001	Fatty acid binding protein	P55050	14.29	14.24	78				5	57		1.7
2705	Carboxylesterase 1	Q8VCC2	61.4	62.68	78				10	21		4.4
3703	Carboxylesterase	Q63880	61.73	61.51	123	2	AISQSGVVISK	+2				1.4
							VTQPEVDTPLGR	+2				
4605	Acetyl CoA acyltransferase	Q8CAY6	42.26	44.81	76				9	29	1.9	2.8
2105	Phosphatidylcholine transfer protein	P53808	26.37	24.79	214	4	YPFPLSNR	+2				1.4
							QSLAIESDGK	+2				
							LLDQPSGLYEYK	+2				
							ESDEQMVAYWEVK + Ox(M)	+2				
3705	2-Hydroxyphytanoyl-CoA lyase	Q9QXE0	60.99	63.66	359	4	QLLEQFDK	+2			0.8	
							ALQSADVIVLFGAR	+2				
							DSFIVSEGANTMDIGR + Ox(M)	+2				
							NQEAMGAFQEFPQVEACR + Ox(M)	+3				
4710	Histidine ammonia-lyase	P35492	76.42	72.89	83	5			12	22	0.5	
glucose metabolism												
0303	Galactokinase	Q9R0N0	39.9	42.18	601	9	MEELEAGR + Ox(M)	+2			1.4	
							VEELLAEAR	+2				
							LAVLITNSNVR	+2				
							HSLGSSEYPVR	+2				
							TDGLVSLLTTSK	+2				
							SLETSLVPLSDPK	+2				
							SLETSLVPLSDPK	+3				
							AFMEEFGAEPELAVSAPGR + Ox(M)	+2				
							VNLIGEHTDYNQGLVLPMALELVTVMVGSPR + 2 Ox(M)	+4				
0610	Glucokinase	Q5SVI6	51.8	51.89	545	8	GILLNWTK	+2			4.9	4.7
							ITVGVDGSVYK	+2				
							VGEGEAGQWSVK	+2				
							FVSQVESDSGDR	+2				
							ASGAEGNNIVGLLR	+3				

Table 2 (Continued)

			MS/MS						peptide mass fingerprinting		fold-change[b]	
SSP	protein identification	accession	Mr Exp	Mr Theor	protein scorae[a]	matched peptides	peptide sequences	peptide charges	matched peptides	sequence coverage	Picual olive oil	Arbequina olive oil
							ASGAEGNNIVGLLR	+2				
							FVSQVESDSGDRR	+3				
							MVDESSVNPGQQLYEK + Ox(M)	+2				
1603	Glycerol kinase	Q8C2M1	53.95	60.56	67				10	25		1.9
2203	Malate dehydrogenase	P14152	34.41	36.38	205	3	LGVTADDVK	+2				1.8
							GEFITTVQQR	+2				
							FVEGLPINDFSR	+2				
3305	Malate dehydrogenase	P14152	37.03	36.38	135	2	DLDVAVLVGSMPR + Ox(M)	+2				present
							FVEGLPINDFSR	+2				
4103a	Hydroxypyruvate isomerase homolog	Q8R1F5	28.09	30.45	533	7	LVLINTPR	+2				1.5
							GEMGLGAVPGR + Ox(M)	+2				
							EGLEQAVLYAK	+2				
							GDTVEGLSWLR	+2				
							GEMEAVFVENLK + Ox(M)	+2				
							ITDPQYFLDTPR	+2				
							EFLPTVGHVQVAQVPDR	+3				
4302	Fructose-1,6-bisphosphatase	Q9QXD6	37.75	36.78	79				8	26		0.8
4306	Fructose-1,6-bisphosphatase	Q9QXD6	37.59	36.78	69				8	27	1.9	2.5
4602	Carbohydrate Kinase	Q8K0F0	54.37	42.28	103				10	35		1.9
Krebs cycle												
1407	Succinyl-CoA ligase β-chain	Q9Z2I9	44.39	50.11	484	8	LYNLFLK	+2				3.8
							LSEIVTLAK	+2				
							IVFSPEEAK	+2				
							SSDEAYAIAK	+2				
							EPIDIVEGIK	+2				
							INFDSNSAYR	+2				
							ICNQVLVCER	+2				
							ILACDDLDEAAK	+2				
1409	Succinate-CoA ligase	Q03184	44.57	34.99	79				6	17		0.7
2302	Isocitrate dehydrogenase 3α	Q6LCB4	40.04	39.63	66				6	19		0.7
methionine cycle/glutathione synthesis												
1507	Glutamate carboxypeptidase	O35409	52.35	84.64	65				7	20		absent
3001	S-adenosyl-L- methionine-dependent methyltransferase	Q9DCS2	24.91	22.68	70				6	34		1.9
3405	S-adenosyl-L-homocysteine hydrolase	P50247	44.74	47.55	69				8	16		3.0
5204	Betaine homocysteine methyltransferase	Q561N0	45.44	45.02	97				10	29	3.0	2.0
4407	Adenosyl homocysteinase	P50247	44.34	47.55	121				10	27	0.9	0.9
4604	Cysteine sulfinic acid decarboxylase	Q8K566	54.47	55.12	79				10	21	0.6	0.7
1506a	Glutathione synthase type A1	Q8R436	50.95	51.95	415	7	FAELQSPNK	+2				2.3
							TGQEIPVNLR	+2				
							LGGSVELVDIGK	+2				
							TVFGVEPDLTR	+2				

Table 2 (Continued)

			MS/MS						peptide mass fingerprinting		fold-change[b]	
SSP	protein identification	accession	Mr Exp	Mr Theor	protein scorae[a]	matched peptides	peptide sequences	peptide charges	matched peptides	sequence coverage	Picual olive oil	Arbequina olive oil
							MMEVAAADVQR + 2 Ox(M)	+2				
							AVFQYIDENQDR	+2				
							AVFQYIDENQDR	+2				
2204	Hydroxyacyl glutathione hydrolase	Q4N922	29.16	31.26	72				7	36		1.8
3603	Glutamate dehydrogenase	P26443	61.64	61.34	132				11	25		1.6
7101	Glutathione S-transferase P 1	GSTP1	23.60	23.48	70				7	40	1.9	1.9
2504	Methionine adenosyltransferase Iα	Q3THS6	49.81	43.69	253	3	SEFPWEVPK	+2			1.4	1.6
							FVIGGPQGDAGVTGR	+2				
							IHTIVISVQHNEDITLEAMQEALK + Ox(M)	+4				
phenylalanine catabolism												
3505	Phenylalanine-4-hydroxylase	P16331	49.53	51.80	101				11	25		0.8
2509	Phenylalanine-4-hydroxylase	P16331	49.09	51.80	78				8	29		2.8
3502	Homogentisate 1,2-dioxygenase	Q7TPP2	50.73	49.96	72				13	35		1.7
urea cycle												
3301	Ornithine carbamoyltransferase	Q8R1A8	39.85	39.36	104				8	24		1.7
methyltransferases												
2104	Thioether S-methyltransferase	P40936	28.08	29.46	177	3	FSGVYLEK	+2				2.1
							VYIGGEDYEK	+2				
							AIQDAGCQVLK	+2				
3109	Thiopurine S-methyltransferase	O55060	29.36	27.59	96				14	59	1.2	
1103	Guanidinoacetate N-methyltransferase	O35969	28.49	26.34	158	2	LQDWALR	+2			1.5	
							VLEVGFGMAIAASR + Ox(M)	+2				
transport proteins												
1710	Albumin 1	Q8C7C7	68.93	68.69	89				7	15		10.7
1718	Albumin 1	Q8C7C7	69.05	68.69	99				7	15		1.4
1721	Albumin 1	Q8C7C7	68.75	68.69	155				16	29	2.0	3.5
2702	Serum albumin	P07724	68.4	68.69	92				7	21		0.6
coagulation												
1406	Serpin B6	Q60854	43.54	42.60	507	8	TGTQYLLR	+2			6.7	13.3
							TNGILFCGR	+2				
							LGMTDAFGGR + Ox(M)	+2				
							MTYIGEIFTK + Ox(M)	+2				
							NEEKPVQMMFK + 2 Ox(M)	+3				
							GTTASQMAQALALDK + Ox(M)	+2				
							LEENYNMNDALYK + Ox(M)	+2				
							AFVEVNEEGTEAAAATAGMMTVR + 2 Ox(M)	+3				
2401	Serpin B6	Q60854	43.61	42.60	223	3	TGTQYLLR	+2				1.5
							MTYIGEIFTK + Oxidation (M)	+2				
							GTTASQMAQALALDK + Ox(M)	+2				

Table 2 (Continued)

			MS/MS						peptide mass fingerprinting		fold-change[b]	
SSP	protein identification	accession	Mr Exp	Mr Theor	protein scorae[a]	matched peptides	peptide sequences	peptide charges	matched peptides	sequence coverage	Picual olive oil	Arbequina olive oil
inflammation												
2709a	Epoxide hydrolase chain B	P34914	59.69	62.52	376	5	SEEALALPR	+2				1.4
							EMVTFLDK + Ox(M)	+2				
							ILVPALMVTAEK + Ox(M)	+2				
							YQIPALAQAGFR	+2				
							ATEIGGILVNTPEDPNLSK	+2				
4102	Acyl-protein thioesterase 1	P97823	25.95	24.69	184	3	ALIDQEVK	+2				1.4
							ALINPANVTFK	+2				
							ASFSQGPINSANR	+2				
4701	Leukotriene A-4 hydrolase	P24527	68.74	69.47	131				14	25		1.5
1806	Valosin containing protein	Q01853	89.42	89.37	72				10	16		1.6
protein metabolism												
1108	Proteasome beta 4 subunit	P99026	26.69	26.92	65				5	25		1.5
2305	Pregnancy zone protein	Q61838	35.97	165.88	2305	8	YNILPVADGK	+2				1.4
							LQDQPNIQR	+2				
							DLSSSDLSTASK	+2				
							LPDLPGNYVTK	+2				
							MVSGFIPMKPSVK + 2 Ox (M)	+3				
							GSGSGCVYLQTSLK	+2				
							EVLVTIESSGTFSK	+2				
							APFALQVNTLPLNFDK	+2				
1716	Lysyl-tRNA synthetase	Q8C1V4	62.59	67.80	254	5	FELFVMK + Ox (M)	+2				4.0
							MLVVGGIDR + Ox (M)	+2				
							ISMVEELEK + Ox (M)	+2				
							GDIIGVEGNPGK	+2				
							LTMFLTDSNNIK + Ox (M)	+2				
3707	Glycyl-tRNA synthetase	Q9CZD3	76.4	81.87	97				13	20		0.5
4202	3-Hydroxyisobutyrate dehydrogenase	Q99L13	30.67	35.44	381	6	DLGLAQDSATSTK	+2				1.2
							EAGEQVASSPAEVAEK	+2				
							GSLLIDSSTIDPSVSK	+2				
							MGAVFMDAPVSGGVGAAR + 2 Ox(M)	+3				
							MGAVFMDAPVSGGVGAAR + 2 Ox(M)	+2				
							TPVGFIGLGNMGNPMAK + 2 Ox(M)''	+2				
miscellaneous												
0101	Rho GDP dissociation inhibitor α	Q5M9P6	26.01	23.41	327	4	TDYMVGSYGPR + Ox(M)	+2			1.7	1.7
							IDKTDYMVGSYGPR + Ox(M)	+3				
							VAVSADPNVPNVIVTR	+2				
							AEEYEFLTPMEEAPK + Ox(M)	+2				
1303	Potassium channel tetramerisation domain containing protein 12	Q6WVG3	36.53	35.89	67				7	26		0.7
2101	Sepiapterin reductase	Q64105	27.36	27.88	871	11	ELPRPEGLQR	+2			1.3	1.2
							EELGAQQPDLK	+2				
							SDGALVDCGTSAQK	+2				
							LLLINNAATLGDVSK	2				

Table 2 (Continued)

			MS/MS						peptide mass fingerprinting		fold-change[b]	
SSP	protein identification	accession	Mr Exp	Mr Theor	protein scorae[a]	matched peptides	peptide sequences	peptide charges	matched peptides	sequence coverage	Picual olive oil	Arbequina olive oil
							miscellaneous					
							VVLAAADLGTEAGVQR	+2				
							QLKEELGAQQPDLK	+3				
							TVVNISSLCALQPYK	+2				
							DMLYQVLAAEEPSVR + Ox(M)	+2				
							MEADGLGCAVCVLTGASR	+2				
							MEADGLGCAVCVLTGASR	+2				
							VLSYAPGPLDNDMQQLAR + Ox(M)	+2				
2106	Purine nucleotide phosphorylase	P23492	29.94	32.28	83				9	40		1.3
2304	Pyridoxal kinase	Q8K183	37.2	35.01	116	2	YDYVLTGYTR	+2			0.7	0.7
							SFLAMVVDIVR + Ox(M)	+2				
2306	Dhdh protein	Q8K0E9	39.5	36.64	315	6	AIGVTFPQDK	+2				2.7
							FFPAMEALR	+2				
							FFPAMEALR + Ox(M)	+2				
							AYGSYEELAK	+2				
							EVLVQGTIGDLR	+2				
							AEFGFDLSHIPR	+3				
2604	Selenium binding protein	Q91X87	52.4	52.51	64				8	19		2.1
3204	Lactamase, beta 2	Q99KR3	31.82	32.75	229	4	SFTVTELR	+2			present	present
							IFYTTTPVK	+2				
							EEQIISLFR	+2				
							NINNDTTYCIK	+2				
3206	Ligninase H2	P14153	34.09	39.52	540	8	LGVTADDVK	+2				1.3
							EVGVYEALK	+2				
							ENFSCLTR	+2				
							GEFITTVQQR	+2				
							DLDVAVLVGSMPR + Ox(M)	+2				
							FVEGLPINDFSR	+2				
							ELTEEKETAFEFLSSA	+2				
							NVIIWGNHSSTQYPDVNHAK	+3				
3203	Sulfotransferase 3A1	O35403	33.16	35.18	219	4	NEMGSFLR + Ox(M)	+2			2.0	2.5
							TENIETIDR	+2				
							DDDIFIVTYPK	+2				
							ELSEEDVDAVVR	+2				
4101	Nucleoside-diphosphate kinase	Q01768	17.46	17.36	70				4	26		1.5

[a] Probability-based Mowse score for MS/MS based identifications: individual ions scores >35 indicate identity or extensive homology ($p < 0.05$). Probability Based Mowse Score for peptide mass fingerprinting based identifications: protein scores greater than 62 are considered significant ($p < 0.05$). The protein score is $-10*\mathrm{Log}(P)$, where P is the probability that the observed match is a random event. [b] Fold-change in hepatic protein levels (as assessed by proteomics) of proteins that were significantly up-or down regulated by Picual and/or Arbequina olive oil, as compared with palm oil.

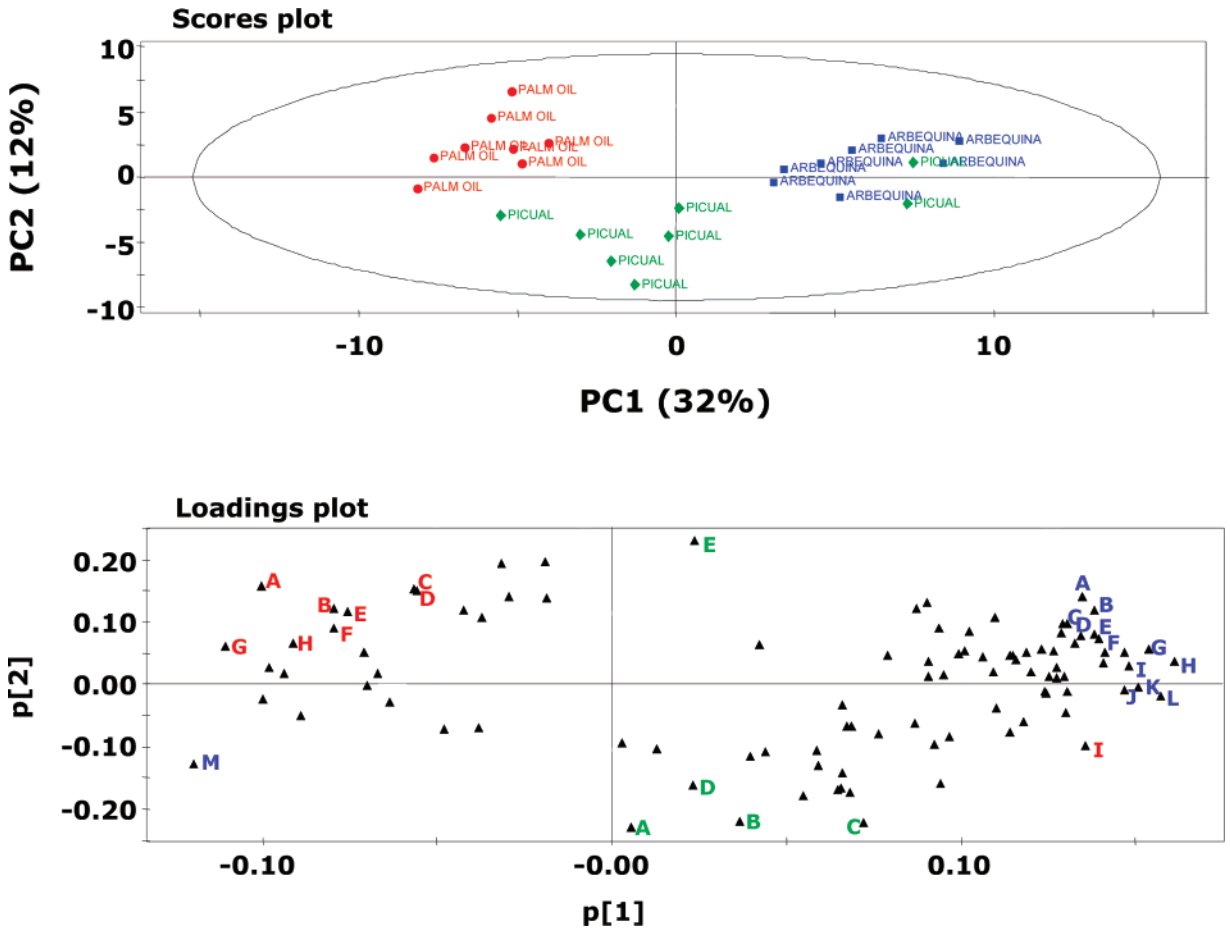

Figure 2. Scores plot (upper panel) and loadings plot (lower panel) of an unsupervised principal component analyses of hepatic cytosolic proteins of which levels were significantly up-or down regulated upon intervention with Picual olive oil or Arbequina olive oil, as compared with palm oil. The scores plot reveals the treatment effects between each of EVOO interventions compared with the palm oil intervention (i.e., the largest distance between the spots representing the dietary intervention groups on the X-axis) on the first and second principal component. The loadings plot reveals the proteins and physiological outcome parameters that provided the largest positive and negative contribution to the dietary treatment effects in the principal component analysis. These were for palm oil (in RED): A = lesion size, B = GPx1 activity, C = cysteinic sulfinic acid decarboxylase, D = succinyl CoA ligase, E = adenosylhomocysteinase, F = revised QUICKI, G = glycyl-tRNA synthetase, H = HSP110, I = lactamase beta; for Picual olive oil (in GREEN): A = adipophilin (Western Blot), B = adipophilin (ELISA), C = liver weight, D = GST activity, E = 2-hydroxyphytanoyl CoA lyase; for arbequina olive oil (in BLUE): A = malate dehydrogenase, b = acyl-protein thioesterase, c = phosphatidylcholine transfer protein, D = superoxide dismutase, E = S-adenosyl-L-methionine dependent methyltransferase, F = S-adenosyl-L-homocysteine hydrolase, G = thioether-S-methyltransferase, H = aldehyde dehydrogenase, I = glutathione synthase, J = thioperoxin peroxidase, K = methionine adenosyltransferase, L = fructose 1,6-bisphosphatase, M = glutamate carboxypeptidase.

Discussion

In this study, both olive oils increased liver size and hepatic fat content, probably through an increased supply of NEFA to the liver and a decreased output of triglycerides from the liver, and no apparent changes in levels of hepatic β-oxidation enzymes as assessed by proteomics. Proteomics identified a range of antioxidant enzymes that were differentially regulated by both olive oils as compared with palm oil.

Mediterranean populations who generally consume a diet high in olive oils that is rich in monounsaturated fatty acids (MUFA) have a low prevalence of CHD and low plasma cholesterol levels.[21] The substitution of a high-MUFA diet for an average American diet lowers total and low-density lipoprotein (LDL) cholesterol in humans,[22] thereby contributing to a reduction in CHD risk. In contrast, both EVOO diets significantly increased plasma cholesterol levels in *Apoe*$^{-/-}$ mice, despite causing a significant decrease in aortic root lesion size and a decreased degree of macrophage infiltration in to the intima.[11] An olive oil diet low in cholesterol, compared with a coconut oil diet, had no significant effects on total cholesterol whereas lesion size was significantly decreased in female *Apoe*$^{-/-}$ animals only.[6] This corroborates our previous observation that dietary cholesterol suppresses the ability of EVOO to improve the lipoprotein profile in an animal model that is extremely sensitive to diet-induced hyperlipidemia because of the absence of the apolipoprotein E protein.[10] However, HDL cholesterol was increased upon consumption of Picual EVOO.[11] Increased HDL concentrations are considered anti-atherogenic because of their ability to promote the efflux of cholesterol from cells, but they may also have antioxidant, anti-inflammatory, and anti-thrombotic properties.[23] In this study, both EVOOs did not significantly affect the HDL related proteins paraoxonase activity and apoA-I levels, but both EVOOs did induce a cholesterol-poor, apoA-IV enriched lipoparticle that had enhanced arylesterase and antioxidant activities.[11] Furthermore, Picual EVOO decreased plasma triglycerides, a finding that has

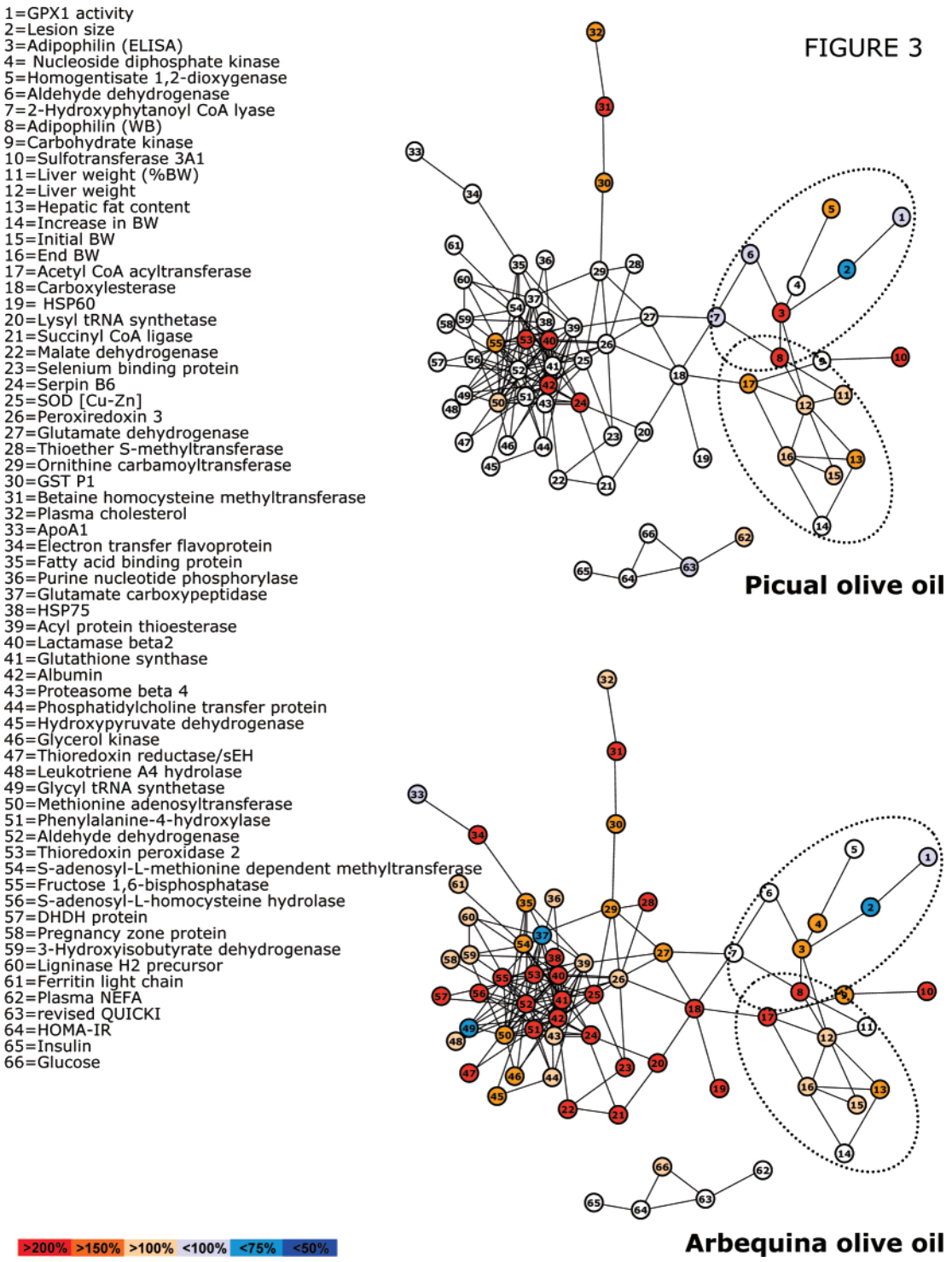

Figure 3. Correlation plot indicating all pairwise correlations between plasma lipid and liver protein levels that had a Pearson correlation higher than 0.66, a *p* value lower than 0.0005, and a *q*-value lower than 0.0025, using the software tool Cytoscape as described in Materials and Methods. The color code indicates the percentage increase or decrease in plasma levels or hepatic protein mass in each dietary olive oil intervention group as compared to the palm oil control group.

been observed before.[6] The decrease in triglycerides in our study was observed despite a significant increase in plasma NEFA (Table 1) and a significant increase in hepatic fat content (Figure 1).

This is, to our knowledge, the first study that reports an increase in hepatic fat accumulation upon consumption of EVOO in this mouse model of atherosclerosis. It has been found previously that diets rich in olive oil generally cause higher concentrations of liver total cholesterol compared with diets containing either saturated or polyunsaturated fatty acids, which may, however, occur only when large amounts cholesterol are fed in the diets.[24,25] Also the amount of dietary fat administered will be crucial since an amount of 10% (w/w) dietary olive oil prevented the development of fatty livers in a previous study.[26] Liver weight was significantly higher upon consumption of Picual EVOO, and the hepatic fat content was

significantly increased upon consumption of both Picual and Arbequina EVOOs. In addition, there were significant five-and 2-fold increases in hepatic adipophilin protein levels upon consumption of Picual and Arbequina EVOO, respectively (Figure 1). Adipophilin (or adipose differentiation-related protein) is bound to the surface of lipid bodies and lipid bilayers.[27] The proposed function of adipophilin appears more complex than merely the packaging of neutral lipids in the cytosol. Adipophilin overexpression in primary liver cells increases the size of cytosolic lipid droplets and reduces the secretion of VLDL, without influencing the rate of β-oxidation, thereby selectively decreasing VLDL assembly.[28] On the other hand, a knockdown of adipophilin decreased the pool of cytosolic lipid droplets, increased the secretion rate of apolipoprotein B-48 $VLDL_1$, and increased β-oxidation.[28] Also, adipophilin knockout in mice had lower levels of hepatic triglycerides without a change in plasma lipid profile, and these animals were protected against the development of a fatty liver.[29] Therefore, down-regulation of adipophilin protein causes a channeling of fatty acids primarily into β-oxidation. Interestingly, we found that plasma triglycerides were lowest and accumulation of hepatic lipid droplets was highest in the Picual EVOO group that had the highest levels of hepatic adipophilin protein, without a noticeable effect on hepatic proteins involved in β-oxidation of fatty acids. Indeed, hepatic adipophilin protein levels and liver weight were two of the main parameters responsible for the treatment effect of Picual olive oil in the principal component analysis (Figure 3), and hepatic adipophilin protein levels and plasma triglycerides were inversely correlated ($r = -0.433$, $p < 0.05$). The two EVOOs produced differential effects on hepatic adipophilin protein, possibly because of a different dietary oleic acid content or a difference in the amount and types of polyphenols. In one of our earlier studies, a decrease in hepatic adipophilin protein was associated with decreased serum and hepatic triglycerides and increased β-oxidation of fatty acids upon consumption of fish oil.[18] Therefore, regulation of adipophilin protein by dietary fatty acids or other compounds may represent an important regulatory pathway in hepatic lipid metabolism, albeit that regulation of adipophilin upon dietary intervention may be species dependent.

Abnormalities in lipid storage in hepatic and adipose tissue gives rise to a multitude of undesirable effects including increased levels of plasma lipids, including NEFA, and insulin resistance.[30] The reason for the increase in plasma NEFA levels in particularly the Picual olive oil group as compared with the palm oil group is not clear, but might involve the disturbance of hormonal regulation of lipolysis in adipose tissue, possibly due to a decrease in insulin sensitivity.[31] Indeed, a significant increase in plasma NEFA and a decrease in insulin sensitivity occurred in the Picual EVOO group that had also the highest levels of hepatic fat and adipophilin protein. In addition, we observed significant increases in several hepatic enzymes involved in the methionine cycle, especially in betaine homocysteine methyl transferase (BHMT), by both EVOOs (Table 2). BHMT, which converts homocysteine into methionine using betaine as a cofactor (Figure 4), is up-regulated during insulin resistance.[32,33] Also, an increase in hepatic BHMT is associated with an increase in hepatic very low-density lipoprotein (VLDL) and apolipoprotein B production rate in rats fed a methionine-deficient diet supplemented with betaine[34] (Figure 4). Increased secretion rates of $VLDL_1$-apolipoprotein B and triglycerides, as assessed by kinetic studies in humans, are a common feature

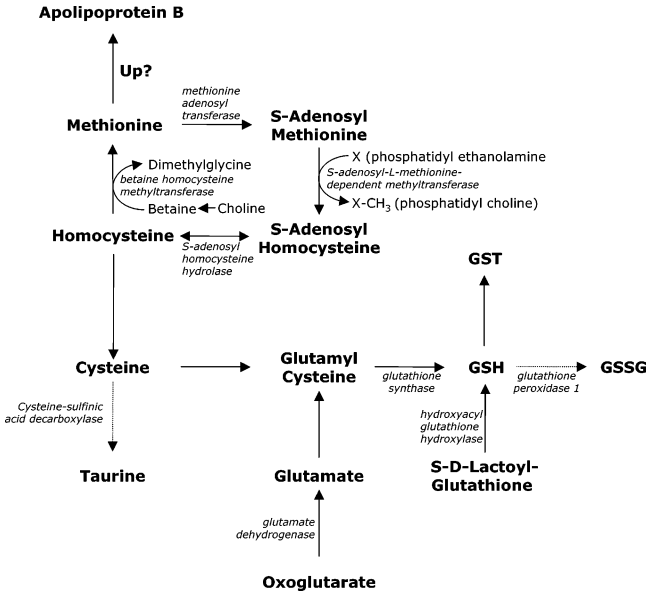

Figure 4. Schematic overview of pathways involved in methionine and glutathione metabolism, of which some were differentially regulated by Picual and Arbequina olive oil intervention, as compared with palm oil intervention.

in insulin resistance. The hepatic VLDL overproduction is believed to be driven by the altered free flow of NEFAs,[35] but BHMT may well be involved in this process. However, despite an increase in plasma NEFA upon consumption of Picual EVOO, plasma triglycerides were actually lower (Table 1), suggesting that VLDL-apolipoprotein B secretion was also likely to be lower. This suggests that an overriding mechanism, possibly involving adipophilin, is preventing the hepatic triglycerides from being secreted.

EVOOs contains a wide range of important minor antioxidant compounds, such as polyphenols, that contribute to the stability of the oil and that can have anti-inflammatory and anti-atherosclerotic properties.[36,37] Consumption of polyphenols has been associated with prevention from and/or treatment of atherosclerosis.[38] Our proteomics approach revealed a range of antioxidant enzymes, like TR, thioredoxin peroxidase 2, peroxiredoxin 3, superoxide dismutase and GST, whose hepatic protein levels were 1.5−4-fold higher upon consumption of both EVOOs compared with palm oil. In addition, hepatic TR activity was significantly higher upon consumption of Picual EVOO, confirming the proteomics findings. A similar coordinated induction of the antioxidant enzymes has been observed in *Apoe*$^{-/-}$ mice in the period preceding lesion formation.[39] Remarkably, most mRNA levels of these antioxidant enzymes started to decline as lesions started to develop. This suggests that the arterial wall delays initial lesion formation by stimulating the expression of antioxidant enzymes, but when this defense capacity collapses a greatly accelerated development of atherosclerosis occurs.[39] Therefore, compounds in EVOO (like for example the polyphenols) may well be able to delay the onset of atherosclerotic lesions by the combat of oxidative stress.

In contrast, protein and activity levels of hepatic GPx1 were lower upon olive oil consumption. In patients with coronary artery disease, a low level of red-cell GPx1 activity is independently associated with an increased risk of cardiovascular events.[40] However, it is questionable whether GPx1 plays a key role in lesion formation at the site of the aortic root. A specific

deficiency in GPx1 was not accompanied by an increase in markers of oxidative damage or increased atherosclerosis.[41] Also, GPx1 expression in the aortic arch of *Apoe*$^{-/-}$ mice, as compared with wild type mice, decreases rapidly in the period preceding lesion development, resulting in hardly any detectable GPx1 expression in atherosclerotic tissue.[39] Moreover, GPx1 enzyme activity was absent in human atherosclerotic lesions.[42] However, based on previous studies we cannot rule out a role for GPx1 in atherogenesis at other sites.[41] In our study, the pairwise correlation analysis revealed a clustering of hepatic GPx1 activity with lesion size, hepatic adipophilin levels, liver weight, hepatic fat content and body weight, indicating for the first time that their regulatory mechanisms may be related to each other (Figure 3). GPx1 may indeed play different roles in different organs,[43] and the lower protein and activity levels of hepatic GPx1 upon olive oil consumption may simply reflect a lower degree of hepatic oxidative stress due to the up-regulation of other antioxidant enzymes.

Proteomics also revealed the up-regulation of glutamate dehydrogenase, glutathione synthase, and hydroxyacyl glutathione hydrolase, as well as the down-regulation of cysteine-sulfinic acid decarboxylase, indicating an increased production of glutathione by predominantly Arbequina EVOO (Table 2 and Figure 4). Other studies in *Apoe*$^{-/-}$ mice showed that depletion of glutathione via a decreased synthesis precedes lipid peroxidation and atherogenesis, and glutathione is severely depleted in the atheroma-prone aortic arch of male compared with wild type mice after 10 weeks.[44] Therefore, glutathione deficiency might be central to the failure of the intracellular antioxidant defenses and is causally implicated in the pathogenesis of atherosclerosis. The apparent increase in glutathione production and other antioxidant defense mechanisms upon consumption of EVOO may thus represent a mechanism by which olive oil components diminish oxidative stress.

In conclusion, olive oil decreased atherosclerosis in Apoe$^{-/-}$ mice despite an increase in plasma total cholesterol and the development of hepatic steatosis. These are conflicting findings, and it would be intriguing if olive oil consumption would affect the interaction between similar mechanisms in humans, as hepatic lipid loading over a longer time period may have detrimental effects. A systems biology approach, as presented here, is crucial in unraveling the complex interactions between pathways that are on one hand involved in the beneficial reduction in plaque formation and on the other hand involved in the potentially less beneficial development of hepatic steatosis. We found, for example, a significant up-regulation of a large array of antioxidant enzymes upon consumption of EVOO that may diminish oxidative stress instigated by hepatic steatosis and in addition, may slow down the development of atherosclerosis. Indeed, the accumulation of triglycerides may not pose a major challenge to the liver, and represent a relatively safe way to store triglycerides, as long as the antioxidant capacity is adequate to prevent lipotoxicity.[45] In addition, our proteomics results revealed, for the first time, that two different EVOOs instigated distinct effects on hepatic lipid metabolism by regulation of hepatic adipophilin. This mechanism may override any regulation by insulin or BHMT to increase the production of VLDL apolipoprotein B that is normally observed upon development of hepatic steatosis and early symptoms of insulin resistance.

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