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Dietary oil modifies the plasma proteome during aging in the rat

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Abstract Fatty acids and other components of the diet may modulate, among others, mechanisms involved in homeostasis, aging, and age-related diseases. Using a proteomic approach, we have studied how dietary oil affected plasma proteins in young (6 months) or old (24 months) rats fed lifelong with two experimental diets enriched in either sunflower or virgin olive oil. After the depletion of the most abundant proteins, levels of less abundant proteins were studied using two-dimensional electrophoresis and mass spectrometry. Our results showed that compared with the sunflower oil diet, the virgin olive oil diet induced significant decreases of plasma levels of acute phase proteins such as inter-alpha inhibitor H4P heavy chain (at 6 months), hemopexin precursor (at 6 and 24 months), preprohaptoglobin precursor (at 6 and 24 months), and α-2-HS glycoprotein (at 6 and 24 months); antioxidant proteins such as type II peroxiredoxin (at 24 months); proteins related with coagulation such as fibrinogen γ-chain precursor (at 24 months), T-kininogen 1 precursor (at 6 and 24 months), and apolipoprotein H (at 6 and 24 months); or with lipid metabolism and transport such as apolipoprotein E (at 6 and 24 months) and apolipoprotein A-IV (at 24 months). The same diet increased the levels of apolipoprotein A-1 (at 6 and 24 months), diminishing in general the changes that occurred with age. Our unbiased analysis reinforces the beneficial role of a diet rich in virgin olive oil compared with a diet rich in sunflower oil, modulating inflammation, homeostasis, oxidative stress, and cardiovascular risk during aging.

Keywords Fatty acids · Olive oil · Plasma proteome · Rat · Sunflower oil

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Introduction

Aging is an endogenous process characterized by a progressive loss of function with decreased fertility,

increased mortality, and augmented susceptibility to the onset of age-related diseases (Kirkwood and Austad 2000). The impact of the diet and dietary components on aging and age-associated degenerative diseases has been widely recognized in recent years (Finkel and Holbrook 2000; Meydani 2001). Therefore, prevention through the introduction of lifestyle and proper nutrition habits ("functional foods") is now considered a primary strategy for what we call healthy aging.

It is well accepted that the Mediterranean diet has many beneficial effects on health, preventing cancer, coronary heart disease, and cognitive impairment, and many of the health effects of this diet have been attributed to virgin olive oil (VOO; Owen et al. 2000; Pérez-Jiménez et al. 2007). Beneficial consequences of VOO consumption can be explained partially on the basis of the antioxidant action of molecules contained in this oil (Owen et al. 2000). In this way, the high content (over 70%) of the monounsaturated fatty acid, oleic acid, is very important because it is much less susceptible to oxidation than the polyunsaturated fatty acid, linoleic acid, which predominates in sunflower oil (SO; Bello et al. 2006). In addition to oleic acid, some of the minor constituents of VOO also behave as antioxidants and are believed to play major roles determining the overall antioxidant effect of this oil (Owen et al. 2000). Recently, it has been recognized that VOO phenols display some pharmacological effects (Waterman and Lockwood 2007). Most of the minor components of VOO show antioxidant, anti-inflammatory, and/or hypolipidemic properties. Their mechanism involves the release of nitric oxide, eicosanoids (prostaglandins and leukotrienes), and adhesion molecules, in most cases by modulating the activation of nuclear factor κB by reactive oxygen species (Perona et al. 2006). It is thus clear that besides its antioxidant role, dietary VOO shows other biological activities that may contribute to a nutritional state that prevents disease by the reduction of inflammatory and autoimmune disorders or by diminution of cancer incidence (Jiménez-Gémez et al. 2009; Materljan et al. 2009). In addition, recent investigations have shown that the Mediterranean diet has beneficial effects on adiposity and type 2 diabetes, and this could help fight against diseases related to chronic inflammation, including metabolic syndrome (Babio et al. 2009).

On the other hand, dietary fatty acids strongly determine the susceptibility of lipoproteins to oxidation, which also has an impact on the activation of adhesion molecules and other pro-inflammatory factors. Moreover, several works have demonstrated a direct effect of fatty acids on the gene expression of many of those factors (Schroeder et al. 2008). Finally, certain aspects of blood platelet function, blood coagulability, and fibrinoliytic activity associated with cardiovascular risk are modulated by dietary fatty acids, *n*-3 fatty acids strongly inhibiting platelet aggregation and activating thrombolytic processes (Vanschoonbeek et al. 2004).

The proteomic approach offers an unbiased way to study changes in protein levels induced by different experimental conditions. This approach has been revealed successful for the identification of novel mechanisms of cell responses against oxidative stress (Martinez-Pinna et al. 2010). A major challenge of proteome research is detecting clinically useful biomarkers of disease, treatment response, and aging (Anderson 2005). Serum and plasma offer particularly promising resources for biomarker discovery because the collection of these samples is minimally invasive and the blood is thought to contain the majority of protein constituents found in the body (Echan et al. 2005). To better understand the possible mechanisms of action of dietary oils in rats, we have initiated a proteomic study to identify plasma proteins whose levels are altered due to different diets during aging. Our results support a beneficial role for VOO modulating inflammation, homeostasis, oxidative stress, and cardiovascular risk during aging.

Methods

Animals, diets, and blood samples

Animals and diets used here were as documented in previous works (Bello et al. 2006; Santos-González et al. 2007; Quiles et al. 2010). Groups of ten male Wistar rats (*Rattus norvegicus*) initially weighing 80–90 g were housed under standard conditions and maintained on a 12-h light/12-h darkness cycle and at a temperature of 22±1°C. Animals were provided ad libitum access to water and were randomly assigned into two experimental groups that were fed from weaning until 24 months of age on a semi-synthetic and isoenergetic diet according to the AIN93 criteria composed of (in grams per 100 g of diet): 26.7 casein,

13.53 starch, 45.29 sucrose, 1.0 vitamin mixture, 3.68 mineral mixture, 1.84 cellulose, 0.09 choline, 0.30 methionine, and 8.0 fat. Experimental diets differed only in the dietary fat source (VOO or SO, see Table 1). Eight rats per group were killed by cervical dislocation and further decapitation at 6 months (young) or 24 months (old) from the start of the experiment. Blood was collected by decantation onto glass tubes coated with lithium heparin, and plasma was then recovered by centrifugation. No evidence of haemolysis was observed. Samples were immediately frozen in liquid nitrogen and then stored at −80°C for later analysis. Animals were handled at the facilities of the University of Granada (Spain) according with guidelines of the Spanish Society for Laboratory Animals, and the experiment had the approval of the Ethical Committee of the University of Granada.

Proteomic analysis

For protein analysis, plasma samples were pooled according to dietary oil (VOO or SO) and to age and duration of dietary intervention (6 or 24 months, n=8). Protein content was measured by the dye binding method described by Stoscheck (1990) with bovine γ-globulin as standard. For proteomic analysis, aliquots of pooled samples (60 μL) were depleted from albumin and immunoglobulins using commercial disposable affinity columns (Proteoextract Albumin/IgG removal kit, Calbiochem) according to the manufacturer's recommendations. Afterwards, lipids and salts were removed from the samples to improve the resolution of two-dimensional gels. Delipidation and salt depletion were accomplished by mixing one volume of plasma sample (that had been depleted previously from albumin and IgG) with four volumes of methanol. After thoroughly mixing with the vortex, one volume of chloroform was added, the mixture was vortexed again, and then three volumes of water (HPLC grade) were incorporated. After centrifugation at 12,000 rpm for 5 min at 4°C, the upper phase was discarded. Three volumes of methanol were then added and the resultant mixture was vortexed again and centrifuged. The supernatant was discarded and the pellet containing precipitated plasma protein was air-dried for 5–10 min. About 0.7 mg of delipidated and salt-depleted plasma proteins were dissolved in 125 μL of sample solubilization solution (8.0 M urea, 50 mM DTT, 4% CHAPS, 0.2% carrier ampholytes, and 0.0002% bromophenol blue). IPG strips (7 cm long, pH 4–7, Bio-Rad, Hercules, CA, USA) were then passively rehydrated for 13 h with the protein lysate. Isoelectrofocusing was carried out at 20°C using a PROTEAN IEF system (Bio-Rad). Focusing

Table 1 Composition of the experimental diets used in this study

	VOO	SO
Fatty acid composition (g/100 g)		
C16:0	8.9	12.6
C16:1n7	1.1	0.2
C18:0	1.9	1.9
C18:1n9	78.7	24.1
C18:2n6	8.4	60.1
C18:3n3	0.9	1
Total saturated	10.9	14.6
Total unsaturated	89.1	85.4
Total monounsaturated	79.8	24.3
Total polyunsaturated	9.3	61.1
C18:1n9/C18:2n6	9.4	0.4
Phenolic profile (mg/kg)		
Hydroxytyrosol	0.17	
Tyrosol	0.23	
Dialdehydic form of elenolic acid linked to hydroxytyrosol	367.2	
Dialdehydic form of elenolic acid linked to tyrosol	25.5	
Oleuropein aglycon	87.3	

Data depicted in this table are taken from Quiles et al. (2010), with permission from the Editorial

was started with a conditioning step of 250 V for 20 min, followed by a voltage ramping step to 4,000 V for 2 h, and a final focusing step of 10,000 Vh. Thereafter, the strips were soaked in equilibration solution (50 mM Tris–HCl, pH 8.8, 6 M urea, 20% glycerol, and 2% SDS) containing 20 mg/mL DTT for 10 min and then in the same solution containing 25 mg/mL iodoacetamide for an additional period of 10 min. The second dimension was carried out in 12.5% polyacrylamide gels at a constant voltage of 200 V using a MINI-PROTEAN 3 Cell (Bio-Rad). Proteins in two-dimensional gels were fixed with 40% methanol–10% acetic acid for 1 h at room temperature. Gels used for peptide identification and analysis were stained with colloidal Coomassie stain (BioSafe, Bio-Rad). Gel images were obtained using a GS-800 Calibrated Densitometer (Bio-Rad) and analyzed with the PDQuest 8.0 2D analysis software (Bio-Rad).

Protein spots of interest were manually excised from preparative gels, transferred to Eppendorf tubes, and automatically digested with trypsin according to standard protocols in a ProGest station (Genomic Solutions). Spots were destained twice for 30 min at 37°C with 200 mM ammonium bicarbonate/40% acetonitrile and dehydrated for 5 min with pure acetonitrile. Then, spots were rehydrated with 25 mM ammonium bicarbonate for 5 min and dehydrated again for 10 min with pure acetonitrile. After that, gel plugs were dried out for 4 h at room temperature. Then, 10 μL of 12.5 ng/μL trypsin solution in 25 mM ammonium bicarbonate was added to each sample, which was then rehydrated with the enzyme for 10-min incubation at room temperature. After that, the temperature was adjusted to 37°C and digestion was carried out for 12 h. Finally, digestion volume was recovered and peptides were extracted with 10 μL of 0.5% trifluoroacetic acid for 15 min. Peptides of each sample were analyzed in a 4700 Proteomics Station (Applied Biosystems, USA) in automatic mode. After drying, samples were analyzed in the *m/z* range 800–4,000. Spectra were internally calibrated with peptides from trypsin autolysis. Proteins were identified and confirmed by matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF/TOF; Reflex IV; Brucker Daltonics, Bremen, Germany, Applied Biosystems mod 4700). MASCOT searching engine (Matrixscience, UK) was used for protein identification over the database (MSDB). Protein identifications with a score value higher than 60 were positively assigned, after considering MW and p*I* values.

Validation by Western blotting

We used immunoblotting to validate some of proteins that showed significant changes in the proteomic study, paying special attention to those proteins closely related to cardiovascular disease risk and other diseases linked to aging. About 30 μg of serum protein was denatured by boiling in SDS dithiothreitol loading buffer (10% sucrose, 2 mM EDTA, 1.5% (*w*/*v*) SDS, 20 mM dithiothreitol, 0.01% (*w*/*v*) bromophenol blue, and 60 mM Tris–HCl (pH 6.8)), separated by SDS-PAGE (10% acrylamide), and then blotted onto nitrocellulose sheets. Blots were stained with Ponceau S for visualization of protein lanes. Then, the membranes were blocked with TBS containing 5% nonfat dry milk and 0.1% Tween-20 for 1 h at room temperature and incubated overnight at 4° C with the following primary antibodies: anti-apolipoprotein E (goat polyclonal, 1:1,000 dilution, Chemicon International Inc.), anti-apolipoprotein A1 (rabbit polyclonal, 1:200 dilution, Santa Cruz Biotech), anti-fetuin-A (goat polyclonal, 1:200 dilution, Santa Cruz Biotech), anti-apolipoprotein A-IV (goat polyclonal, 1:200 dilution, Santa Cruz Biotech), anti-fibrinogen (goat polyclonal, 1:200 dilution, Santa Cruz Biotech), and anti-apolipoprotein J (goat polyclonal, 1:7,500 dilution, US Biological). Blots were washed three times for 10 min in TBS containing 0.1% Tween-20 and then incubated for 1 h at room temperature with a secondary antibody (anti-goat or anti-rabbit IgG, at 1:2,000 dilution in both cases) coupled to horseradish peroxidase (Sigma). Blots were developed using a peroxidase reaction with enhanced chemiluminescent immunoblotting detection system (ECL-PLUS, Sigma). Density of staining with Ponceau S was used as a control for protein loading as described (Bello et al. 2003). Photographic films and Ponceau S-stained blots were scanned in a GS-800 calibrated densitometer (Bio-Rad) to obtain digital images. Quantification of intensity reaction was carried out using Quantity One software (Bio-Rad). Data obtained from the quantification of stained bands (in arbitrary units) were normalized to that of the corresponding lane stained with Ponceau S in order to correct for any difference in protein loading between samples.

Statistical analysis

Proteomic data were analyzed with PDQuest 8.0 2D software (Bio-Rad). To accurately compare spot quantities between gels, image spot quantities were normalized by the local regression model. Fluctuations in the protein expression levels among the control maps were monitored in densitometric analysis, and a coefficient of variation (CV) was generated for the mean value of each spot. Significant differences between proteins were assessed by Student's test. Only those changes that were statistically significant ($p<0.05$) and that involved at least twofold increases or half decreases of protein levels were considered in our study.

Results

Proteomic study

To study how dietary oils affected the levels of plasma proteins during aging, rats were fed lifelong with two experimental diets enriched in SO or VOO, and then we carried out a proteomic study of rat plasma after the depletion of albumin and IgG. At least four replicates were performed for all samples, similar protein spot patterns being obtained within each sample type. After automatic detection, the matching of spots in each of the gels, and normalization of the data using the PDQuest image analysis software, a mean of 144 spots were resolved on the two-dimensional electrophoresis gels. Nearly 100% of all spots were matched on duplicate gels (the gels of the sample were produced, assayed, and processed in parallel), and the intensity of the same spot from different gels showed no significant change. A representative gel showing those proteins whose levels in plasma were altered in animals fed with different diets is depicted in Fig. 1. Only proteins that increased or decreased significantly ($p<0.05$) by a factor of at least 2 were considered in this study. This gel is annotated to show the location of spots excised and identified by peptide mass fingerprinting, and the proteins identified are listed in Table 2. The predicted molecular weight and p*I* of protein spots were used to corroborate protein identifications.

Comparing all results obtained in each of the studies, we were able to identify successfully a total of 54 spots, corresponding to 16 different proteins, with significant changes with respect to aging or diet. These proteins can be grouped into four categories as follows (see Table 2 for details): *acute phase proteins*—β-Properdin factor, serine protease inhibitor III, inter-α-inhibitor H4P heavy chain, α-1-antitrypsin precursor, α-2-HS glycoprotein (fetuin-A), and β-Fetuin;

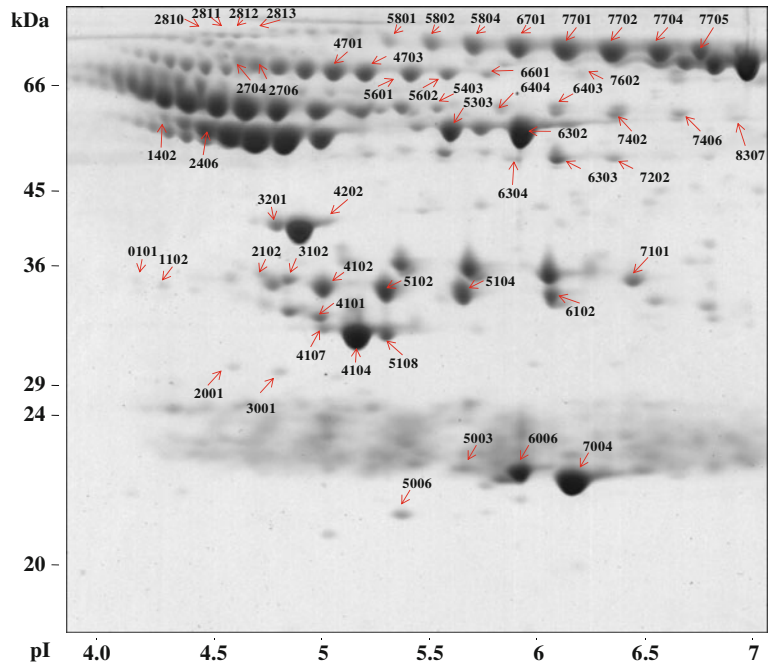

Fig. 1 Representative two-dimensional gel electrophoresis image showing the most abundant proteins rat plasma depleted from albumin and IgG. The scanned image of the Coomassie-stained gel was used to detect and compare protein spots. Those proteins whose levels were significantly altered in rats fed on SO or VOO diets are *numbered* and indicated by *arrows* (see Table 1). Gradient of pH (first dimension) is indicated at the *bottom* of the gel, and the positions of molecular mass standards (in kilodaltons) are shown on the *left*

Table 2 List of proteins identified by MALDI-TOF/TOF that showed significantly al+tered levels in plasma after the proteomic study

Spot	Protein name	Score	p*I*	M_r (Da)	No. of matched peptides	Sequence coverage (%)	Accession no. (NCBI)
Acute phase proteins							
101	Beta properdin factor	183	6.57	86.435	9	15	CAE83972
1102	Beta properdin factor	99	6.57	86.435	5	9	CAE83972
1402	Serine protease inhibitor 3	185	5.39	45.639	16	35	S11320
2001	Inter-alpha-inhibitor H4P heavy chain (fragment)	125	6.08	103.884	10	15	NP_062242
2406	Alpha-1-antitrypsin precursor	346	5.7	46.277	17	43	AAA40788
2810	Inter-alpha inhibitor H4P heavy chain	263	6.08	103.884	23	15	NP_062242
2811	Inter-alpha inhibitor H4P heavy chain	263	6.08	103.884	23	15	NP_062242
2812	Inter-alpha inhibitor H4P heavy chain	263	6.08	103.884	23	15	NP_062242
2813	Inter-alpha inhibitor H4P heavy chain	263	6.08	103.884	23	15	NP_062242
3001	Inter-alpha inhibitor H4P heavy chain (fragment)	162	6.08	103.884	11	14	NP_062242
6403	α-2-HS glycoprotein (Fetuin-A)	321	6.71	42.361	17	48	CAB62543
6404	α-2-HS glycoprotein (Fetuin-A)	236	6.71	42.361	14	43	CAB62543
7402	α-2-HS-glycoprotein (Fetuin-A)	281	6.71	42.361	16	43	CAB62543
7406	α-2-HS-glycoprotein (Fetuin-A)	148	6.71	42.361	15	47	CAB62543
8307	Beta fetuin	60	6.71	42.361	7	25	EDL78063
Lipid metabolism and transport							
2102	Apolipoprotein E precursor	155	5.23	35.788	12	37	NP_620183
3201	Apolipoprotein A-IV	652	5.12	44.428	33	82	AAA40747
4101	Apolipoprotein E precursor	114	5.23	35.788	12	38	NP_620183
4104	Apolipoprotein E precursor	254	5.23	35.788	18	37	NP_620183
4107	Apolipoprotein E precursor	254	5.23	35.788	18	37	NP_620183
4202	Apolipoprotein A-IV	219	5.12	44.428	20	56	AAA40747
5003	Apolipoprotein A-I	312	5.52	30.101	17	51	NP_036870
5108	Apolipoprotein E precursor	254	5.23	35.788	18	37	NP_620183
6006	Apolipoprotein A-I	284	5.51	29.869	15	44	NP_036870
7004	Apolipoprotein A-I precursor	374	5.52	30.1	21	65	NP_036870
Blood coagulation							
2704	T-kininogen 1 precursor	224	6.29	48.757	10	25	AAA41489
2706	T-kininogen 1 precursor	382	6.29	48.817	17	43	AAA41489

4701	T-kininogen 1 precursor	307	6.29	48.757	20	52	AAA41489
4703	T-kininogen 1 precursor	307	6.29	48.757	20	52	AAA41489
5601	T-kininogen 1 precursor	170	6.28	48.577	7	23	AAA41489
5602	T-kininogen 1 precursor	321	6.29	48.757	16	42	AAA41489
6303	Fibrinogen γ-chain precursor	180	5.57	50.247	16	48	NP_036691
6304	Fibrinogen γ-chain precursor	141	5.62	51.227	16	49	NP_036691
6601	Apolipoprotein H precursor	66	8.59	34.315	8	12	CAJ29887
7202	Fibrinogen γ-chain precursor	296	5.85	50.247	22	58	NP_036691
7602	Apolipoprotein H precursor	118	8.59	34.315	14	39	CAJ29887
Heme metabolism, antioxidant protection, and transport							
3102	Preprohaptoglobin precursor	149	6.1	43.075	15	34	NP_036714
4102	Preprohaptoglobin precursor	149	6.1	43.075	15	34	NP_036714
5006	Type II peroxiredoxin	244	5.2	21.968	6	27	AAC35744
5102	Preprohaptoglobin precursor	149	6.1	43.075	15	34	NP_036714
5104	Preprohaptoglobin precursor	149	6.1	39.037	14	51	NP_036714
5303	Vitamin D binding protein	493	5.76	55.078	26	59	AAA41082
5403	Vitamin D binding protein	305	5.65	55.079	17	46	AAA41082
5801	Hemopexin precursor	279	7.58	52.059	15	36	NP_445770
5802	Hemopexin precursor	212	7.58	51.999	20	43	NP_445770
5804	Hemopexin precursor	196	7.58	52.059	13	31	NP_445770
6102	Preprohaptoglobin precursor	129	6.1	39.037	13	49	NP_036714
6302	Vitamin D binding protein	228	5.76	55.078	20	55	AAA41082
6701	Hemopexin precursor	197	7.58	51.999	20	36	NP_445770
7101	Preprohaptoglobin precursor	194	6.1	39.051	14	41	AAH89816
7701	Hemopexin precursor	196	7.58	51.999	20	30	NP_445770
7702	Hemopexin precursor	279	7.58	512.059	15	36	NP_445770
7704	Hemopexin precursor	119	7.58	51.999	16	36	NP_445770
7705	Hemopexin precursor	349	7.58	51.999	22	45	NP_445770

Proteins are grouped by role: (a) Acute phase proteins; (b) lipid metabolism and transport; (c) blood coagulation; and (d) heme metabolism, antioxidant protection, and transport

lipid metabolism and transport—apolipoprotein E precursor, apolipoprotein A-IV precursor, and apolipoprotein A-I precursor; *blood coagulation*—T-kininogen 1 precursor, fibrinogen γ-chain precursor, and apolipoprotein H (β2-glycoprotein I, β2GPI); and *Heme metabolism, antioxidant protection, and transport*—preprohaptoglobin precursor, hemopexin precursor, type II peroxirredoxin 1, and vitamin D binding protein. Details of two-dimensional gels showing those proteins whose levels in plasma were changed significantly as a function of the experimental diets are depicted in Fig. 2.

Proteins decreased with VOO in comparison with the SO diet

Most of the proteins categorized as *acute phase proteins* decreased in the VOO group compared with the SO group. Levels of inter-alpha inhibitor H4P heavy chain decreased significantly only at 6 months. In addition, this protein decreased significantly in old rats as compared with young rats in the SO group. Fetuin-A was decreased both at 6 and 24 months with the VOO diet. During aging, most of the isoforms of this protein were increased with SO diet and, to a lesser extent (only two spots), with VOO diet. Finally, three proteins—β-Properdin, α-1-Antitrypsin, and β-Fetuin—increased significantly in old rats when compared with young rats only in the SO group, although no significant differences were found for these proteins when comparisons were carried out between dietary groups (see Tables 2 and 3).

Regarding the proteins categorized within the group of *lipid metabolism and transport*, apolipoprotein A-IV precursor increased at 6 months in the VOO

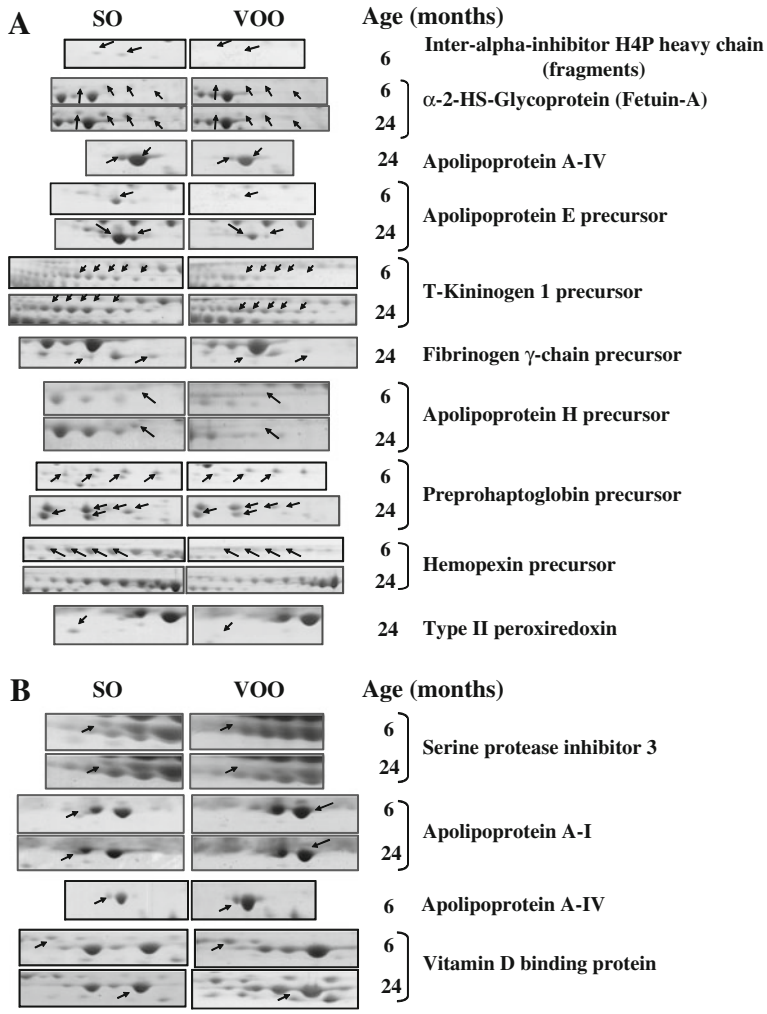

Fig. 2 Close-up sections of protein spots of proteins whose levels were significantly altered in plasma of rats fed on SO or VOO diet. **a** Proteins whose levels were decreased. **b** Proteins whose levels were increased in the VOO group relative to the SO group

Table 3 Proteomic data obtained after analysis with PDQuest 8.0 2D software (Bio-Rad)

	6 mo *vs* 24 mo (SO)					6 mo *vs* 24 mo (VOO)					SO *vs* VOO (6 months)					SO *vs* VOO (24 months)				
	6 mo		24 mo			6 mo		24 mo			SO		VOO			SO		VOO		
SPOT	OD	CV	OD	CV	Effect	OD	CV	OD	CV	Effect	OD	CV	OD	CV	Effect	OD	CV	OD	CV	Effect
101	0.1	0.0	0.8	25.5	**8** ▲	n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s	
1102	0.1	0.0	1.0	11.6	**10** ▲	n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s	
1402	n.s.	n.s.	n.s.	n.s.		47.5	45.4	10.9	67.6	**4.4** ▼	5.4	15.7	35.9	30.6	**6.7** ▲	4.5	44	14.9	35.5	**3.3** ▲
2001	3.3	9.1	0.9	11.3	**3.7** ▼	n.s.	n.s.	n.s.	n.s.		1.9	15.4	0.4	53.5	**4.8** ▼	n.s.	n.s.	n.s.	n.s.	
2102	0.1	0.0	3.5	23.6	**35** ▲	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.		15.5	7.8	0.4	53.5	**38.7** ▼
2406	2.7	90.8	6.9	19.4	**2.6** ▲	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.	
2704	6.1	58.8	0.1	0.0	**61** ▼	n.s.	n.s.	n.s.	n.s.		3.5	6.4	2.1	20.6	**1.7** ▼	4.8	16.3	11.9	26.7	**2.5** ▲
2706	14.7	21.9	9.5	21.6	**1.5** ▼	n.s.	n.s.	n.s.	n.s.		12.1	29.3	4.4	50.7	**2.8** ▼	n.s	n.s	n.s	n.s	
2810	5.2	37.2	1.1	16.9	**4.7** ▼	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s	
2811	7.0	27.9	1.1	26.2	**6.4** ▼	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s	
2812	3.9	12.2	1.2	29.7	**3.3** ▼	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s	
2813	2.6	29.1	0.7	4.1	**3.7** ▼	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s	
3001	4.1	13.4	1.5	11.5	**2.7** ▼	0.3	31.2	0.8	27.1	**2.7** ▲	2.6	24.5	2.4	47.2	**1.1** ▼	n.s	n.s	n.s	n.s	
3102	2.7	17.0	5.2	15.6	**1.9** ▲	n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s	
3201	n.s.	n.s.	n.s.	n.s.		12.6	12.5	3.8	2.0	**3.3** ▼	n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s	
4101	3.1	13.4	6.4	22.3	**2.1** ▲	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.		8.8	4.8	1.4	78.7	**6.3** ▼
4102	n.s.	n.s.	n.s.	n.s.		5.3	24.8	25.7	42.7	**4.9** ▲	17.9	29.8	5.4	20.6	**3.3** ▼	n.s	n.s	n.s	n.s	
4104	30.3	5.2	88.7	22.1	**2.9** ▲	2.7	15.5	32.4	31.6	**12** ▲	45.1	36.9	2.6	9.1	**17.4** ▼	132.8	20.2	35	25.8	**3.8** ▼
4107	1.4	39.5	4.5	7.9	**3.2** ▲	0.6	90.2	2.9	41.5	**4.8** ▲	n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s	
4202	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.		0.5	71.6	2.9	38.5	**5.8** ▲	26.7	17.8	2.2	63.8	**12.1** ▼
4701	15.2	14.7	22.4	3.0	**1.5** ▲	5.4	25.5	16.1	3.8	**3** ▲	13.4	24.1	6.3	37.9	**2.1** ▼	29.7	6.3	20.4	15.5	**1.5** ▼
4703	12.9	10.7	19.4	0.8	**1.5** ▲	2.4	32.2	7.8	8.3	**3.3** ▲	9.6	15.5	2.5	28.6	**3.8** ▼	25.9	3.4	13.5	6.4	**1.9** ▼
5003	n.s.	n.s.	n.s.	n.s.		20.2	19.0	6.5	63.4	**3.1** ▼	3.70	33.9	22.20	28.70	**6** ▲	6.5	26.2	18.8	29.4	**2.9** ▲
5006	0.8	26.3	3.4	12.6	**4.3** ▲	0.8	24.8	0.2	37.1	**4** ▼	n.s.	n.s.	n.s.	n.s.		3.2	14.1	0.7	18.9	**4.6** ▼
5102	n.s.	n.s.	n.s.	n.s.		6.0	25.0	54.2	49.8	**9** ▲	35.1	37.6	6.3	23.4	**5.6** ▼	20.3	14.1	4.2	63.4	**4.8** ▼
5104	n.s.	n.s.	n.s.	n.s.		3.6	5.1	36.4	32.2	**10** ▲	24.8	26.5	3.8	8.9	**6.5** ▼	n.s	n.s	n.s	n.s	
5108	5.2	28.7	13.5	31.7	**2.6** ▲	n.s.	n.s.	n.s.	n.s.		3.1	25.1	0.7	25.1	**4.5** ▼	n.s	n.s	n.s	n.s	
5303	n.s	n.s	n.s	n.s		n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s		49.1	13.5	24	21.6	**2** ▼
5403	n.s	n.s	n.s	n.s		n.s.	n.s.	n.s.	n.s.		0.8	32.6	2.1	17.9	**2.6** ▲	n.s	n.s	n.s	n.s	
5601	8.6	5.4	14.1	7.3	**1.6** ▲	0.8	40.6	3.6	32.4	**4.5** ▲	5.5	14.9	0.8	48.5	**6.9** ▼	18.8	2.8	6.4	43.4	**2.9** ▼
5602	n.s	n.s	n.s	n.s		n.s.	n.s.	n.s.	n.s.		1.4	23.2	0.3	13.3	**4.7** ▼	8.5	5.6	2.4	67.1	**3.5** ▼
5801	11.5	13.9	3.6	6.8	**3.2** ▼	n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s	
5802	19.6	11.6	7.8	14.4	**2.5** ▼	n.s.	n.s.	n.s.	n.s.		19	30.4	6.3	13.5	**3** ▼	n.s	n.s	n.s	n.s	
5804	29.4	4.8	15.1	12.4	**2** ▼	n.s.	n.s.	n.s.	n.s.		40.5	29	7.8	24.4	**5.2** ▼	20.6	7.5	12.3	21.2	**1.7** ▼
6006	n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s		62.4	13.1	130.6	25.3	**2.1** ▲
6102	10.5	14.0	17.1	22.5	**1.6** ▲	1.5	13.5	13.7	13.3	**9.1** ▲	6.6	27.8	1.6	24.7	**4.2** ▼	25	7.3	20.9	8.3	**1.2** ▼
6302	n.s.	n.s.	n.s.	n.s.		95.2	62.9	302.4	20.4	**3.2** ▲	n.s	n.s	n.s	n.s		11.9	17.3	163.1	12.2	**13.7** ▲
6303	n.s.	n.s.	n.s.	n.s.		0.4	57.4	4.5	43.9	**11.3** ▲	n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s	
6304	0.1	0.0	1.1	50.6	**11** ▲	n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s	
6403	2.2	31.5	4.7	22.5	**2.2** ▲	0.1	124.3	1.1	22.1	**11** ▲	1	38.8	0.1	0.0	**10** ▼	n.s	n.s	n.s	n.s	
6404	1.4	24.9	2.5	30.3	**1.8** ▲	n.s.	n.s.	n.s.	n.s.		0.3	23.6	0.1	0.33	**3** ▼	3	6.6	1.2	55.4	**2.3** ▼
6601	n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s		1.3	37.2	0.3	16.6	**4.4** ▼	2.3	14.7	0.9	12.6	**2.6** ▼
6701	38.8	5.9	26.6	16.0	**1.5** ▼	n.s.	n.s.	n.s.	n.s.		67.1	39.4	8.2	77.8	**8.2** ▼	37.1	13.6	19.9	18.1	**1.8** ▼
7004	n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s		135.4	6.8	237.7	24	**1.7** ▲
7101	6.2	8.6	8.1	6.0	**1.3** ▲	n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s		10.3	8.3	5.7	13.9	**1.8** ▼
7202	0.8	45.5	2.0	39.6	**2.5** ▲	0.2	51.2	0.8	19.3	**4** ▲	n.s	n.s	n.s	n.s		2	14.4	1.3	14.2	**1.5** ▼
7402	1.5	21.7	5.7	25.8	**3.8** ▲	0.1	72.5	0.6	41.6	**6** ▲	0.5	29.4	0.1	0.0	**5** ▼	8	8.1	2	31.8	**4** ▼
7406	0.7	21.5	3.8	17.2	**5.4** ▲	n.s.	n.s.	n.s.	n.s.		n.s.	n.s.	n.s.	n.s.		4.5	10.6	1.1	11.4	**4.1** ▼
7602	3.3	16.3	1.5	17.2	**2.2** ▼	n.s.	n.s.	n.s.	n.s.		1.9	8.5	0.8	58.1	**2.4** ▼	1.2	5.9	0.6	2.2	**2** ▼
7701	n.s.	n.s.	n.s.	n.s.		8.8	55.8	23.3	20.0	**2.7** ▲	n.s.	n.s.	n.s.	n.s.		53.8	11.7	28	11.2	**1.9** ▼
7702	n.s.	n.s.	n.s.	n.s.		10.9	52.8	23.4	16.5	**2.2** ▲	n.s.	n.s.	n.s.	n.s.		59.8	18.4	27.1	11.8	**2.2** ▼
7704	n.s.	n.s.	n.s.	n.s.		5.5	52.0	20.1	27.2	**3.7** ▲	59.6	49.9	5.7	66.1	**10.5** ▼	59.6	19	24.8	13.6	**2.4** ▼
7705	n.s.	n.s.	n.s.	n.s.		1.7	103.5	9.5	42.9	**5.6** ▲	19.7	28.2	1.8	59.6	**10.9** ▼	n.s	n.s	n.s	n.s	
8307	0.1	0.0	1.0	44.9	**10** ▲	n.s.	n.s.	n.s.	n.s.		n.s	n.s	n.s	n.s		n.s	n.s	n.s	n.s	

"Effect" columns depict aging-dependent changes in protein spots for each dietary group (24 vs. 6 months) or diet-induced changes for each group of age (VOO vs. SO diet) for those cases when statistically significant differences were observed with Student's test ($p < 0.05$). Arrows represent the sense of the observed change. Upward arrows represent increases at 24 months with respect to 6 months within a given dietary group, or in VOO diet with respect to SO diet within a given age group. Downward arrows represent decreases at 24 months with respect to 6 months within a given dietary group, or in VOO diet with respect to SO diet within a given age group. See Table 2 for identification of spot numbers

OD optical density, *CV* coefficient of variation (in percent), *n.s.* non-significant differences

group, whereas another isoform of this protein was decreased at 24 months. During aging, the more acidic isoform was decreased with the VOO diet. Apolipoprotein E precursor was decreased at 6 months in the VOO group, although this change was observed in only two isoforms; this protein was also decreased at 24 months in the VOO group, and this change involved more isoforms. During aging, most of the apolipoprotein E isoforms were increased with both experimental diets. All the identified proteins listed in the category *blood coagulation* decreased significantly in the VOO group when compared with the SO group. T-kininogen 1 precursor was decreased both at 6 and 24 months with the VOO diet. On the other hand, most of spots of the protein were increased in old animals for both experimental diets. Fibrinogen

gamma chain precursor was decreased significantly at 24 months, although no significant effect was observed at 6 months. During aging, this protein increased with both diets. Finally, β2GPI was decreased in the VOO group compared with the SO group both at 6 and 24 months. During aging, this protein did not show significant changes with the VOO diet, although it was decreased with the SO diet. Most of the proteins categorized in the last group, *Heme metabolism, antioxidant protection, and transport*, also decreased with the VOO diet. A decrease of preprohaptoglobin precursor was detected both at 6 and 24 months. During aging, some isoforms were significantly increased with the VOO diet. Hemopexin precursor was also decreased at 6 and 24 months with the VOO diet. During aging, some of the most acidic isoforms decreased significantly with the SO diet, while other basic isoforms increased significantly with the VOO diet. Finally, type II peroxiredoxin levels decreased at 24 months with the VOO diet compared with the SO diet, but no significant difference between both diets was observed at 6 months. During aging, type II peroxiredoxin was increased with the SO diet, but it was decreased with the VOO diet.

Proteins increased with VOO in comparison with the SO diet

One of the proteins categorized as acute phase protein, serine protease inhibitor III, was increased significantly after consumption of the VOO diet, both at 6 and 24 months. During aging, this protein was decreased with the VOO diet. Levels of apolipoprotein A-I precursor also increased at 6 at 24 months in the VOO group when compared with the SO group, and as shown for serine protease inhibitor III, levels of apolipoprotein A-I precursor decreased with the VOO diet during aging. Finally, vitamin D binding protein levels were increased in the VOO group both at 6 and 24 months when compared with SO group. During aging, one isoform was increased with the VOO diet.

All proteomic data, with indication of optical densities of the identified spots, coefficients of variation, and effects of age or diet are depicted in Table 3.

Western blotting validation of two-dimensional results

To confirm some results obtained in our proteomic study and to better understand the role of diet in the development of diseases associated with aging, immunoblotting analyses were performed on plasma samples depleted of the most abundant proteins by affinity chromatography (see "Methods"). The proteins chosen for immunologic validation were proteins closely related to cardiovascular disease risk and other diseases linked to aging. These proteins were: apolipoprotein A-I, apolipoprotein A-IV, apolipoprotein E, alpha-2-HS glycoprotein (fetuin-A), and fibrinogen gamma chain precursor (Fig. 3).

About 30 μg of the depleted plasma sample was used for Western blotting detection of apolipoprotein A-I. Blots obtained from one-dimensional gels showed a band of 25 kDa (Fig. 3) whose size coincided with that in two-dimensional gels. Band quantification confirmed our proteomic results, i.e., an increase of the protein with the VOO diet when compared with the SO diet in both groups of age. The decrease observed in Western blots in the VOO group during aging was also in accordance with the changes observed in the proteomic analysis, although we also observed a decrease of the protein with the SO diet in old rats when compared with young rats.

About 30 μg of the depleted plasma sample was also used for the immunodetection of apolipoprotein E. The stained blot showed a band of 36 kDa (Fig. 3), which coincided with the size observed in two-dimensional gels. We observed an increase of this protein during aging for both experimental diets, although a decrease was detected with the VOO diet when compared with the SO diet for both groups of age (Fig. 3). Therefore, the results obtained by immunostaining also matched the proteomic analysis.

About 5 μg of the depleted plasma sample was used to quantify alpha 2-HS glycoprotein (fetuin-A). The band of 59 kDa detected in blots (Fig. 3) coincided with the size of the protein in two-dimensional gels, which was separated in several isoforms (see Fig. 2). The results obtained by immunodetection were equivalent to those of the proteomic analysis in all cases, i.e., a slight decrease of the protein with the VOO diet when compared with the SO diet at 6 and 24 months and, on the other hand, an increase of the protein during aging for both experimental diets (Fig. 3).

The results of immunoblotting analyses for apolipoprotein A-IV did not follow those of the proteomic analysis. After immunoblotting, we obtained a band of 46 kDa whose size was the same as that of the protein identified in two-dimensional gels. Quantification of the band detected in immunostained blots

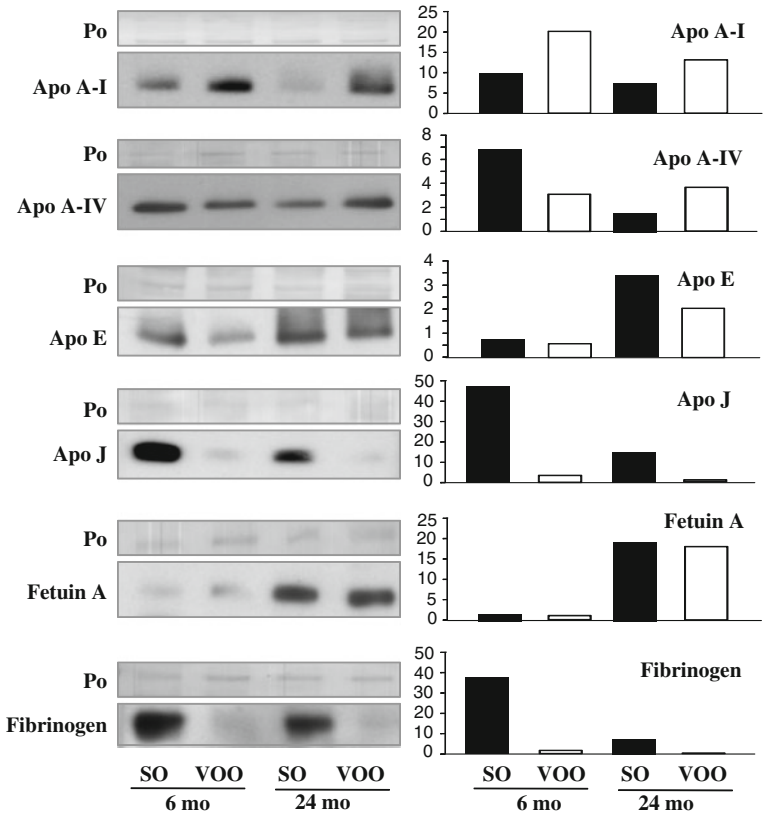

Fig. 3 Western blot validation of protein changes detected in bidimensional gels of rat plasma from rats fed on SO or VOO diets. The panel depicts representative areas of immunostained blots obtained from one-dimensional gels. Results of Ponceau S staining (Po) are also shown *above* each immunostained blot to demonstrate equal protein loading for all samples. *Bar graphs* shown on the *right* represent the densitometric quantification of the corresponding immunostained band (in OD × mm^2) referred to the amount of protein loading measured from the densitometric quantification of the corresponding lane stained with Ponceau S

indicated a decrease of apolipoprotein A-IV with the VOO diet when compared with the SO diet at 6 months. However, an increase with the same diet was observed at 24 months (Fig. 3). Apparent discrepancies observed with both techniques could be due to the existence of changes in different isoforms due to posttranslational modifications.

Finally, about 30 μg of the depleted plasma sample was used to quantify fibrinogen gamma chain by Western blotting. The blot showed a band of 57 kDa which coincided with the size observed in two-dimensional gels. In this case, we observed a marked decrease of the protein with the VOO diet when compared with the SO diet both at 6 and 24 months of age. On the other hand, we also observed a decrease of the protein during aging for both dietary intervention groups (Fig. 3).

Inmunodetection of apolipoprotein J/clusterin

Recent discoveries have supported an important role for apolipoprotein J/clusterin in the development of some neurodegenerative diseases (Thambisetty et al. 2010). Taking into account the existing relationship between the development of neurodegenerative diseases and nutrition, we found it interesting to study the putative changes of this protein in rats fed lifelong with our two experimental diets. Although we did not detect changes of clusterin in our proteomics analysis, presumably due to the existence of other proteins masking clusterin in the two-dimensional gels, we carried out immunoblotting analysis for analyzing putative changes in clusterin as a function of diet or age. This analysis yielded a band of approximately 50 kDa which was markedly decreased in the VOO group when compared with the SO group both at 6 and 24 months. During aging, the protein was decreased with both experimental diets (Fig. 3).

Discussion

The change of various serum proteins and antioxidant components is associated with age (Chlebovska and

Chlebovsky 1999; Kim et al. 2002). Dietary oils, through their fatty acid composition and minor constituents, might modulate, among others, those mechanisms involved in homeostasis, aging, and age-related diseases. In this study carried out in rats fed lifelong on diets enriched in either SO or VOO, we have detected significant changes in the plasma proteomic profile, providing new information about the benefits of VOO on aging.

Effects of dietary oils on inflammation

Fatty acids can directly or indirectly influence some of the factors mediating the immune response. Some of proteins identified in our work related with these processes are alpha 2-HS glycoprotein (fetuin-A), inter-alpha inhibitor H4P heavy chain, alpha 1 anti-trypsin precursor, serine protease inhibitor III, and beta properdin factor. Fetuin-A was decreased with the VOO diet both at 6 and 24 months. During aging, most of the fetuin-A isoforms increased with the SO diet, and to a lesser extent with the VOO diet. Fetuin-A is a prominent serum glycoprotein and a major non-collagenous component of mineralized bone in mammals which plays important biological functions, including those involved in the mineralization of bone (Triffitt 1976) and in immune response (Lebreton et al. 1979). In rats, this protein inhibits osteogenesis and may promote artery calcification (Binkert et al. 1999; Price et al. 2004). In this sense, mice deficient in fetuin-A showed a systemic calcification in their tissues (Yoshida et al. 2006). It is important to note that high concentrations of fetuin-A are strongly associated with metabolic syndrome and a pro-atherogenic lipid profile in humans (Ix et al. 2006). On the other hand, serum alpha 2-HS glycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level (Srinivas et al. 1993); thus, it might be related with states of insulin resistance and an increase in type II diabetes. The fact that the VOO diet decreased the levels of this protein in young and old rats may indicate that this type of fat may influence several processes such as osteogenesis, atherosclerosis, or states of insulin resistance.

Inter-alpha inhibitor H4P heavy chain was decreased significantly with the VOO diet when compared with the SO diet only at 6 months. This protein was decreased significantly with aging after intake of the SO diet. This protein belongs to a family of plasma protease inhibitor of hepatic origin, collectively termed as the inter-α-inhibitor family. Inter-α-inhibitor H4P levels increase significantly under acute inflammatory conditions (Daveau et al. 1998). Changes in the levels of acute phase proteins at 6 months due to VOO intake support that the type of fat plays an important role in the regulation of inflammation at early ages. On the other hand, anti-inflammatory properties of polyunsaturated fatty acids are well documented (Calder 2009), which agrees with the decrease of this protein after prolonged intake of SO diet. However, alpha 1 antitrypsin precursor, another acute phase protein, was increased after intake of the same diet during aging. This protein is also considerably increased during the acute phase response (Sandford et al. 1999). Another protein identified in our work was serine protease inhibitor III, which was increased in the VOO group when compared with the SO group both at 6 and 24 months. During aging, this protein was decreased after intake of the VOO diet. Serine protease inhibitor III is an acidic glycoprotein which inhibits serine proteases by forming irreversible complexes with proteinases. Its gene is barely expressed in healthy animals, seems to escape the regulation by growth hormone, and is strongly induced during the acute phase reaction by interferon γ via the interleukin 6-responsive element (Kordula and Travis 1995).

Finally, beta properdin factor was strongly increased with aging after intake of the SO diet. This protein is a regulator of the alternative complement pathway whose function depends on multiple interactions between its subunits and ligands (Muller-Eberhard and Schreiber 1980). The increase observed after prolonged intake of the SO diet indicates that the type of fat may regulate the complement system in this group of rats.

Effects of dietary oils on lipid metabolism and transport

Dietary lipid quality can affect the lipoprotein metabolism, altering their concentrations in the blood, permitting their greater or lesser recruit in the artery wall, thus modulating the susceptibility to vascular damage. We observed changes in three proteins involved in these processes. Apolipoprotein A-I precursor was increased in the VOO group both at 6 and 24 months. On the other hand, the protein was decreased with the VOO diet during aging. Apo A-I is the main apolipoprotein of HDL particles, being strongly related to plasma levels of these anti-

atherogenic complexes (Chan and Watts 2006). An inverse relationship between plasma levels of Apo A-I and coronary heart disease has been suggested (Duverger et al. 1996a). Significantly elevated levels of Apo A-I are associated with lower mortality and myocardial infarction 5 years after coronary artery bypass graft surgery in humans (Anderson 2005). Thus, the increase of this protein in both age groups after intake of the VOO diet is reflective of anti-atherogenic status. Apolipoprotein A-IV precursor was decreased with the VOO diet when compared with the SO diet at 6 months, but it was increased with the same experimental diet at 24 months. During aging, the protein was increased with the VOO diet. It has been shown that Apo A-IV overexpression has a protective effect on mice susceptible to atherosclerosis, reducing injuries (Duverger et al. 1996b; Ostos et al. 2001). Interestingly, a recent study has analyzed the impact of the different isoforms of this protein on its lipid-binding capacity and efficiency in cholesterol metabolism (Gomaraschi et al. 2010). In this sense, the results obtained in our proteomic analysis showed a different processing of the Apo A-IV isoforms with the VOO diet in both age groups, which might be related with a differential lipid-binding capacity.

Apolipoprotein E precursor was decreased at 6 and 24 months with the VOO diet when compared with the SO diet. During aging, the protein was increased with both experimental diets. Apo E plays an important role in lipoprotein metabolism and cardiovascular disease. In addition, this protein has emerged as an important molecule in several biological processes not directly related to its lipid transport function, including Alzheimer's disease and cognitive function, immunoregulation, and, possibly, even infectious diseases (Mahley and Rall 2000). The fact that compared with the SO diet intake of the VOO diet decreased the levels of this protein in both age groups, as well as during aging, reinforces the cardioprotective effect of this diet.

Apolipoprotein J/clusterin was decreased in the VOO group when compared with the SO group, both at 6 and 24 months. During aging, the protein was decreased in both dietary intervention groups. Clusterin is a 70- to 80-kDa heterodimeric sulfated glycoprotein which is constitutively synthesized and secreted by a variety of tissues and found in most biological fluids (French et al. 1993). The protein has been reportedly implicated in diverse physiological processes such as lipid transport (Rosenberg and Silkensen 1995), complement inhibition, tissue remodeling, membrane recycling, cell–cell and cell–substratum interactions (Silkensen et al. 1994), stabilization of stressed proteins in a folding-competent state (Trougakos and Gonos 2004), and promotion or inhibition of apoptosis (Trougakos and Gonos 2002). In human plasma, Apo J is associated with Apo A-I in high- and very high-density lipoproteins (Jenne et al. 1991), and its expression stimulates the proliferation and migration of smooth muscle cells (Miyata et al. 2001). In vivo, Apo J/clusterin is up-regulated in many severe physiological disturbances, including accumulation in the artery wall during the development of atherosclerosis (Trougakos et al. 2002). Interestingly, Apo J/clusterin has been recently related with the development of Alzheimer's disease, being presented as a marker of this disease (Thambisetty et al. 2010), and has also been associated with diabetes, probably through an increase in insulin resistance primarily and through an impairment of insulin secretion secondarily (Daimon et al. 2011). The decrease of Apo J/clusterin levels by the VOO diet in both age groups likely supports an important role for this diet on endothelium function and in preventing the development of neurodegenerative diseases during aging.

Effects of dietary oils on coagulation and hemostatic processes

Certain aspects of blood platelet function, blood coagulability, and fibrinolytic activity associated with cardiovascular risk are modulated by dietary fatty acids. In our work, some proteins related with these processes showed significant changes with the experimental diets.

Fibrinogen gamma chain precursor is strongly, consistently, and independently related to cardiovascular risk as it is involved in the coagulation cascade, mainly in fibrin formation (Kruskal et al. 1987). Therefore, this protein is closely related with thrombus formation and plasma viscosity. Many studies have related high levels of fibrinogen with cardiovascular disease (Danesh et al. 2005). A recent study suggests that fibrinogen gamma chain precursor is an excellent marker of cardiovascular risk (Lovely et al. 2010). Interestingly, in this work, we have shown that fibrinogen gamma chain was strongly decreased with

the VOO diet compared with the SO diet at 6 and 24 months, reinforcing again the beneficial effects of this type of fat in the prevention of cardiovascular diseases.

Apolipoprotein H precursor (β2-glycoprotein I, β_2GPI), another protein related with blood coagulation, was decreased with the VOO diet compared with the SO diet both at 6 and 24 months. During aging, β_2GPI was decreased after intake of the SO diet, but no significant changes were observed after intake of the VOO diet. β_2GPI is the main target antigen for antiphospholipid antibodies in patients with antiphospholipid syndrome and has been shown to regulate the activation of plasminogen. Although the exact physiological role of β_2GPI is not well understood, it is known that β_2GPI forms complexes with oxidized LDL, which represents a common metabolic product relevant to atherogenesis and a risk factor or an indirect but significant contributor for atherothrombotic complications in autoimmune patients (Matsuura et al. 2006). On the other hand, anticoagulant properties have also been attributed to this protein interceding in the coagulation cascade (Miyakis et al. 2004). Finally, β_2GPI has also been proposed as a marker of cerebral infarction (Yasuda et al. 2004). The decrease of β_2GPI in animals fed with the VOO diet regardless of age again reinforces the benefits of prolonged consumption of a diet rich in VOO.

Finally, we observed that T-kininogen 1 precursor was decreased significantly after intake of the VOO diet compared with the SO diet both at 6 and 24 months. On the other hand, the majority of spots for this protein in two-dimensional gels were increased with aging for both experimental diets. Kininogens are multifunctional proteins that act as precursor of kinins, small vasoactive peptides that also promote endothelial cell proliferation through kinin receptors (Colman and Schmaier 1997). On the other hand, T-kininogen 1 also functions as a potent inhibitor of cysteine proteinases (Sueyoshi et al. 1985). Plasma levels of kininogens increase with age in rats, which agrees with our work, increasing dramatically during the last few months of life (Walter et al. 1998). Some studies suggest that an age-related increase in serum kininogens might play a role in modulating the changes in proliferative capacity of blood-exposed cells, including endothelial cells (Torres et al. 2001). The proteolytic processing of kininogen involves the release of the nonapeptide bradykinin, a vasoactive and pro-inflammatory mediator, while the rest of the molecule has anti-adhesive properties (Asakura et al. 1992) inhibiting platelet aggregation (Chavakis et al. 2002). Therefore, the decrease of T-kininogen 1 after intake of a diet rich in VOO in both age groups could contribute to the decline of an inflammatory state.

Effects of dietary oils on heme metabolism and antioxidant protection

Some animal studies suggest that VOO protects certain molecules from oxidation, as indicated by decreased LDL susceptibility to oxidation or other markers of oxidation (Visioli et al. 2002). In our work, we observed that levels of preprohaptoglobin precursor and hemopexin precursor were significantly decreased with the VOO diet when compared with the SO diet, both at 6 and 24 months of age. Haptoglobin is a serum protein that binds hemoglobin released into plasma following intravascular hemolysis and allows its metabolization in the liver (Hanley et al. 1983). In addition, this protein has antioxidant functions because it prevents oxidative damage mediated by free hemoglobin (Melamed-Frank et al. 2001). Our observations support VOO as having a protective effect in both age groups, reflected by the lower levels of the protein, although some isoforms increased with aging. On the other hand, hemopexin is a plasma glycoprotein with a high binding affinity to heme, which is potentially toxic because it has the ability to intercalate between the lipid membranes and produce hydroxyl radicals. Therefore, hemopexin inhibits some of the oxidative reactions catalyzed by heme-protecting tissues from oxidation (Tolosano et al. 1999). Previous studies have documented the protective effect of hemopexin–haptoglobin binding avoiding intravascular hemolysis and its associated diseases (Tolosano et al. 2002). Since both proteins increase in rat serum with aging and under stress conditions (Chlebovska and Chlebovsky 1999), we consider it interesting that a diet rich in VOO decreases the levels of both proteins in young and old rats, which would help keep a healthy state at an early age and throughout aging.

Type II peroxirredoxin was decreased at 24 months with the VOO diet when compared with the SO diet, although no change was observed at 6 months. Interestingly, aging resulted in an increase of this protein with the SO diet, but a decrease was observed

with the VOO diet. Peroxiredoxins are a family of thiol-specific peroxidases found in all species that can reduce a wide range of peroxides, thus playing a major role in antioxidant protection (Wong et al. 2004). This protein is induced by oxidative stress conditions provoked by alimentary vitamin E deficiency (Tolle et al. 2005). The aging-related increase of type II peroxiredoxin observed in plasma of rats fed with the SO diet might reflect a pro-oxidant effect of this diet in the body. It is plausible to interpret that the decreased levels of peroxiredoxin in plasma obtained from animals fed with the VOO diet (as well as decreased levels of hemopexin and prepro-haptoglobin) may be indicative of a better antioxidant status compared with the animals fed with the SO diet.

Vitamin D binding protein was increased at 6 and 24 months with the VOO diet when compared with the SO diet. During aging, one spot was increased with the VOO diet. Vitamin D binding protein is a serum glycoprotein which transports vitamin D sterols and binds to monomeric actin (Cooke 1986), which allows it to act as a scavenging molecule, helping to eliminate and prevent the polymerization of actin in the plasma (Haddad 1995). Our observations agree with previous studies carried out on rats fed throughout their lives with VOO or SO diets, showing that prolonged consumption of olive oil improved the total bone mineral content (Mataix 2002).

Interestingly, a significant number of protein changes identified in this work as the result of consumption of the VOO in comparison with the SO diet were also highlighted in a previous study focused on changes induced by dietary CoQ_{10} supplementation of a diet containing SO compared to an SO alone diet (Santos-González et al. 2007). The fact that a VOO diet and a CoQ_{10}-supplemented SO diet increase the levels of proteins, such as serine protease inhibitor III, apolipoprotein A-I, or vitamin D binding protein, and decrease other proteins, such as hemopexin, prepohaptoglobin, fibrinogen gamma chain, alpha-2-HS glycoprotein, type II peroxiredoxin, inter-alpha inhibitor H4P heavy chain, or apolipoprotein H, when compared with the SO alone diet indicates that CoQ_{10} and VOO may have similar effects on processes related to inflammation, blood coagulation, lipid metabolism, transport, and bone metabolism. In accordance, a recent work has reported that these two diets also produced similar effects on a number of parameters relative to oxidative stress (Quiles et al. 2010).

Overall, our study strongly supports the intake of a diet rich in VOO, through oleic acid and/or its minor constituents, as providing greater benefits than a diet rich in SO both in young and old rats, improving and maintaining an antioxidant status, an anti-inflammatory state, and anti-atherogenic lipid profile during aging.

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References

Anderson L (2005) Candidate-based proteomics in the search for biomarkers of cardiovascular disease. J Physiol 563:23–60

Asakura S, Hurley RW, Skorstengaard K, Ohkubo I, Mosher DF (1992) Inhibition of cell adhesion by high molecular weight kininogen. J Cell Biol 116:465–476

Babio N, Bullo M, Salas-Salvado J (2009) Mediterranean diet and metabolic syndrome: the evidence. Public Health Nutr 12:1607–1617

Bello RI, Alcaín FJ, Gómez-Díaz C, López-Lluch G, Navas P, Villalba JM (2003) Hydrogen peroxide- and cell-density-regulated expression of NADH-cytochrome b_5 reductase in HeLa cells. J Bioenerg Biomembr 35:169–179

Bello RI, Gómez-Díaz C, Burón MI, Navas P, Villalba JM (2006) Differential regulation of hepatic apoptotic pathways by dietary olive and sunflower oils in the aging rat. Exp Gerontol 41:1174–1184

Binkert C, Demetriou M, Sukhu B, Szweras M, Tenenbaum HC, Dennis JW (1999) Regulation of osteogenesis by fetuin. J Biol Chem 274:28514–28520

Calder PC (2009) Polyunsaturated fatty acids and inflammatory processes: new twists in an old tale. Biochimie 91:791–795

Chan CD, Watts GF (2006) Apolipoproteins as markers and managers of coronary risk. QJM 99:277–287

Chavakis T, Boeckel N, Santoso S, Voss R, Isordia-Salas I, Pixley RA, Morgenstern E, Colman RW, Preissner KT (2002) Inhibition of platelet adhesion and aggregation by a defined region (Gly-486-Lys-502) of high molecular weight kininogen. J Biol Chem 277:23157–23164

Chlebovska K, Chlebovsky O (1999) Favourable effect of K, Mg aspartate on serum proteins in aging rats. Mech Ageing Dev 108:127–138

Colman RW, Schmaier AH (1997) Contact system: a vascular biology modulator with anticoagulant, profibrinolytic,

antiadhesive, and proinflammatory attributes. Blood 90:3819–3843

Cooke NE (1986) Rat vitamin D binding protein. Determination of the full-length primary structure from cloned cDNA. J Biol Chem 261:3441–3450

Daimon M, Oizumi T, Karasawa S, Kaino W, Takase K, Tada K, Jimbu Y, Wada K, Kameda W, Susa S, Muramatsu M, Kubota I, Kawata S, Kato T (2011) Association of the clusterin gene polymorphisms with type 2 diabetes mellitus. Metabolism PMID: 20850846 (in press)

Danesh J, Lewington S, Thompson SG, Lowe GD, Collins R, Kostis JB, Wilson AC, Folsom AR, Wu K, Benderly M, Goldbourt U, Willeit J, Kiechl S, Yarnell JW, Sweetnam PM, Elwood PC, Cushman M, Psaty BM, Tracy RP, Tybjaerg-Hansen A, Haverkate F, de Maat MP, Fowkes FG, Lee AJ, Smith FB, Salomaa V, Harald K, Rasi R, Vahtera E, Jousilahti P, Pekkanen J, D'Agostino R, Kannel WB, Wilson PW, Tofler G, Arocha-Pinango CL, Rodriguez-Larralde A, Nagy E, Mijares M, Espinosa R, Rodriquez-Roa E, Ryder E, Diez-Ewald MP, Campos G, Fernandez V, Torres E, Marchioli R, Valagussa F, Rosengren A, Wilhelmsen L, Lappas G, Eriksson H, Cremer P, Nagel D, Curb JD, Rodriguez B, Yano K, Salonen JT, Nyyssonen K, Tuomainen TP, Hedblad B, Lind P, Loewel H, Koenig W, Meade TW, Cooper JA, De Stavola B, Knottenbelt C, Miller GJ, Cooper JA, Bauer KA, Rosenberg RD, Sato S, Kitamura A, Naito Y, Palosuo T, Ducimetiere P, Amouyel P, Arveiler D, Evans AE, Ferrieres J, Juhan-Vague I, Bingham A, Schulte H, Assmann G, Cantin B, Lamarche B, Despres JP, Dagenais GR, Tunstall-Pedoe H, Woodward M, Ben-Shlomo Y, Davey Smith G, Palmieri V, Yeh JL, Rudnicka A, Ridker P, Rodeghiero F, Tosetto A, Shepherd J, Ford I, Robertson M, Brunner E, Shipley M, Feskens EJ, Kromhout D, Dickinson A, Ireland B, Juzwishin K, Kaptoge S, Lewington S, Memon A, Sarwar N, Walker M, Wheeler J, White I, Wood A (2005) Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. JAMA 294:1799–1809

Daveau M, Jean L, Soury E, Olivier E, Masson S, Lyoumi S, Chan P, Hiron M, Lebreton JP, Husson A, Jegou S, Vaudry H, Salier JP (1998) Hepatic and extra-hepatic transcription of inter-alpha-inhibitor family genes under normal or acute inflammatory conditions in rat. Arch Biochem Biophys 350:315–323

Duverger N, Kruth H, Emmanuel F, Caillaud JM, Viglietta C, Castro G, Tailleux A, Fievet C, Fruchart JC, Houdebine LM, Denefle P (1996a) Inhibition of atherosclerosis development in cholesterol-fed human apolipoprotein A-I-transgenic rabbits. Circulation 94:713–717

Duverger N, Tremp G, Caillaud JM, Emmanuel F, Castro G, Fruchart JC, Steinmetz A, Denefle P (1996b) Protection against atherogenesis in mice mediated by human apolipoprotein A-IV. Science 273:966–968

Echan LA, Tang HY, Ali-Khan N, Lee K, Speicher DW (2005) Depletion of multiple high-abundance proteins improves protein profiling capacities of human serum and plasma. Proteomics 5:3292–3303

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247

French LE, Chonn A, Ducrest D, Baumann B, Belin D, Wohlwend A, Kiss JZ, Sappino AP, Tschopp J, Schifferli JA (1993) Murine clusterin: molecular cloning and mRNA localization of a gene associated with epithelial differentiation processes during embryogenesis. J Cell Biol 122:1119–1130

Gomaraschi M, Putt WE, Pozzi S, Iametti S, Barbiroli A, Bonomi F, Favari E, Bernini F, Franceschini G, Talmud PJ, Calabresi L (2010) Structure and function of the apoA-IV T347S and Q360H common variants. Biochem Biophys Res Commun 393:126–130

Haddad JG (1995) Plasma vitamin D-binding protein (Gc-globulin): multiple tasks. J Steroid Biochem Mol Biol 53:579–582

Hanley JM, Haugen TH, Heath EC (1983) Biosynthesis and processing of rat haptoglobin. J Biol Chem 258:7858–7869

Ix JH, Shlipak MG, Brandenburg VM, Ali S, Ketteler M, Whooley MA (2006) Association between human fetuin-A and the metabolic syndrome: data from the Heart and Soul Study. Circulation 113:1760–1767

Jenne DE, Lowin B, Peitsch MC, Bottcher A, Schmitz G, Tschopp J (1991) Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. J Biol Chem 266:11030–11036

Jiménez-Gémez Y, López-Miranda J, Blanco-Colio LM, Marín C, Pérez-Martínez P, Ruano J, Paniagua JA, Rodríguez F, Egido J, Pérez-Jiménez F (2009) Olive oil and walnut breakfasts reduce the postprandial inflammatory response in mononuclear cells compared with a butter breakfast in healthy men. Atherosclerosis 204:e70–e76

Kim JW, No JK, Ikeno Y, Yu BP, Choi JS, Yokozawa T, Chung HY (2002) Age-related changes in redox status of rat serum. Arch Gerontol Geriatr 34:9–17

Kirkwood TB, Austad SN (2000) Why do we age? Nature 408:233–238

Kordula T, Travis J (1995) Activation of the rat serine proteinase inhibitor 3 gene by interferon gamma via the interleukin 6-responsive element. Biochem J 309:63–67

Kruskal JB, Commerford PJ, Franks JJ, Kirsch RE (1987) Fibrin and fibrinogen-related antigens in patients with stable and unstable coronary artery disease. New Engl J Med 317:1361–1365

Lebreton JP, Joisel F, Raoult JP, Lannuzel B, Rogez JP, Humbert G (1979) Serum concentration of human alpha 2 HS glycoprotein during the inflammatory process: evidence that alpha 2 HS glycoprotein is a negative acute-phase reactant. J Clin Invest 64:1118–1129

Lovely RS, Kazmierczak SC, Massaro JM, D'Agostino RB Sr, O'Donnell CJ, Farrell DH (2010) Gamma' fibrinogen: evaluation of a new assay for study of associations with cardiovascular disease. Clin Chem 56:781–788

Mahley RW, Rall SC Jr (2000) Apolipoprotein E: far more than a lipid transport protein. Annu Rev Genomics Hum Genet 1:507–537

Martinez-Pinna R, Barbas C, Blanco-Colio LM, Tunon J, Ramos-Mozo P, Lopez JA, Meilhac O, Michel JB, Egido J, Martin-Ventura JL (2010) Proteomic and metabolomic

profiles in atherothrombotic vascular disease. Curr Atheroscl Rep 12:202–208

Mataix J (ed) (2002) Lípidos, vol I. Nutrición y alimentación humana. Ergon, Madrid

Materljan E, Materljan M, Materljan B, Vlacic H, Baricev-Novakovic Z, Sepcic J (2009) Multiple sclerosis and cancers in Croatia—a possible protective role of the "Mediterranean diet". Coll Antropol 33:539–545

Matsuura E, Kobayashi K, Tabuchi M, Lopez LR (2006) Oxidative modification of low-density lipoprotein and immune regulation of atherosclerosis. Prog Lipid Res 45:466–486

Melamed-Frank M, Lache O, Enav BI, Szafranek T, Levy NS, Ricklis RM, Levy AP (2001) Structure–function analysis of the antioxidant properties of haptoglobin. Blood 98:3693–3698

Meydani M (2001) Nutrition interventions in aging and age-associated disease. Ann NY Acad Sci 928:226–235

Miyakis S, Giannakopoulos B, Krilis SA (2004) Beta 2 glycoprotein I—function in health and disease. Thromb Res 114:335–346

Miyata M, Biro S, Kaieda H, Eto H, Orihara K, Kihara T, Obata H, Matsushita N, Matsuyama T, Tei C (2001) Apolipoprotein J/clusterin is induced in vascular smooth muscle cells after vascular injury. Circulation 104:1407–1412

Muller-Eberhard HJ, Schreiber RD (1980) Molecular biology and chemistry of the alternative pathway of complement. Adv Immunol 29:1–53

Ostos MA, Conconi M, Vergnes L, Baroukh N, Ribalta J, Girona J, Caillaud JM, Ochoa A, Zakin MM (2001) Antioxidative and antiatherosclerotic effects of human apolipoprotein A-IV in apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol 21:1023–1028

Owen RW, Giacosa A, Hull WE, Haubner R, Wurtele G, Spiegelhalder B, Bartsch H (2000) Olive-oil consumption and health: the possible role of antioxidants. Lancet Oncol 1:107–112

Pérez-Jiménez F, Ruano J, Pérez-Martínez P, López-Segura F, López-Miranda J (2007) The influence of olive oil on human health: not a question of fat alone. Mol Nutr Food Res 51:1199–1208

Perona JS, Cabello-Moruno R, Ruíz-Gutiérrez V (2006) The role of virgin olive oil components in the modulation of endothelial function. J Nutr Biochem 17:429–445

Price PA, Williamson MK, Nguyen TM, Than TN (2004) Serum levels of the fetuin–mineral complex correlate with artery calcification in the rat. J Biol Chem 279:1594–1600

Quiles JL, Pamplona R, Ramirez-Tortosa MC, Naudi A, Portero-Otin M, Araujo-Nepomuceno E, Lopez-Frias M, Battino M, Ochoa JJ (2010) Coenzyme Q addition to an *n*-6 PUFA-rich diet resembles benefits on age-related mitochondrial DNA deletion and oxidative stress of a MUFA-rich diet in rat heart. Mech Ageing Dev 131:38–47

Rosenberg ME, Silkensen J (1995) Clusterin: physiologic and pathophysiologic considerations. Int J Biochem Cell Biol 27:633–645

Sandford AJ, Chagani T, Spinelli JJ, Pare PD (1999) alpha1-Antitrypsin genotypes and the acute-phase response to open heart surgery. Am J Respir Crit Care Med 159:1624–1628

Santos-González M, Gómez Díaz C, Navas P, Villalba JM (2007) Modifications of plasma proteome in long-lived rats fed on a coenzyme Q_{10}-supplemented diet. Exp Gerontol 42:798–806

Schroeder F, Petrescu AD, Huang H, Atshaves BP, McIntosh AL, Martin GG, Hostetler HA, Vespa A, Landrock D, Landrock KK, Payne HR, Kier AB (2008) Role of fatty acid binding proteins and long chain fatty acids in modulating nuclear receptors and gene transcription. Lipids 43:1–17

Silkensen JR, Schwochau GB, Rosenberg ME (1994) The role of clusterin in tissue injury. Biochem Cell Biol 72:483–488

Srinivas PR, Wagner AS, Reddy LV, Deutsch DD, Leon MA, Goustin AS, Grunberger G (1993) Serum alpha 2-HS-glycoprotein is an inhibitor of the human insulin receptor at the tyrosine kinase level. Mol Endocrinol 7: 1445–1455

Stoscheck CM (1990) Quantitation of protein. Methods Enzymol 182:50–68

Sueyoshi T, Enjyoji K, Shimada T, Kato H, Iwanaga S, Bando Y, Kominami E, Katunuma N (1985) A new function of kininogens as thiol-proteinase inhibitors: inhibition of papain and cathepsins B, H and L by bovine, rat and human plasma kininogens. FEBS Lett 182:193–195

Thambisetty M, Simmons A, Velayudhan L, Hye A, Campbell J, Zhang Y, Wahlund LO, Westman E, Kinsey A, Guntert A, Proitsi P, Powell J, Causevic M, Killick R, Lunnon K, Lynham S, Broadstock M, Choudhry F, Howlett DR, Williams RJ, Sharp SI, Mitchelmore C, Tunnard C, Leung R, Foy C, O'Brien D, Breen G, Furney SJ, Ward M, Kloszewska I, Mecocci P, Soininen H, Tsolaki M, Vellas B, Hodges A, Murphy DG, Parkins S, Richardson JC, Resnick SM, Ferrucci L, Wong DF, Zhou Y, Muehlboeck S, Evans A, Francis PT, Spenger C, Lovestone S (2010) Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. Arch Gen Psychiatry 67:739–748

Tolle A, Schlame M, Charlier N, Guthmann F, Rustow B (2005) Vitamin E differentially regulates the expression of peroxiredoxin-1 and -6 in alveolar type II cells. Free Radic Biol Med 38:1401–1408

Tolosano E, Hirsch E, Patrucco E, Camaschella C, Navone R, Silengo L, Altruda F (1999) Defective recovery and severe renal damage after acute hemolysis in hemopexin-deficient mice. Blood 94:3906–3914

Tolosano E, Fagoonee S, Hirsch E, Berger FG, Baumann H, Silengo L, Altruda F (2002) Enhanced splenomegaly and severe liver inflammation in haptoglobin/hemopexin double-null mice after acute hemolysis. Blood 100:4201–4208

Torres C, Li M, Walter R, Sierra F (2001) T-kininogen inhibits fibroblast proliferation in the G(1) phase of the cell cycle. Exp Cell Res 269:171–179

Triffitt JT (1976) Plasma proteins present in human cortical bone: enrichment of the alpha2HS-glycoprotein. Calcif Tissue Res 22:27–33

Trougakos IP, Gonos ES (2002) Clusterin/apolipoprotein J in human aging and cancer. Int J Biochem Cell Biol 34:1430–1448

Trougakos IP, Gonos ES (2004) Functional analysis of clusterin/apolipoprotein J in cellular death induced by severe genotoxic stress. Ann NY Acad Sci 1019:206–210

Trougakos IP, Stathatos M, Chalikia A, Melidonis A, Gonos ES (2002) Serum levels of the senescence biomarker clus-

terin/apolipoprotein J increase significantly in diabetes type II and during development of coronary heart disease or at myocardial infarction. Exp Gerontol 37: 1175–1187

Vanschoonbeek K, Feijge MA, Paquay M, Rosing J, Saris W, Kluft C, Giesen PL, de Maat MP, Heemskerk JW (2004) Variable hypocoagulant effect of fish oil intake in humans: modulation of fibrinogen level and thrombin generation. Arterioscler Thromb Vasc Biol 24:1734–1740

Visioli F, Poli A, Gall C (2002) Antioxidant and other biological activities of phenols from olives and olive oil. Med Res Rev 22:65–75

Walter R, Murasko DM, Sierra F (1998) T-kininogen is a biomarker of senescence in rats. Mech Ageing Dev 106:129–144

Waterman E, Lockwood B (2007) Active components and clinical applications of olive oil. Altern Med Rev 12:331–342

Wong CM, Siu KL, Jin DY (2004) Peroxiredoxin-null yeast cells are hypersensitive to oxidative stress and are genomically unstable. J Biol Chem 279:23207–23213

Yasuda S, Atsumi T, Ieko M, Matsuura E, Kobayashi K, Inagaki J, Kato H, Tanaka H, Yamakado M, Akino M, Saitou H, Amasaki Y, Jodo S, Amengual O, Koike T (2004) Nicked beta2-glycoprotein I: a marker of cerebral infarct and a novel role in the negative feedback pathway of extrinsic fibrinolysis. Blood 103:3766–3772

Yoshida Y, Takahashi Y, Yoshikawa T, Nonomura A, Yoshioka A (2006) Suppressive effect of alpha2 Heremans–Schmid glycoprotein on in vitro calcification of osteogenesis. Pediatr Int 48:11–16