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Microarray analysis of hepatic genes differentially expressed in the presence of the unsaponifiable fraction of olive oil in apolipoprotein E-deficient mice

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The hypothesis that the unsaponifiable fraction of olive oil dramatically influences hepatic gene expression was tested in mice. Two olive oils, obtained from the same olive cultivar but by different technological procedures, were characterized to show that they differed mainly in terms of the composition/quantity of this unsaponifiable fraction. Using DNA microarrays, hepatic gene expression was analysed in apoE-deficient mice fed one of two isoenergetic, isonitrogenous diets containing either 10 % (w/w) olive oil or unsaponifiable fraction-enriched olive oil. To provide an initial screening of potential candidate genes involved in a differential response, only genes with remarkably modified expression (signal $\log_2$ ratio >3 or <-3) were further considered. The eleven genes fulfilling these prerequisites were confirmed by quantitative RT–PCR, and then analysed in apoE-deficient mice with a C57BL/6J genetic background. *Orosomucoid* and *serum amyloid A2* were upregulated (to variable extents depending on the genetic background) in the absence of hepatic steatosis and inflammation. *Fabp5* and *Mt2* were also strongly upregulated. Several proteases were highly suppressed by the unsaponifiable-enriched olive diet, independent of the genetic background. The findings indicate that change in the expression of these genes is a good marker of the intake of the unsaponifiable fraction of olive oil. The results highlight the important biological effects of the unsaponifiable fraction of olive oil. The term 'monounsaturated fatty acid-enriched oil' no longer appears appropriate for describing all the oils to which it is currently applied since it does not adequately reflect that they have different biological effects.

Apolipoprotein E-deficient mice: Olive oil: Unsaponifiable fraction

The 'Seven Countries' study showed that the so-called Mediterranean diet is associated with a reduced risk of cardiovascular mortality despite its associated high intake of fat, mainly derived from olive oil (Keys *et al.* 1986; Keys, 1995). It has also been shown that MUFA-containing oils reduce total and LDL cholesterol levels with no proportionate reduction of HDL cholesterol levels (Mattson & Grundy, 1985; Mensink & Katan, 1989; Mata *et al.* 1992). A whole panoply of experimental work has been designed to explain these epidemiological and clinical data. However, MUFA-enriched diets have selective physiological effects in man, indicating that other components, e.g. TAG species or non-fatty acids, rather than the oleic acid content alone, might be responsible for the benefits of virgin olive oil (Ruiz-Gutierrez *et al.* 1996; Kris-Etherton *et al.* 1999; Abia *et al.* 2001; Perona *et al.* 2003, 2006). Similar findings have also been described in animal studies dealing with the effects of olive oil on the vascular wall (Calleja *et al.* 1999; Herrera *et al.* 2001). When studying the biological effects of olive oil it should be remembered that it is more than a simple mixture of fatty acids, and that it contains other biologically active substances such as tocopherols, polyphenols and phytosterols, some of which have antioxidant and anti-inflammatory activities (Visioli *et al.* 2000, 2003; de la Puerta *et al.* 2001; de la Puerta-Vazquez *et al.* 2004; Perona *et al.* 2006).

In Spain, unsaponifiable fraction-enriched olive oil is known as *orujo* or olive-pomace oil, and is extracted by the secondary centrifugation of the residue left after obtaining virgin olive oil. Improved procedures for the extraction of this oil now allow it to be obtained with higher concentrations of terpenoids, tocopherols, phytosterols and waxes from the epicarp of the olive – compounds that are in low concentration in virgin olive oil (Pérez-Camino & Cert, 1999). The nutritional value of this innovative olive oil preparation has not yet been studied, although the biological activity of most of these compounds is now being unveiled. We recently reported the vasorelaxant effects of oleanolic acid and erythrodiol, two triterpenoids found in *orujo*, on rat aorta

Abbreviations: OO diet, diet supplemented with olive oil; qRT–PCR, quantitative real-time RT–PCR; UEOO diet, diet supplemented with unsaponifiable fraction-enriched olive oil.

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(Rodriguez-Rodriguez *et al.* 2004), as well as the improvement obtained with the unsaponifiable fraction of virgin olive oil in the balance between the vasoprotective and prothrombotic factors released by endothelial cells (Sanchez Perona *et al.* 2004).

The liver produces a number of lipoproteins (VLDL and HDL), apo and enzymes. The latter (hepatic lipase, lecithin cholesterol acyltransferase and phospholipid transfer protein) are involved in the plasma transformation of lipoproteins (den Boer *et al.* 2004). ApoE-deficient mice develop spontaneous atherosclerosis (Osada *et al.* 2000) and cognitive deficits resembling those of Alzheimer's disease and ageing, and therefore provide a model for all these problems (Masliah *et al.* 1995; Oitzl *et al.* 1997; Krzywkowski *et al.* 1999). When fed high-fat diets these mice show slight hepatic steatosis that responds to dietary manipulations such as changing the type of linoleic acid isomer provided (Arbonés-Mainar *et al.* 2006*a*). These steatotic variations have been associated with the development of atherosclerotic lesions (Arbonés-Mainar *et al.* 2006*b*). Thus, the liver may undergo important metabolic changes under the influence of olive oil, mediated through changes in plasma lipoprotein concentration, and the steatotic-prone liver of apoE-deficient mice offers an excellent model in which to verify this. To test the hypothesis that the unsaponifiable fraction of olive oil significantly influences hepatic gene expression, apoE-deficient mice of different genetic backgrounds were fed diets supplemented with either 10 % (w/w) olive oil or 10 % unsaponifiable fraction-enriched olive oil. Gene expression was then determined by microarray analysis and confirmed by real-time RT–PCR.

Methods and material

Animals

The experimental animals were seventeen two-month-old male, homozygous apoE KO mice with a C57BL6J × OLA129 genetic background, and twelve homozygous apoE KO mice with a C57BL6J genetic background, all bred at the Unidad Mixta de Investigación, University of Zaragoza. To confirm that the initial plasma cholesterol and TAG concentrations of these animals were the same, blood samples were taken (after overnight fasting) from the retroorbital plexus after isofluorane anaesthetization. All mice were housed in sterile filter-top cages in rooms maintained under a 12 h light–12 h dark cycle. All had access *ad libitum* to food and water. The study protocol was approved by the Ethics Committee for Animal Research of the University of Zaragoza. Body weights and food intake were recorded throughout the experiment.

Diets

Two study groups were established: (1) one received a chow diet (Teklad Mouse/Rat Diet no. 2014; Harlan Teklad, Harlan Ibérica, Barcelona, Spain) supplemented with 10 % (w/w) olive oil obtained by a conventional procedure (OO diet) (*n* 9 and *n* 6 for the first and second experiments respectively; see later), and (2) the other received the same chow diet but supplemented with 10 % (w/w) unsaponifiable fraction-enriched olive oil (UEOO diet; *n* 8 and *n* 6 for the first and second experiments respectively; see later). This unsaponifiable fraction-enriched olive oil was manufactured by an innovative procedure (Pérez-Camino & Cert, 1999). Both olive oils were obtained from the same cultivar, contained the same TAG species and both were refined to eliminate the influence of soluble phenol compounds. All diets were prepared weekly and stored in an N_2 atmosphere at -20°C. Fresh food was provided daily. The animals were fed the experimental diets for 11 weeks; both were well tolerated. The unsaponifiable fraction was characterized as previously described (de la Puerta-Vazquez *et al.* 2004).

Plasma analyses

At the end of the experimental period and after an overnight fast, the animals were killed by suffocation with CO_2 and blood was drawn from their hearts. Plasma serum amyloid A concentrations were evaluated by immunoassay using a rat monoclonal anti-mouse serum amyloid A antibody (BioSource International, Camarillo, CA, USA) as the primary antibody (intra-day CV of this assay was 15 % for positive control). Plasma α1-acid glycoprotein (orosomucoid) concentrations were determined by the single radial immunodiffusion test using the mouse α1-AG plate kit (Cardiotech Services, Louisville, KY, USA) and were processed the same day (intra-day CV of this assay was 4 % for positive control).

Analysis of hepatic lipids

To determine hepatic cholesterol and TAG concentrations, lipids were extracted from 100 mg tissue using the method of Folch *et al.* (1957), employing 2,6-di-*tert*-butyl-*p*-cresol as an antioxidant. The extracted lipids were redissolved in 1 ml of chloroform–methanol (2 : 1, v/v) and stored at -20°C until analysis. The lipid classes were determined by HPLC as described by Perona & Ruiz-Gutierrez (2004). Standard solutions of cholesteryl oleate, triolein, cholesterol and phosphatidylcholine, prepared in chloroform–methanol (2 : 1, v/v), were used for the identification and quantification of cholesteryl esters, TAG, free cholesterol and phospholipids, respectively.

RNA isolation, Affymetrix oligonucleotide array hybridization and data analysis

Immediately after the animals were killed the livers were removed and frozen in liquid nitrogen. RNA from each liver was isolated using Trigent reagent (MRC, Cincinatti, OH, USA). DNA contaminants were removed by TURBO DNAse treatment using the DNA removal kit from AMBION (Austin, TX, USA). RNA was quantified by absorbance at $A_{260/280}$ (the $A_{260/280}$ ratio was greater than 1·75). The integrity of the 28S and 18S ribosomal RNA was verified by agarose formaldehyde gel electrophoresis followed by ethidium bromide staining. Images were captured and analysed using a Bio-Rad image analysis system and Molecular Analyst (Bio-Rad, Madrid, Spain). The 28S/18S ratio was greater than 2.

Aliquots (20 μg) of total liver RNA from each mouse of each group (*n* 9 for the OO group and *n* 8 for the UEOO group) were pooled to avoid problems of individual variation,

and purified using the RNeasy system (Quiagen, Barcelona, Spain). Total liver RNA (8 μg) was then used for biotin labelling. Hybridization, washing, scanning and analysis with the Affymetrix GeneChip Murine Genome MOE430A array (Affymetrix, Santa Clara, CA, USA) were performed according to the standard Affymetrix protocols used at Progenika Biopharma (Derio, Spain). Fluorometric data were generated by Affymetrix software, and the fluorimetric signal adjusted so that all the probe sets provided intensities within a manageable range. Transcripts with signal intensities that were lower than the noise of the matrix (± 3 SD) were not taken into account. The data obtained in the microarray hybridizations were processed with Microarray Suite 5.0 (Affymetrix) software. The identification of genes that were up- or down-regulated by the unsaponifiable fraction was performed by comparing gene expressions in the livers of animals from the two diet groups (significance set at $P<0.01$). Of these we selected only those whose signal $\log_2$ ratio was higher than 1·5 (up-regulated genes) or lower than -1.5 (down-regulated genes). Signal $\log_2$ ratio is now recommended by Affymetrix software and several authors (Gilsbach *et al.* 2006) because of the linear response observed in contrast to fold change. The complete datasets were deposited in the Gene Expression Omnibus (GEO) database (accession number GSE2261).

Quantification of mRNA

The differences in mRNA expression observed with the microarrays was confirmed by Northern blotting and quantitative real-time RT–PCR (qRT–PCR) analysis of individual samples. Northern blot analysis was performed as previously described (Acín *et al.* 2005). The mouse clones for *apoa5* (4196296 IMAGE Clone) and *pon1* (4158951 IMAGE Clone) were obtained from MGC Geneservice (Cambridge, UK). The probes used were those previously described: *apoa5* (Arbonés-Mainar *et al.* 2006*a*) and *pon1* (Acín *et al.* 2005). A mouse *β-actin* fragment (Acín *et al.* 2005) was used to normalize the amount of RNA. Labelling and quantification were performed as previously described (Acín *et al.* 2005).

Equal amounts of DNA-free RNA from each sample of each animal were used in qRT–PCR analyses. First-strand cDNA synthesis and the PCR reactions were performed using the SuperScript III Platinum Two-Step qRT–PCR Kit with SYBR Green (Invitrogen, Madrid, Spain), according to the manufacturer's instructions and as previously described (Arbonés-Mainar *et al.* 2006*a*). The primers used were designed by Primer Express® (Applied Biosystems, Foster City, CA, USA): for *chymotrypsinogen* – sense, 5′-CAG CAC CAT GGC ATT CCT TT-3′, antisense, 5′-GCA TCC TCT CCG TTG ACG AT-3′; for *elastase 2* – sense, 5′-ACT GAA TTG CCG GGC ATC TA-3′, antisense, 5′-GTT CCT TGC CAT CAC CGA GT-3′; for *fatty acid binding protein 5* – sense, 5′-GAC GGT CTG CAC CTT CCA AG-3′, antisense 5′-CAG GAT GAC GAG GAA GCC C-3′; for *glucokinase* – sense, 5′-ACG ACC CCT GCT TAT CCT CA-3′, antisense, 5′-CAC GGT CCA TCT CCT TCT GC-3′; for *leptin receptor* – sense, 5′-TGA CTT GCA GAT GGT CAC CC-3′, antisense, 5′-AAG CCG TCT CTC TGT AAG ACG C-3′; for *metallothionein 2* – sense, 5′-TCG GAA TCT TCA CTC TTC AAA CC-3′, antisense, 5′-CCA TCG GAG GCA CAG GAG-3′; for *nicotinamide N-methyltransferase* – sense, 5′-CCT GGG CAG TCT GCT CAA G-3, antisense, 5′-AAT GGT GTA ACC GGC CTC TTC-3; for *orosomucoid 2* – sense, 5′-TTG GAA GCT CAG AAC CCA GAA-3′, antisense, 5′-TCG AAG CTC CAT CGT GTC ATT-3′; for *pancreatic lipase 2* – sense, 5′-GGA GCC CAG CAC AGA TCA AC-3′, antisense, 5′-GCC AGT TTT CTT CTC CCT TGT C-3′; for *serum amyloid A 2* – sense, 5′-CTG GCT GGA AAG ATG GAG AC-3′, antisense, 5′-TGT CCT CGT GTC CTC TGC-3′; for *trypsin 4* – sense, 5′-CTT CAA TGC CTG GAT GCC C-3′, antisense, 5′-CAG GAG ACA ATG CCC TGG AG-3′; and for *β-actin* – sense, 5′-CTG ACT GAC TAC CTC ATG AAG ATC CT-3′, antisense, 5′-CTT AAT GTC ACG CAC GAT TTC C-3′. The specificity of the PCR reaction was confirmed by sequencing the products after their electrophoretic separation in agarose gels. Real-time RT–PCR reactions were performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) following the standard procedure. The relative amount of all mRNA was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. *β-actin* mRNA was used as the invariant control.

Liver histology analysis

Aliquots of liver were stored in neutral formaldehyde and embedded in paraffin. Sections (4 μm) were stained with haematoxylin and eosin and observed with a Nikon microscope.

Statistical analysis

The Mann–Whitney *U* test, according to the Algoritm of Microarray Suite 5.0 (Affymetrix), was used to compare the mean fluorimetric signal intensities of eleven to twenty perfect match probe per probe set hybridized to liver RNA from the OO group with those obtained for the UEOO group and in either case subtracted the intensities of a similar number of mismatched probes (background hybridization). Significance was set at $P<0.01$. Correlations between variables were tested by calculating the Spearman's rank-order correlation coefficient (r_s); tests were performed using Instat 3.02 software for Windows (GraphPad, San Diego, CA, USA).

Results

Dietary characteristics

Tables 1 and 2 show the lipid composition of the two olive oils used. Their composition in linolenic fatty acid, SFA and MUFA was similar (Table 1), but the composition of the unsaponifiable fraction was different (except for squalene) (Table 2): the unsaponifiable fraction-enriched olive oil had greater quantities of phytosterols, waxes, triterpenes (erythrodiol, uvaol and maslinic) and tocopherols.

No significant difference in weight gain (4·4 (SD 0·2) for the OO group compared to 4 (SD 0·3) g for the UEOO group) or food intake (3·9 (SD 0·3) for the OO group compared to 3·8 (SD 0·2) g for the UEOO group) was observed between the animals of the two diet groups. Similarly, the weight of

Table 1. Fatty acid composition of the olive oils used (g/100 g, w/w)

Fatty acids	Olive oil	Unsaponifiable fraction-enriched olive oil
Myristic (14 : 0)	0·02	0·02
Palmitic (16 : 0)	10·98	10·29
Stearic (18 : 0)	3·53	2·95
Arachidic (20 : 0)	0·42	0·45
Behenic (22 : 0)	0·12	0·17
Lignoceric (24 : 0)	0·05	0·07
Palmitoleic (16 : 1)	0·82	0·76
Oleic (18 : 1)	77·80	74·27
Gadoleic (20 : 1)	0·25	0·33
Linoleic (18 : 2*n*-6)	4·52	8·07
Linolenic (18 : 3*n*-3)	0·62	0·70
SFA	15·14	13·95
MUFA	78·87	75·36

the liver appeared unaltered by the administration of either diet (0·79 (SD 0·05) and 0·76 (SD 0·02) g, respectively).

Gene expression in livers of apoE-deficient mice fed the different diets

To determine the changes in hepatic gene expression induced by the unsaponifiable fraction of olive oil, the expression of 22 690 transcripts represented on the Affymetrix GeneChip Murine Genome MOE430A array was quantified in pooled liver samples of nine animals that received the OO diet and another eight that received the UEOO diet. The livers of OO animals expressed 10 455 transcripts, while those of the UEOO animals expressed 10 675 (identified as ‘present’ by Affymetrix software). Using the Mann–Whitney ranking feature of the Affymetrix software to determine significant differences in gene expression ($P<0·01$), the increased expression of 920 sequences plus the reduced expression of 420 sequences was identified in samples from the animals on the UEOO diet compared to those on the OO diet when no multiple test correction was applied. When the latter was taken into consideration by removing expressions with similar gene symbols, identical accession number and identical UNIGENE number, the number of genes with increased and repressed expression was reduced to 660 and 324, respectively. To select the most relevant, only differentially regulated genes with a signal $\log_2$ ratio higher than 1·5 (for those genes up-regulated) or lower than $-1·5$ (for those repressed) were taken into account. Tables 3 and 4 list the genes whose mRNA reflected these expressions. Thirty genes fulfilled the criterion of showing increased expression as a response to the unsaponifiable fraction of olive oil (Table 3). Five of these genes coded for acute phase proteins (*Orm1*, *Orm2*, *Orm3*, *Saa1* and *Saa2*), four coded for proteins belonging to the extracellular space (*Apcs*, *Fgl1*, *Prg4* and *Sdc4*), three were involved in signal transduction (*Egfr*, *Lepr* and *Sesn1*), three in electron transport (*Cyp17a1*, *Cyp2b20* and *Qscn6*) and two in protein metabolism (*Ren1* and *Serpina3n*). A further three were enzymes involved in acetyl-CoA (*Pdk4*), fatty acid (*Scd1*) and bile acid (*Cyp7a1*) biosynthesis, two coded for ion binding proteins (*Mt1* and *Mt2*), two for metabolite transport proteins (*Fabp5* and *Lcn2*), four for proteins with miscellaneous functions (e.g. one was involved in apoptosis (*Bcl2 l*), another was an enzyme involved in nicotinamide metabolism (*Nnmt*), one was a member of the complement cascade (*Cfh*) and one was a transcription factor (*Gadd45 g*). Finally, two genes coded for unknown expressed sequence tags. Twenty-four genes met the criterion of showing a reduced expression as a response to the presence of the unsaponifiable fraction of olive oil (Table 4). Of these, eight were involved in proteolysis (*Cpa1*, *Cpb1*, *Chym*, *Ela1*, *Ela2*, *Ela3b*, *Prss2* and *Try4*), four in lipid metabolism (*Clps*, *Cel*, *Pnlip* and *Pnliprp1*), three in glutathione conjugation (*Gsta2*, *Gsta4* and *Gstm3*), two coded for heat shock proteins (*Hspa1a* and *Hspb1*), two for transcription factors (*Nr1d1* and *Dbp*), one for an enzyme involved in carbohydrate metabolism (*Gck*), three for proteins with miscellaneous functions (one extracellular receptor (*Dmbt1*), one enzyme involved in RNA metabolism (*Rnase1*) and one cell surface protein (*Sycn*)), and finally one for an unknown expressed sequence tag.

To validate the results obtained with the microarray, the expressions of eleven genes – *Chym*, *Ela2*, *Fabp5*, *Gck*, *Lepr*, *Mt2*, *Nnmt*, *Orm2*, *Pnlip*, *Saa2*, *Try4* – that were strikingly up- or down-regulated (signal $\log_2$ ratio >3 or <-3) were individually studied by specific qRT–PCR assays. *β-actin* was used to normalize the results that are shown in Table 5. The six up-regulated genes included in the validation analysis – *Fabp5*, *Lepr*, *Mt2*, *Nnmt*, *Orm2*, *Saa2* – appeared

Table 2. Composition of the unsaponifiable fraction of the olive oils used (mg/kg, w/w)

Component	Olive oil	Unsaponifiable fraction-enriched olive oil
Erythrodiol + uvaol	17	500
Maslinic acid	0	110
Squalene	2600	2500
Total phytosterols	1120	2240
Total tocopherols	220	981
Waxes	120	3400

Table 3. Hepatic genes up-regulated by the unsaponifiable fraction of olive oil*

Biological process	GenBank	Affymetrix ID	Name	Gene symbol	Olive oil	UE olive oil	Signal $\log_2$ ratio
Acetyl-CoA biosynthesis	NM_013743	1417273_at	Pyruvate dehydrogenase kinase 4	*Pdk4*	20·6	96·1	1·9
Acute phase response	BE628912	1451054_at	Orosomucoid 1	*Orm1*	702	3087·5	2
Acute phase response	NM_011016	1420438_at	Orosomucoid 2	*Orm2*	71·5	1496·9	3·9
Acute phase response	NM_013623	1450611_at	Orosomucoid 3	*Orm3*	20·7	115·1	2·3
Acute phase response	NM_009117	1450788_at	Serum amyloid A 1	*Saa1*	2017·4	5175·9	1·5
Acute phase response	NM_011314	1449326_x_at	Serum amyloid A 2	*Saa2*	858·1	4291·9	3·3
Apoptosis	NM_009743	1420887_a_at	Bcl-xl	*Bcl2l*	20·1	103	2·9
Bile acid metabolism	NM_007824	1422100_at	Cytochrome P450, 7a1	*Cyp7a1*	109·9	495·4	2·5
Metal binding protein	BC027262	1451612_at	Metallothionein 1	*Mt1*	15·1	88·1	2·9
Metal binding protein	AA796766	1428942_at	Metallothionein 2	*Mt2*	420·7	4657	3·1
Complement cascade	AI987976	1450876_at	Complement component factor h	*Cfh*	70·4	234·3	1·8
Electron transport	NM_007809	1417017_at	Cytochrome P450, 17a1	*Cyp17a1*	34·2	146·1	1·9
Electron transport	AF128849	1451787_at	Cytochrome P450, 2b20	*Cyp2b20*	185·3	615·6	1·8
Electron transport	AK004880	1420832_at	Quiescin Q6	*Qscn6*	313·2	688·9	1·5
Extracellular space	NM_011318	1419059_at	Serum amyloid P	*Apcs*	902·2	4829·2	2·2
Extracellular space	BC005679	1417654_at	Syndecan 4	*Sdc4*	275·6	1978·7	2·9
Extracellular space	NM_021400	1449824_at	Proteoglycan 4	*Prg4*	83·8	373·2	2·1
Extracellular space	BC021946	1424599_at	Fibrinogen-like protein 1	*Fgl1*	1698·1	4164	1·6
Fatty acid synthesis	NM_009127	1415965_at	Stearoyl-coenzyme A desaturase	*Scd1*	195·3	721·1	1·8
Methyl transferase	AK006371	1432517_a_at	Nicotinamide *N*-methyltransferase	*Nnmt*	107·1	1158·3	3·4
Protease inhibitor	NM_009252	1419100_at	Proteinase inhibitor, A3N	*Serpina3n*	935·4	2959·6	1·5
Proteolysis	NM_031192	1448975_s_at	Renin 1	*Ren1*	48·6	269·9	2·7
Signal transduction	U42467	1425644_at	Leptin receptor	*Lepr*	2·8	166·1	5·8
Signal transduction	U03425	1424932_at	Epidermal growth factor receptor	*Egfr*	148·6	446·8	1·5
Signal transduction	BG076140	1433711_s_at	Sestrin 1	*Sesn1*	63·4	166·9	1·5
Transcription factor	AK007410	1453851_a_at	Growth arrest and DNA-damage-inducible 45	*Gadd45g*	96·5	240·3	1·5
Transport (fatty acids)	BC002008	1416022_at	Fatty acid binding protein 5	*Fabp5*	32·6	238·5	3
Transport (fatty acids)	X14607	1427747_a_at	Lipocalin 2	*Lcn2*	400·9	3304·6	2·9
	AK017926	1428306_at	Expressed sequence tag	–	80·9	359·2	2·4
	AV291259	1429144_at	Expressed sequence tag	–	341·9	1280·7	1·7

UE, unsaponifiable fraction-enriched.

* Data represent intensity of signal for each condition with the Affymetrix chip. For details of procedures, see p. 630.

Table 4. Hepatic genes down-regulated by the unsaponifiable fraction of olive oil*

Biological process	GenBank	Affymetrix ID	Name	Gene symbol	Olive oil	UE olive oil	Signal $\log_2$ ratio
Carbohydrate metabolism	BC011139	1419146_a_at	Glucokinase	*Gck*	63·8	7·9	−3·2
Extracellular receptor	NM_007769	1418287_a_at	Endoglin	*Dmbt1*	319·6	52·1	−2·2
Glutathione conjugation	NM_008182	1421040_a_at	Glutathione *S*-transferase, α2	*Gsta2*	269	59·2	−2·3
Glutathione conjugation	NM_010357	1416368_at	Glutathione *S*-transferase, α 4	*Gsta4*	219	47·1	−2·1
Glutathione conjugation	J03953	1427473_at	Glutathione *S*-transferase, mu 3	*Gstm3*	217·2	45·3	−1·9
Heat shock protein	M12573	1427126_at	Heat shock protein 1A	*Hspa1a*	634	220·9	−1·5
Heat shock protein	U03561	1425964_x_at	Heat shock protein 1	*Hspb1*	458·1	71·3	−2·4
Lipid metabolism	NM_025469	1415805_at	Colipase, pancreatic	*Clps*	393·1	51·3	−2·8
Lipid metabolism	BC006872	1417257_at	Carboxyl ester lipase	*Cel*	282·7	52	−2·5
Lipid metabolism	AI326372	1433431_at	Pancreatic lipase	*Pnlip*	858·3	78·6	−3·3
Lipid metabolism	NM_018874	1415777_at	Pancreatic lipase related 1	*Pnliprp1*	246·5	16·6	−2·7
Nuclease activity	NM_011271	1416523_at	Pancreatic ribonuclease A1	*Rnase1*	305·3	73·4	−1·6
Proteolysis	AK003088	1428062_at	Carboxypeptidase A1	*Cpa1*	800·3	70·6	−3·3
Proteolysis	AK003061	1428102_at	Carboxypeptidase B1	*Cpb1*	536·6	84·1	−2·6
Proteolysis	NM_025583	1448220_at	Chymotrypsinogen	*Chym*	778	59·2	−3·3
Proteolysis	BC011218	1423693_at	Pancreatic elastase 1	*Ela1*	690·8	231	−1·9
Proteolysis	NM_007919	1448281_a_at	Elastase 2	*Ela2*	666	79·6	−3·3
Proteolysis	NM_026419	1415884_at	Pancreatic elastase 3B	*Ela3b*	470·5	77·6	−2
Proteolysis	BI348548	1433459_x_at	Trypsin 2	*Prss2*	549·3	86·1	−2·6
Proteolysis	AB009661	1415954_at	Trypsin 4	*Try4*	436·3	29·4	−4·1
Surface protein	BC019567	1451228_a_at	Syncollin	*Sycn*	242·7	14·5	−3
Transcription factor	W13191	1426464_at	Nuclear receptor 1 D 1	*Nr1d1*	262·3	59·8	−1·8
Transcription factor	BB550183	1438211_s_at	Albumin promoter binding protein	*Dbp*	116·5	46·1	−1·5
	BF581690	1428359_s_at	Expressed sequence tag	–	203·8	19·5	−2·5

UE, unsaponifiable fraction-enriched.

* Data represent intensity of signal for each condition with the Affymetrix chip. For details of procedures, see p. 630.

Table 5. Hepatic genes regulated by the unsaponifiable fraction of olive oil*

	Olive oil (*n* 9)		UE olive oil (*n* 8)			
	Mean	SD	Mean	SD	Fold change	Signal $\log_2$ ratio
Genes up-regulated						
Fabp5	1·0[a]	0·8	5·5[b]	0·4	5·3	2·4
Lepr	0·4[a]	0·5	4·1[b]	0·2	10·9	3·4
Mt2	0·5[a]	0·6	13·6[b]	1·5	29·1	4·8
Nnmt	0·1[a]	0·3	2·4[b]	0·4	16·8	4·1
Orm2	2·4[a]	0·3	75·2[b]	2	31·8	5·0
Saa2	3·4[a]	0·3	108·5[b]	1	32·2	5·0
Genes down-regulated						
Chym	35·1[a]	0·1	5·7[b]	0·3	0·16	−2·6
Ela2	16·2[a]	0·1	4·2[b]	0·3	0·26	−1·9
Gck	3·7[a]	0·8	0·8[b]	0·6	0·21	−2·2
Pnlip	12·2[a]	3	2·0[b]	0·2	0·16	−2·6
Try4	15·2[a]	0·1	4·6[b]	0·6	0·30	−1·7

UE, unsaponifiable fraction-enriched.

[a,b] Mean values within a row with unlike superscript letters were significantly different (Mann–Whitney *U* test; $P<0·001$).

* Data represent arbitrary units normalized to the *β-actin* expression for each condition with the quantitative real-time RT–PCR. For details of procedures, see p. 630.

significantly increased in their expressions by the presence of the unsaponifiable fraction of olive oil. The five down-regulated genes selected – *Chym*, *Ela2*, *Gck*, *Pnlip*, *Try4* – were significantly decreased in mice receiving the diet enriched in the unsaponifiable fraction of olive oil. Fig. 1 shows the correlation between the mean values of signal $\log_2$ ratio for the eleven genes with the microarray assay performed with pooled samples (Tables 3 and 4), and the mean of each group obtained after the analysis of samples from each animal in both experimental groups (Table 5). Good agreement between these procedures was obtained (*r* 0·9382, $P<0·0001$) and all samples were correctly classified, although in two cases the magnitude of the response differed between both methods. Two genes showing mild expression change (signal $\log_2$ ratio $<1·5$), *apolipoprotein 5* and *paraoxonase 1*, were also studied by Northern blotting, which confirmed their microassay results (data not shown). This indicates that pooled samples can be successfully used to provide an initial screening of gene expression in studies of this type, with the attending economic and time savings although with the limitation of no information on biological variability.

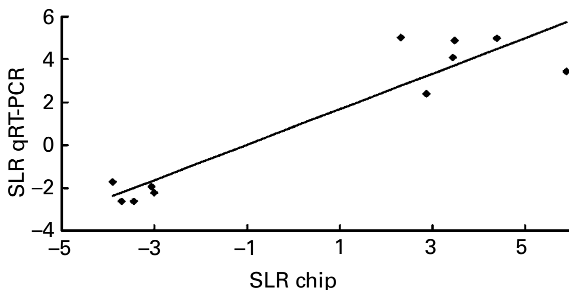

Fig. 1. Correlation analysis between microarray and quantitative real-time RT–PCR (qRT–PCR) data. The expression of eleven genes – *Chym*, *Ela2*, *Fabp5*, *Gck*, *Lepr*, *Mt2*, *Nnmt*, *Orm2*, *Pnlip*, *Saa2* and *Try4* – was individually studied by qRT–PCR and normalized to the invariant *β-actin* gene. For details of procedures, see p. 630. The mean values obtained for signal $\log_2$ ratio (SLR) from individual analyses (see Table 5) were plotted against the microarray values which used pooled samples (see Tables 3 and 4). Good agreement between the procedures was seen (*r* 0·9382, $P<0·0001$).

Plasma presence of unsaponifiable-activated gene products

Since five of the overexpressed genes (*Orm1*, *Orm2*, *Orm3*, *Saa1* and *Saa2*) coded for circulating proteins that may influence general homeostasis, their plasma levels were determined. If the remarkable changes observed in the hepatic expression of these proteins were reflected in plasma to the same degree, this would make them highly attractive markers of the consumption of the unsaponifiable fraction of olive oil. Serum amyloid proteins are well-known modifiers of plasma lipoproteins but no physiological role for orosomucoid has been proposed besides its role in the acute phase response (Hochepied *et al.* 2003). Fig. 2(*a*) shows the results of Western analysis of serum amyloid protein concentration. The observed increase of *Saa* mRNA expression (Table 3) was not reflected in plasma in either the OO or UEOO mice, suggesting the absence of an acute phase reaction. To confirm this lack of acute phase response, the livers of animals consuming the different diets were examined histologically. Fig. 2(*b*) shows representative images of livers from animals of the two diet groups. For comparison, an image of an ApoE-deficient mouse of the same age that was fed the chow diet with no added oil of any type is included (marked as control group). The liver of this control animal shows marked steatosis, in agreement with the described accumulation of lipids in this model (Sehayek *et al.* 2000). Interestingly, both experimental diets alleviated these steatotic features. No inflammatory cells were detected in the liver of any animal, suggesting that the UEOO diet does not induce an acute phase response. However, the increased hepatic *Orm2* mRNA levels observed in the chip analysis were confirmed by qRT–PCR (Fig. 3(A)) and were reflected in the plasma concentration of animals consuming the UEOO diet (Fig. 3(C)). The present results indicate that the increase in hepatic *Orm2* mRNA leads to increased concentrations of the protein product in plasma.

To determine whether this induction of *Orm2* also occurred in apoE-deficient mice with the C57BL/6J genetic background, their livers were studied for the expression of this gene. Although the UEOO diets led to a reduction in hepatic

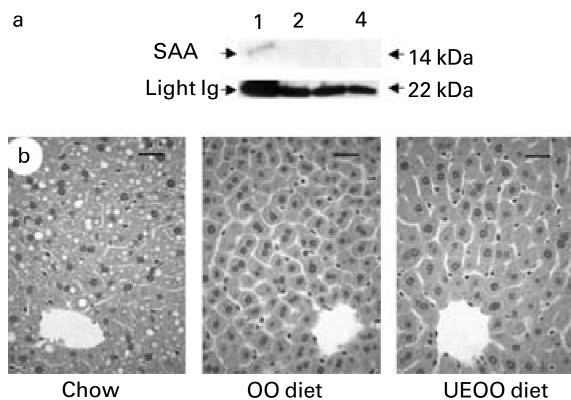

Fig. 2. Absence of acute phase reaction in mice fed the different diets (OO diet, diet supplemented with olive oil; UEOO diet, diet supplemented with unsaponifiable fraction-enriched olive oil). (*a*), Plasma serum amyloid A (SAA) Western blot analysis. Lane 1, a positive control corresponding to rat plasma from an animal treated with turpentine to induce the acute phase reaction. Lanes 2–4, plasma from mice consuming the different diets: chow, OO diet and UEOO diet, respectively. Light chain Ig detection was used as a loading control. (*b*), Representative liver micrographs from apoE-deficient mice consuming the different diets (bars = 25 μm). For details of procedures, see p. 630.

Orm2 mRNA levels (Fig. 3(B)), surprisingly this was not reflected at the plasma level (data not shown). The increase in hepatic *Orm2* mRNA expression is probably a specific response elicited by the unsaponifiable fraction of olive oil, and is not related to an acute phase response but conditioned by the genetic background of the mice.

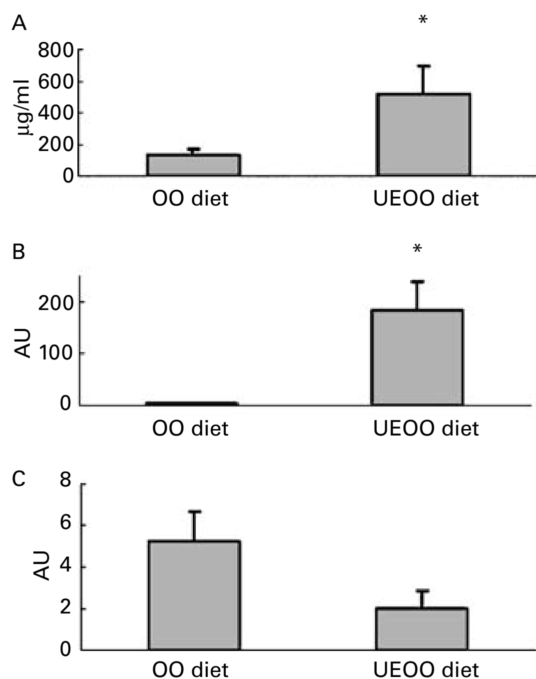

Fig. 3. Influence of genetic background on orosomucoid expression in apoE-deficient mice consuming the different diets (OO diet, diet supplemented with olive oil; UEOO diet, diet supplemented with unsaponifiable fraction-enriched olive oil). (A), Plasma orosomucoid 2 levels in apoE-deficient mice with C57BL/6J × Ola129 genetic background. (B, C), mRNA expression for orosomucoid 2 in livers of apoE-deficient mice with C57BL/6J × Ola129 (B) and C57BL/6J (C) genetic backgrounds (determined by quantitative real-time RT–PCR). For details of procedures, see p. 630. Values are means with their standard errors depicted by vertical bars. Mean values were significantly different from those of the OO diet (Mann–Whitney *U* test): $^*P < 0{\cdot}001$.

Effects of mouse genetic background on the response to unsaponifiable fraction-enriched olive oil

To investigate which of the selected genes – *Chym*, *Ela2*, *Fabp5*, *Gck*, *Lepr*, *Mt2*, *Nnmt*, *Pnlip*, *Saa2*, *Try4* – were influenced by the genetic background, their expressions in apoE-deficient mice (C57BL/6J genetic background) receiving both diets were assayed by qRT–PCR (normalized to the invariant gene *β-actin*). The results are expressed as signal $\log_2$ ratios in both genetic backgrounds (Fig. 4). Interestingly, no *Pnlip* expression was detected in the livers of mice with the C57BL/6J background and lesser variability of response was observed in these mice. For the genes *Lepr* and *Saa2*, a significant opposite response was seen in mice of different genetic background. In contrast, for seven other genes the influence of the UEOO diet was similar in both types of mice (*Mt2*, *Chym* and *Ela2* expressions), although the magnitude of response significantly differed (more pronounced for the *Fabp5* gene in the C57BL/6J background, and less so in genes *Gck*, *Nnmt* and *Try4* in the C57BL/6J × Ola129 background). The present results further illustrate that genetic background may influence the response to the unsaponifiable fraction of olive oil.

Discussion

The unsaponifiable fraction of olive oil remarkably influenced the activity of several genes. The nutrigenomic approach of the present work clearly shows the important effect of this fraction. Some of its components, such as phytosterols (Plosch *et al.* 2004) and vitamin E (Barella *et al.* 2004), have been shown to affect the expression of certain genes.

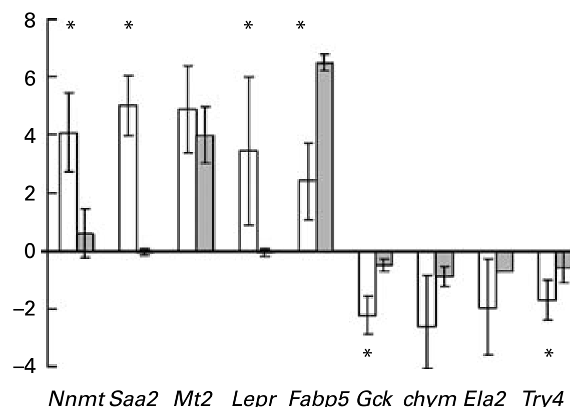

Fig. 4. Influence of genetic background of apoE-deficient mice on the pattern of gene expression in response to the diet supplemented with unsaponifiable fraction-enriched olive oil (UEOO diet). Data are expressed as signal $\log_2$ ratios of hepatic mRNA expression (as determined by quantitative real-time RT–PCR) for each gene in apoE-deficient mice with C57BL/6J × Ola129 (□) and C57BL/6J (■) genetic backgrounds consuming either the diet supplemented with olive oil (OO diet) or the UEOO diet. Animals receiving the OO diet were used as the reference against which to compare the effects of the UEOO diet. For details of procedures, see p. 630. Values are means with their standard deviations depicted by vertical bars. $^*P < 0{\cdot}001$ between genetic backgrounds according to the Mann–Whitney *U* test.

Others, such as maslinic acid (Montilla *et al.* 2003; Márquez-Martín *et al.* 2006) and erythrodiol (Rodriguez-Rodriguez *et al.* 2004), are biologically active compounds but it is not known whether they influence the expression of any gene. Independent of the mechanism involved, the present results reveal the additional effects of the unsaponifiable fraction with respect to the influence of olive oil, and support the idea that not all MUFA-containing oils behave in the same way (Kritchevsky *et al.* 1984; Kris-Etherton *et al.* 1999).

The huge amounts of information provided by microarrays requires further action be undertaken if meaningful and manageable data are to be obtained, such as selecting only the genes with the highest expression changes (Dutta *et al.* 2003; Vergnes *et al.* 2003; Artieda *et al.* 2005; Calpe-Berdiel *et al.* 2005) or those involved in a certain metabolic pathway (Horton *et al.* 2003; Kreeft *et al.* 2005). In the present work, analysis was performed by subjecting the microarray data to two rounds of selection. First, a restrictive step was performed, selecting only those genes with a signal $\log_2$ ratio of $\pm 1{\cdot}5$. With this criterion, only thirty genes were found to be remarkably up-regulated and twenty-four notably down-regulated. Second, only those genes whose expression was strongly modified were deemed to be potential markers of the intake of the unsaponifiable fraction. The strong modification of the expression of these genes was individually confirmed by qRT–PCR. Good agreement was observed between the Affymetrix chip and qRT–PCR data (Fig. 1). In high-density microarrays, such as the Affymetrix MOE430A gene chip, several probes are used for the same gene, therefore the main concern regarding repeatability is the natural individual variability of mRNA expression. Minimum information about a Microarray experiment (MIAME) standards recommend using several chips for this reason (http://www.mged.org/index.html), but this is not the only option and sometimes it may be too stringent in terms of sample demands, time, cost and the manageability of information. The present data clearly show that pooling RNA from different animals and using this in microarray analysis is a reliable screening method for the search of biological effects in terms of saving samples, time and economic resources, as other authors have found (Napoli *et al.* 2002; Dutta *et al.* 2003; Artieda *et al.* 2005; Calpe-Berdiel *et al.* 2005; Kreeft *et al.* 2005). However, the main drawback of this approach is the lack of information on biological variability of individual samples. This limitation, also observed in other areas (Peng *et al.* 2003), may be particularly important in the nutrition field in order to distinguish dietary responders and non-responders. Therefore, the experimenter should be aware of this caveat before deciding to pool samples.

The eleven genes whose expression was strongly modified – *Chym*, *Ela2*, *Fabp5*, *Gck*, *Lepr*, *Mt2*, *Nnmt*, *Orm2*, *Pnlip*, *Saa2*, *Try4* – were tested for their suitability as markers of the consumption of the unsaponifiable fraction. Their dependency on genetic background in apoE-deficient mice was also analysed. Seven of these genes – *Chym*, *Ela2*, *Fabp5*, *Gck*, *Mt2*, *Nnmt* and *Try4* – were representative markers of the presence of the unsaponifiable fraction of olive oil in the diet, independent of the genetic background. Three of these genes – *Chym*, *Ela2* and *Try4* – are involved in proteolysis and showed reduced expression in the UEOO animals. *Gck*, an enzyme involved in glucose metabolism and also repressed in animals receiving high-fat diets, showed similar behaviour (Dutta *et al.* 2003). The opposite (up-regulation) was observed for the expressions of *Fabp5*, *Mt2* and *Nnmt*. *Fabp5* (*mal1*) is considered to be an epidermal protein although it is also expressed in adipocytes (Maeda *et al.* 2003) and the liver (see GenBank accession AK167389 for a clone isolated from a liver cDNA library, and the present data). The exact role of this protein is not yet completely known, although it has been proposed to bind leukotriene A4 (Zimmer *et al.* 2004) and to play a role in systemic insulin sensitivity (Maeda *et al.* 2003). The change in its expression induced by the UEOO diet was particularly dramatic in the C57BL/6J animals. *Mt2* is thought to be associated with obesity since knock-out mice lacking this gene develop this problem (Miura & Koizumi, 2005). In both studied substrates, the expression of this gene was up-regulated (Fig. 4). *Nnmt* has been recently associated with plasma homocysteine levels (Souto *et al.* 2005). Its genetic background-dependent response might explain the variation in homocysteine levels in different strains of mice. Taken as a whole, the present results suggest that the unsaponifiable components of olive oil play an important role in controlling the expression of genes with roles in obesity, insulin sensitivity and cardiovascular risk factors, and that it deserves further attention.

The expression levels of *Lepr*, *Orm2* and *Saa2* act as markers of the presence of the unsaponifiable fraction of olive oil in the diet, but in a genetic background-dependent fashion. No increase in plasma serum amyloid A was seen, but circulating levels of orosomucoid appeared to be elevated in mice with the hybrid genetic background (C57BL/6J × OLA129). The induction of orosomucoids has to date been attributed to acute phase reactions (Hochepied *et al.* 2003). In this regard, the absence of any hepatic steatosis or inflammation plus a lack of change in serum amyloid A after the administration of the unsaponifiable fraction suggests that increased orosomucoid plasma levels are a unique response elicited by these compounds via the induction of *Orm2* expression. Recent studies have found that subjects with increased plasma concentrations of orosomucoid have higher levels of vitamin A (Thurnham *et al.* 2003). In addition, in transgenic mice overexpressing *srebp1* and *srebp2*, transcriptional factors involved in lipid metabolism, increased expression of this gene has also been described (Horton *et al.* 2003) although at a lower intensity than in mice consuming the UEOO diet. Together, the data suggest an unknown role for orosomucoid that might vary depending on the genetic background.

The absence of hepatic steatosis and inflammation after the administration of either diet is an interesting finding that confirms previous data showing hepatic TAG (in other experimental models) to be reduced when olive oil is consumed (Deng *et al.* 2004). This effect is lost, however, when a higher percentage olive oil intake is provided or when the intake is accompanied by cholesterol (Acín *et al.* 2005). Overall, the results indicate that in dyslipidaemic apoE-deficient mice, olive oil provides protection against hepatic steatosis until a certain fat intake is reached. This would be especially true in low-cholesterol diets such as with the traditional Mediterranean diet.

In conclusion, this nutrigenomic approach clearly illustrates the important effects of the unsaponifiable fraction of olive oil. The present results suggest that it is no longer appropriate to

speak of MUFA-enriched oils (avocado, oleic acid-enriched safflower, oleic acid-enriched sunflower, olive and peanut oils) as though all had the same effects. Future studies should be aware of this to avoid confusion – both to researchers and consumers. The present approach also shows new connections between nutrition and gene expression. A gene product with unknown biological function, orosomucoid, was up-regulated to an extent depending on the genetic background of the mice. *Fabp5* and *Mt2* were strongly up-regulated while the expression of several proteases was repressed by the UEOO diet. These modifications in expression could be used as markers of the intake of the unsaponifiable fraction of olive oil. The present results also show the usefulness of Affymetrix chip technology for characterizing gene expression levels in response to nutritional components in intact animal systems.

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