REVIEW ARTICLE

Knowledge of the Biological Actions of Extra Virgin Olive Oil Gained From Mice Lacking Apolipoprotein E

Natalia Guillén,[a,b] Sergio Acín,[b] María A. Navarro,[a,b] Joaquín Carlos Surra,[a,b] Carmen Arnal,[a,c] José Manuel Lou-Bonafonte,[d] Pedro Muniesa,[a,e] María Victoria Martínez-Gracia,[a] and Jesús Osada[a,b]

[a]CIBER de Fisiopatología de la Obesidad y Nutrición, Instituto de Salud Carlos III, Madrid, Spain
[b]Departamento de Bioquímica, Biología Molecular y Celular, Facultad de Veterinaria, Instituto Aragonés de Ciencias de la Salud, Universidad de Zaragoza-Dirección Salud del Gobierno de Aragón, Zaragoza, Spain
[c]Departamento de Patología Animal, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain
[d]Departamento de Farmacología y Fisiología, Facultad de Ciencias de la Salud y el Deporte, Universidad de Zaragoza, Zaragoza, Spain
[e]Departamento de Anatomía y Embriología y Genética Animal, Facultad de Veterinaria, Universidad de Zaragoza, Zaragoza, Spain

The low incidence of cardiovascular disease in countries bordering the Mediterranean basin, where olive oil is the main source of dietary fat, has stimulated interest in the chemical composition of olive oil and in the production of other oils enriched with its minor components. This review summarizes what has been learned about the effects of different olive oil preparations on the development of atherosclerosis and about the prognostic value of associated plasma variables in the disease from experiments on genetically modified mice that spontaneously develop atherosclerosis. The limitations of this animal model associated with its morphological and physiological differences with humans are minimized by the similarity of the two genomes and by the potential for increased understanding attainable, given that the dietary interventions reported here would have taken 400 years to achieve in humans. As observed in traditional Mediterranean populations, it has been confirmed that extra virgin olive oil is beneficial when consumed judiciously and in a diet that is low in cholesterol due to the relative scarcity of animal products. Furthermore, the use of genomic techniques has led to the identification of new markers of response to olive oil. In conclusion, multidisciplinary research into extra virgin olive oil is expanding our knowledge of the substance's biological properties.

Key words: *Apolipoprotein E deficient mice. Olive oil. Atherosclerosis. Lipoproteins.*

Conocimiento de la acción biológica del aceite de oliva virgen extra mediante el uso del ratón carente de la apolipoproteína E

La baja incidencia de enfermedades cardiovasculares en los países de la cuenca mediterránea, donde el aceite de oliva es la principal fuente de grasa en la alimentación, ha motivado un mejor conocimiento de su composición química y el desarrollo de aceites enriquecidos en sus componentes minoritarios. En esta revisión se recopilan los efectos de diferentes preparaciones del aceite de oliva sobre el desarrollo de la aterosclerosis y el valor pronóstico para la enfermedad de los parámetros plasmáticos mediante el empleo de un ratón modificado genéticamente en el que ésta se desarrolla espontáneamente. Las limitaciones del modelo por sus diferencias morfológicas y fisiológicas con el hombre se minimizan ante la similitud de ambos genomas y el avance de conocimiento que posibilita, ya que efectuar en humanos las intervenciones recopiladas habría requerido 400 años. Confirmando la tradición de los pueblos mediterráneos, se ha verificado la eficacia del aceite de oliva virgen consumido prudentemente y en dietas con bajo contenido en colesterol por la relativa escasez de productos de origen animal. Además, la exploración con herramientas de genómica ha identificado nuevos marcadores de respuesta al aceite. En conclusión, la investigación multidisciplinaria del aceite de oliva virgen extra permite ampliar el conocimiento de sus propiedades biológicas.

Palabras clave: *Ratón carente de apolipoproteína E. Aceite de oliva. Aterosclerosis. Lipoproteínas.*

A NEW TOOL IN BIOMEDICAL RESEARCH

The recent award of the Nobel Prize in Medicine to the "parents" of gene-deficient mice (Capechi, Evans, and Smithies), has led to the widespread recognition of a powerful tool to advance knowledge on the molecular basis of disease and to assess the

The work conducted by the present research group was financed by FEDER-CICYT (SAF2007-60173), Redes DGA (B-69), and FISS for cooperative research C03-01 and G03-140, and by the Spanish Heart Foundation. Natalia Guillén and Sergio Acín received grants from the Regional Government of Aragón, Spain, and from the Cuenca Villoro foundation.

Correspondence: Dr. J. Osada.
Departamento de Bioquímica y Biología Molecular y Celular.
Facultad de Veterinaria. Universidad de Zaragoza.
Miguel Servet, 177. 50013 Zaragoza. España.
E-mail: Josada@unizar.es

ABBREVIATIONS

apoE: apolipoprotein E
HDL: high-density lipoprotein
LDL: low-density lipoprotein
VLDL: very low-density lipoprotein

efficacy and safety of treatment before use in humans.[1] Furthermore, the sequencing of the mouse genome,[2] which has an astonishing similarity to the human genome, has shown that the genes involved in the main vital functions are highly conserved and thus rapid progress may be achieved by using such a versatile animal.

The present review attempts to summarize some of the lessons learned in the last 10 years regarding the use of these types of animals in research on virgin olive oil and atherosclerosis.

APOLIPOPROTEIN E-DEFICIENT MICE

The generation of the apolipoprotein E-deficient mouse has been one of the most important contributions to progress in research on atherosclerosis in the last 15 years.[3] This was achieved by researchers at 3 independent laboratories who inactivated the apolipoprotein E (apoE) gene in totipotent embryonic stem cells and generated the corresponding gene-deficient mouse.[4-6] This mutation segregated according to the Mendelian model and the animals homozygous for the inactivated gene were 100% viable.

The absence of the apoE gene prevents the remnant chylomicron and very low density lipoprotein (VLDL) particles from being eliminated by the liver, thus leading to their accumulation in the plasma and finally being deposited in the subendothelial space of the arterial walls. Thus, when the apoE-deficient mouse is fed a normal mouse diet, extensive fibroproliferative atherosclerosis spontaneously develops[7] which presents the same lesion formation sequence established in other animal models and in humans. The complexity of the lesions and the ease with which they spontaneously generate over a short period in the apoE-deficient mouse, together with the similarity of the model to human disease, make it an attractive system to study the factors, both environmental and genetic, that predispose to atherosclerosis.[8,9]

Among the environmental factors, diet is one of the most relevant. In this field, the use of apoE-deficient mouse provides a valuable biological assay of the influence of the different components of diet on the development of atherosclerosis and the implicated mechanisms, as reported in previous reviews.[10,11]

EXTRA VIRGIN OLIVE OIL AND OILS AND FATS CONSUMED BY HUMANS

Figure 1 shows the average composition of fatty acids present in the triglycerides of some oils and fats used in human consumption. Currently, the ones most consumed in the world are as follows: soybean oil (used by 26% of the world population), palm oil (18%), sunflower oil (13%), and rapeseed/canola oil (12%). All these oils are obtained from the seeds by chemical extraction and, with the exception of vitamin E which is dissolved during extraction procedure, the triglycerides are the determinants of biological activity. Depending on the degree of saturation of the predominant fatty acids in these compounds, they are classified as saturated fats, with saturated fatty acids (solid at room temperature), and monounsaturated and polyunsaturated oils due to the presence of these types of fatty acids (liquids). This simple method of grouping fats and oils into 3 categories (Figure 1) is widely used and relatively accurately explains properties such as the hypercholesterolemia-inducing effect of saturated fat and the hypocholesterolemia-inducing effect of polyunsaturated oils. Better knowledge of the structures and properties of fatty acids has confirmed that not all saturated fatty acids (long chain) or all polyunsaturated oils (the position of the last double bond in the chain, n-3 or n-6, and the cis or trans configuration of some double bonds) have the same effect. These aspects have been reviewed by Rubio[12] and Surra et al.[13]

Although the worldwide consumption of olive oil is low, it has been the main source of fat in the Mediterranean basin and forms part of the classic Mediterranean diet. The healthy properties of this diet were shown in the Seven Countries study, which was designed to investigate the relationship between diet and cardiovascular disease in 14 populations from 7 different countries (the United States, Finland, the Netherlands, Yugoslavia, Italy, Greece, and Japan). Individuals who were studied in Crete presented lower rates of cardiovascular disease and cancer compared to the other study regions,[14] and it was concluded that this could be due low intake of saturated fats and high intake of oleic acid, due to olive oil consumption. More recently, the results obtained after following up the study participants for 25 years indicated that the rate of these diseases remains lower in southern European countries than in northern ones.[15,16]

The Lyon Diet Heart Study[17,18] was the first clinical trial to confirm the benefits of the Mediterranean diet and included 605 myocardial infarction

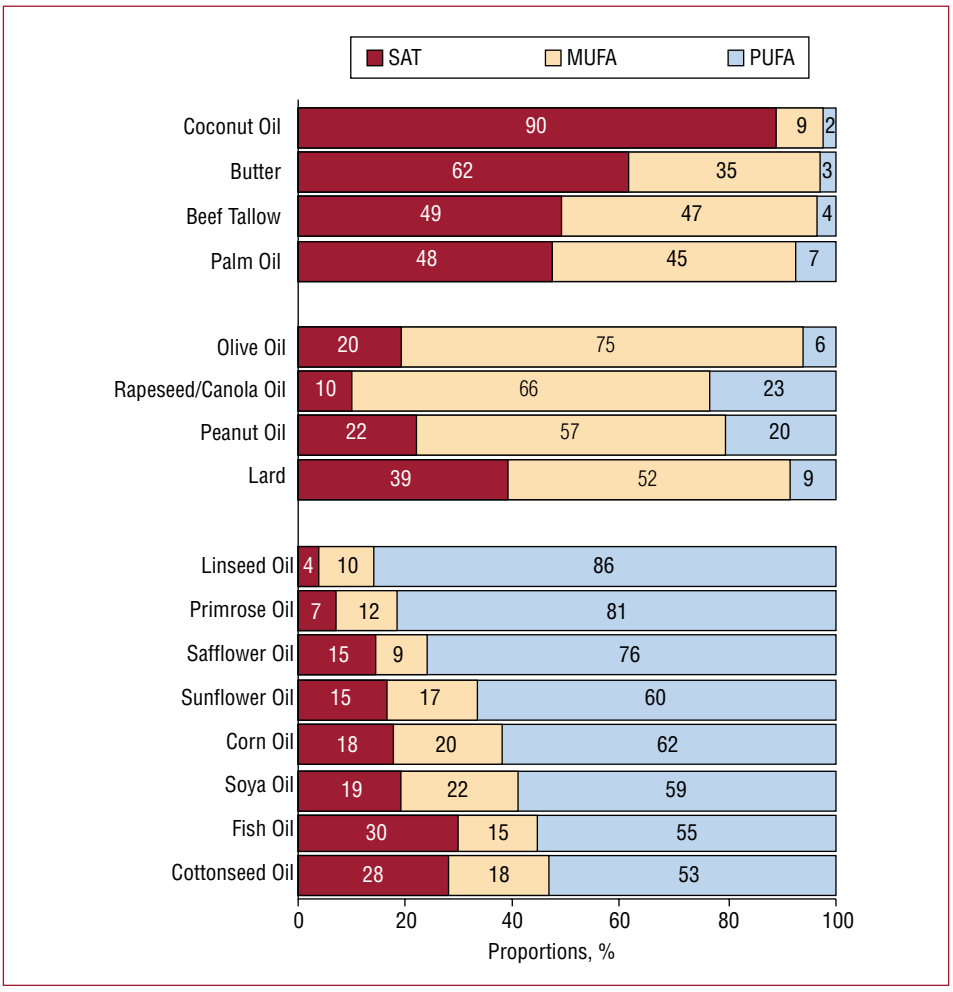

Figure 1. Percentage of the different types of fatty acids present in the different oils and fats. The most frequently consumed oils in the world are as follows: soybean (used by 26% of the world population), palm (18%), sunflower (13%), and colza/canola (12%). Adapted from Surra et al,[13] with permission from the Spanish Society of Obesity. MUFA indicates monounsaturated fat; PUFA, polyunsaturated fat; SAT, saturated fat.

patients who were randomized to 2 groups. The control group followed a diet similar to the one recommended by the American Heart Association and the other group followed a Mediterranean diet. After 27 months, the results showed decreased rates of coronary events (73%) and total mortality (70%) in the Mediterranean diet group. Another study, the Indo-Mediterranean trial, investigated the effect of a Mediterranean-type diet on the progression of coronary artery disease in high-risk patients.[19] The study also concluded that a Mediterranean-type diet could be more effective in the primary and secondary prevention of cardiovascular disease than the diet recommended by the United States cholesterol education program.

In a study conducted in Greece that included 22 000 people, Trichopoulou et al[20] also found that following a Mediterranean diet was associated with lower total mortality and lower mortality due to cardiovascular disease.

An interventional study is currently underway in Spain on the primary prevention of cardiovascular disease using the Mediterranean diet (PREDIMED), which included 772 asymptomatic people at high cardiovascular risk who were randomly assigned to 1 of 3 diet groups: a low-fat diet, a Mediterranean diet with extra virgin olive oil, and a Mediterranean diet supplemented with nuts. After the first 3 months of intervention, it was found that the groups receiving the Mediterranean diets presented lower plasma glucose concentrations, a lower cholesterol/high-density lipoprotein cholesterol (HDL-C) ratio and lower systolic blood pressure compared to the low-fat diet group.[21]

All this evidence has led to the Mediterranean diet becoming a model diet despite its high fat content.[22] This has led to increased gastronomic interest due to its greater palatability and because it encourages the consumption of vegetable products rich in low glycemic carbohydrates.[23] Unfortunately, this well-regarded Mediterranean diet is currently being influenced and modified by a group of factors, such as globalization and technological progress, that have led to food being more easily available and to decreased physical activity.[24] Dietary patterns in Mediterranean countries are rapidly changing,

with an increased intake of saturated fats and refined carbohydrates, with a consequent increase in obesity in these regions. Different authors[25-27] have highlighted the need to return to more moderate and balanced eating habits, and they emphasize the importance of preserving certain dietary traditions and a way of life that will ensure the health of future generations.

Since olive oil is the component that contributes the most calories in this type of diet, increased interest has been shown regarding its properties. Virgin olive oil is juice from the fruit of Olea europea obtained by physical procedures and does not require the type of extraction process used for seed oil. When the preparation has certain organoleptic characteristics verified by professional tasters and its acidity is <1, it is denominated extra virgin olive oil.

The constituents of virgin olive oil can be divided into 2 fractions, saponifiable and unsaponifiable.[28-31]

The saponifiable fraction generally ranges from 98.5% to 99.5%, mainly formed by triglycerides and, to a lesser extent, free fatty acids together with other minority components such as monoglycerides or diglycerides, phosphatidates, waxes, and sterol esters.[28-30] Olive oil is rich in oleic acid (monounsaturated), and contains moderate quantities of palmitic and linoleic acids and a low percentage of stearic and linolenic acids,[28,31] as shown in Table 1. The composition of fatty acids and other components differs from one sample to another, depending on olive oil production area, climate, how mature the olives are, and the variety.[28,32]

The unsaponifiable fraction is formed by the compounds that can be obtained after saponification of the oil.[33] This fraction, also known as the minority components of olive oil, contains a great variety of compounds that fulfill a wide range of functions and maintain the stability of the oil and its organoleptic characteristics. These minority components are lost to a great extent during the refining processes[34] and thus are not present in current olive oils if they are not enriched with virgin olive oil. Currently, the characterization and determination of these compounds is a very active, and highly complex, research field. Table 2 shows the main groups of compounds, together with their range of concentrations in virgin olive oil, and Figure 2 shows those that may have biological activity.[31,35]

The total content of terpene compounds represents one of the most abundant fractions and they can have a tetracyclic or pentacyclic structure. Among these there are 2 alcohols, uvaol, and erythrodiol, and their corresponding acids, oleanolic and maslinic.[35] The most abundant sterols are β-sitosterol (75%-90% of total sterols), Δ^5-avenasterol (5%-36%), and campesterol (3%).[36]

TABLE 1. **Percentage of the Different Fatty Acids Present in Olive Oil**[a]

Common Name (Numerical Symbol)	Percentage
Myristic (14:0)	0.0-0.05
Palmitic (16:0)	7.5-20
Palmitoleic (16:1n7)	0.3-3.5
Margaric (17:0)	0-0.3
Heptadecenoic (17:1)	0-0.3
Stearic (18:0)	0.5-5
Oleic (18:1n9)	55-83
Linoleic (18:2n6)	3.5-21
α-linolenic (18:3n3)	0-0.9
Arachidic (20:0)	0-0.6
Eicosenoic (20:1n9)	0-0.4
Behenic (22:0)	0-0.2
Lignoceric (24:0)	0-0.2

[a]See Bosku[28] and Montedoro et al.[31]

TABLE 2. **Minority Components of Virgin Olive Oil**[a]

Components	Concentration, mg/100 g Oil
Terpene compounds	100-350
Sterols	100-250
Hydrocarbons	
Squalene	150-800
Carotenes	0.5-1
Phenol compounds	5-100
Aliphatic alcohols	10-70
Tocopherols	0.5-30
Esters	10-20
Aldehydes and ketones	4-8
Chlorophyls	0.1-2

[a]See Montedoro et al[31] and Jiménez et al[34]

The main constituent of the hydrocarbons is squalene followed by the carotenes (lutein and β-carotene) in far smaller quantities.[34]

The phenolic compounds form the polar fraction and affect the stability and flavor of olive oil.[37,38] Among the most frequent phenolic compounds 4 main groups can be distinguished: a) simple phenols, either alcohols (tyrosol and hydroxytyrosol) or acids (p-cumaric, vanillic, caffeic, synaptic, protocatechuic, gallic, syringic); b) polyphenols (flavonoids: luteolin and apigenin); c) secoiridoids (esterized derivatives of elenolic acid—glycosylated oleuropein or nonglycosylated—with hydroxytyrosol and tyrosol); and d) lignans: (+)-pinoresinol and (+)-1-acetoxypinoresinol. Many of these compounds have antioxidant properties and high concentrations are associated with increased resistance to oil oxidation.[39] Hydroxytyrosol contributes the most to this effect,[27] and its concentration depends on the

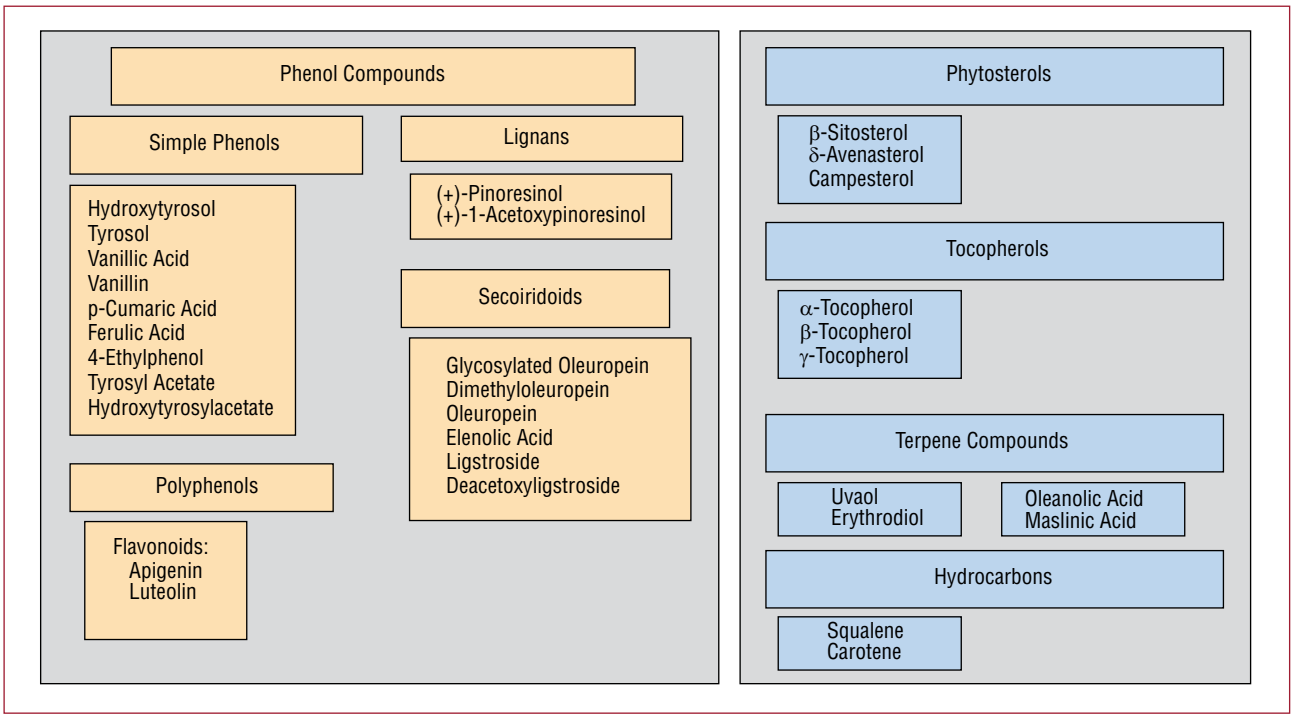

Figure 2. The main chemical compounds in extra virgin olive oil with biological properties grouped by categories.

combination of several factors, such as the variety of olive, how mature they are, climate, and the method of preparation,[37,38] since can be lost in the refining processes.[24]

The aliphatic alcohol fraction is made up of linear-chain saturated alcohols with an even number of carbon atoms (^{18}C to ^{28}C). The main ones are hexacosanol, octacosanol, and tetracosanol.[24] These alcohols, as well as the sterols and triterpene alcohols, can combine with fatty acids and form nonglyceride esters.

Tocopherols, which are also part of the unsaponifiable fraction, are present in different quantities depending on the variety of olive.[40] The most abundant of these is α-tocopherol (52%-87%), with β-tocopherol (15%-20%), and γ-tocopherol (7%-23%) also being present.[28]

More than 100 different types of aldehydes and ketones have been identified.[32] Similar to other compounds, their presence in the oils depends on the variety of olive, climatic conditions, and the quality of the oil.[30] The aroma and flavor of virgin olive oil are partly due to the presence of these types of chemical compounds.[28,30]

Chlorophylls, although in smaller proportions, give virgin olive oil its color, together with lycopene and β-carotene.[32]

EXTRA VIRGIN OLIVE OIL IN MEDITERRANEAN-TYPE DIETS

To demonstrate the effect of extra virgin olive oil on the development of atherosclerosis, we fed apoE-deficient mice a diet containing 10% (w/w) extra virgin olive oil without cholesterol, in an attempt to reproduce the classic Mediterranean diet in which this oil is the main source of fat and there is low consumption of animal products.[41] This percentage was chosen by taking 2 criteria into account: first, research on rats fed diets with different percentages of olive oil showed that 10% (w/w) of this oil was the minimum necessary dose to obtain a significant increase in plasma concentrations of apolipoprotein A-I (the main protein component of HDL)[42]; second, this percentage was selected to provide a metabolic model reflecting human consumption. Thus, taking into account that a mouse consumes around 3 g of feed per day of which 10% (w/w) is oil, our mice ingested 0.3 g of oil per day. As each animal weighs around 30 g, the dose consumed would be of 10 g of oil per kilogram of body weight. Extrapolating without transformation to the average human weight (70 kg), if the mice weighed as much a human they would be consume 700 g of oil per day. If we take into account that the metabolic rate of the mouse is 10 times higher than that in humans,[43]

the adjusted quantity would be 70 g of oil per day in humans. If we assess this in terms of energy, the consumption of 70 g of oil/day implies an intake of 630 kcal (at a rate of 9 kcal/g of fat) representing 25% of a 2500-kcal diet for an average person, and this is lower than olive oil intake in the classic Mediterranean diet, where 35% (875 kcal) of the total calories comes from this oil.[41] In order to reach this energy level, an approximate intake of 100 g/d would be needed in a diet where the exclusive source of fat was olive oil. Given that this condition is not fulfilled in current consumer society, the USA Food and Drug Administration has been more prudent in recommending the daily consumption of 25 mL of olive oil.[44]

Apo E-deficient mice were fed with a diet containing 10% (w/w) extra virgin olive oil without cholesterol for 12 weeks, then killed, and the extent of atherosclerotic lesions was quantified. In this study, this percentage of dietary olive oil delayed the development of atherosclerosis, to which the females were more sensitive (Figure 3), and this was associated with increased plasma concentrations of apolipoprotein A-I.[45]

The same study included a group of animals fed with a similar percentage of high oleic sunflower oil, and thus these animals received the same percentage of oleic acid as the animals fed with extra virgin olive oil. Despite the similar content of this fatty acid in the diets, the animals which were fed the high oleic sunflower oil diet did not present reduced levels of atherosclerosis (Figure 3). This observation led to the conclusion that the triglyceride constituent of extra virgin olive oil was not the only cause of its activity.

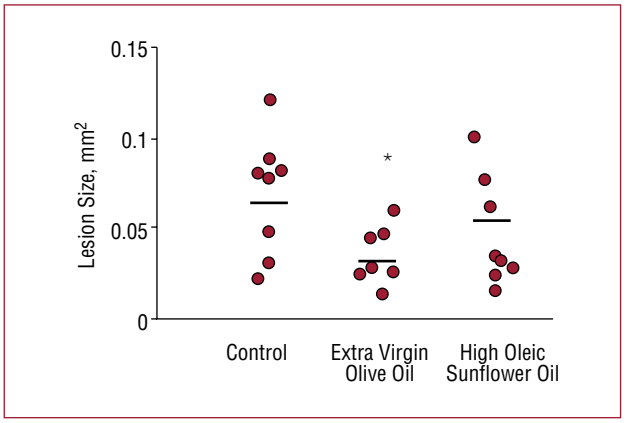

Figure 3. Size of atherosclerotic lesions in apo E-deficient mice fed with control diet, control diet enriched with 10% (w/w) extra virgin olive oil or high oleic sunflower oil. The values for each animal and group mean are shown. Statistical analysis was conducted using Mann-Whitney U test. A *P* value of <.05 was used as a cutoff for statistical significance. Adapted from Calleja et al,[45] with permission from Lippincott Williams & Wilkins.

DOES THE EFFECT OCCUR IN RELATION TO LEVEL OF INTAKE?

Once the beneficial effect of the aforementioned diet (10% [w/w] extra virgin olive oil without cholesterol) was demonstrated, we investigated whether there was a tolerance dose. Thus, another experiment was conducted in which extra virgin olive oil formed 20% (w/w) of the feed. In this new study, and in contrast to the effect of the 10% dose,[45] no reductions in lesions were observed (Figure 4). The animals following this higher olive oil content diet did not obtain greater benefit than those fed with the carbohydrate-enriched diet.[46] Furthermore, it was observed that whereas the intake of 10% fat in the form of virgin olive oil did not affect body weight or plasma cholesterol concentrations and did lead to a reduction in triglyceride concentrations,[45] doubling the quantity of olive oil in the diet led, on the contrary, to an increase in body weight and plasma cholesterol and triglyceride concentrations.[46] These results indicate that there are margins within which olive oil offers better protection.

EXTRA VIRGIN OLIVE OIL IN WESTERN-TYPE DIETS

Currently, our diets are rich in animal products and it is estimated that daily cholesterol intake forms 0.15% (w/w) of diets.[41] With the aim of understanding the outcome of including this percentage of cholesterol in the diet on the effect of extra virgin olive oil, an experiment was conducted in which animals were fed extra virgin olive oil (20% of the diet) with or without cholesterol. The results indicated that the inclusion of dietary cholesterol increased lesions in both the animals fed with the control diet and in those fed with a virgin olive oil-enriched diet (Figure 4). When investigating the possible mechanisms underlying these results, it was observed that, whereas dietary cholesterol reduced apolipoprotein A-I in females, there was reduced serum paraoxonase activity in males. These parameters are both associated with HDL and contribute to its antiatherogenic properties. In fact, the size of the aortic lesion in males was inversely related to such activity even after statistically adjusting for apolipoprotein A-I and HDL-C levels. These results demonstrate the association between the nutritional regulation of paraoxonase and atherosclerosis dependent on sex.[46]

To compare the efficacy of different olive oils in western-type diets—20% (w/w) of the fat content and 0.15% (w/w) cholesterol—several diets were prepared with extra virgin olive oil obtained from

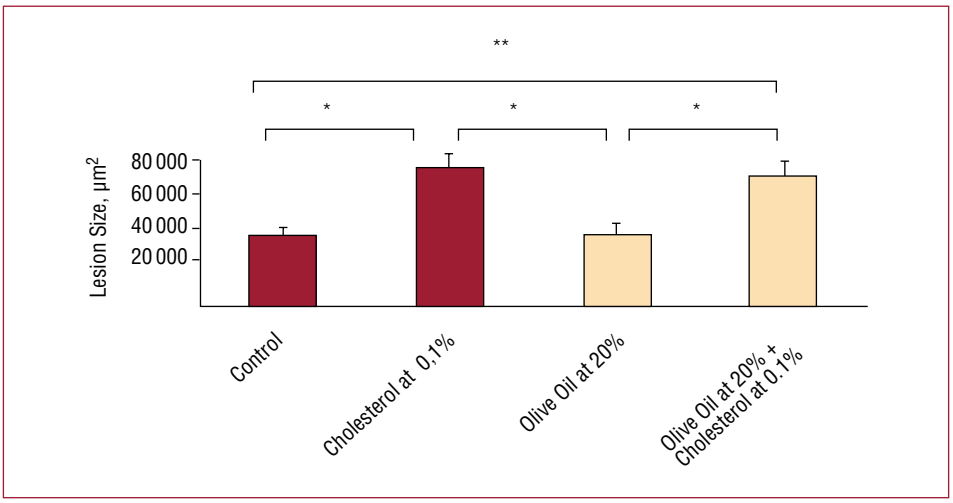

Figure 4. Size of the atherosclerotic lesions in apo E-deficient mice fed with different diets. The mean value and SEM are presented for each group. Statistical analysis was conducted using the Mann-Whitney U test. *P<.05; **P<.02. Adapted from Acín et al,[46] with permission from Elsevier.

the arbequina, cornicabra, empeltre, and picual cultivars and these were compared to a group of animals that received palm oil. The animals fed with diets containing olive oil presented reductions in atherosclerotic lesions compared to those fed with palm-oil enriched diets (Figure 5) despite presenting higher concentrations of total cholesterol and triglycerides in blood. In this experiment, the development of atherosclerosis could not be explained by apolipoprotein A-I concentrations or total paraoxonase activity. On the other hand, the diets enriched with extra virgin olive oil induced an increase of small, dense HDL enriched with apo A-IV tightly bound to paraoxonase. These apolipoprotein A-IV-enriched particles were very effective in inactivating the peroxides present in the low-density lipoproteins (LDL) which are thought to initiate atherosclerosis. In this study, the increase in small, dense HDL particles explained the development of atherosclerotic lesions, since these were inversely correlated with their development.[47]

In conclusion, and based on the results obtained, we can state that virgin olive oil is more effective in preventing the development of atherosclerosis if it is consumed in Mediterranean-type diets with a low cholesterol content, and that even in western-type diets its use is more beneficial than saturated palm oils.

WHAT COMPONENTS ARE INVOLVED IN THE DEVELOPMENT OF ATHEROSCLEROSIS?

To determine the role of the minority components of olive oil, the following experiment was designed. Extra virgin olive oil was obtained and a portion underwent a washout process that eliminated the hydrosoluble minority components, which mainly consisted of phenolic compounds. Diets were prepared containing 10% of both types of oils, and fed to apoE-deficient mice. It was observed that the elimination of the hydrosoluble fraction led to the oil losing its antiatherosclerotic properties (results not yet published).

A second experiment, also aimed at demonstrating the relevance of the minority components, was conducted using oil from the same cultivar and that had been prepared using two different procedures: by pressing and by centrifugation. Both types of oils were refined to eliminate the hydrosoluble component. Centrifugation produced an oil rich in water-insoluble compounds of the unsaponifiable fraction, such as phytosterols, tocopherols, triterpenes, and waxes. The 2 types of oils (10% w/w) were fed to the genetically modified mice. Oil obtained by pressing and refining increased atherosclerotic lesions, whereas oil produced by centrifugation—rich in water-insoluble minority components and lacking hydrosoluble compounds—reduced atherosclerosis.[48] The presence of the unsaponifiable fraction referred to led to decreases in lipid parameters, such as plasma triglycerides and LDL-C and very low density lipoprotein cholesterol (VLDL-C), as well as parameters of oxidative stress, such as isoprostane (8-iso-prostaglandin $F_{2\alpha}$).

These 2 experiments resolve the contradictory results obtained by authors based in the USA, who used a standard olive oil and found increases in atherosclerotic lesions,[49,50] as occurred in both experiments when washed-out or refined olive oil was used. The finding that virgin olive oil delayed the development of atherosclerosis was corroborated by other Spanish authors who fed rabbits with this oil.[51]

The hydrosoluble fraction is highly relevant, as indicated by the results of our experiments, but even in its absence, the development of new methods of preparing of olive oil that enrich it in other minority components still offers great potential, since these

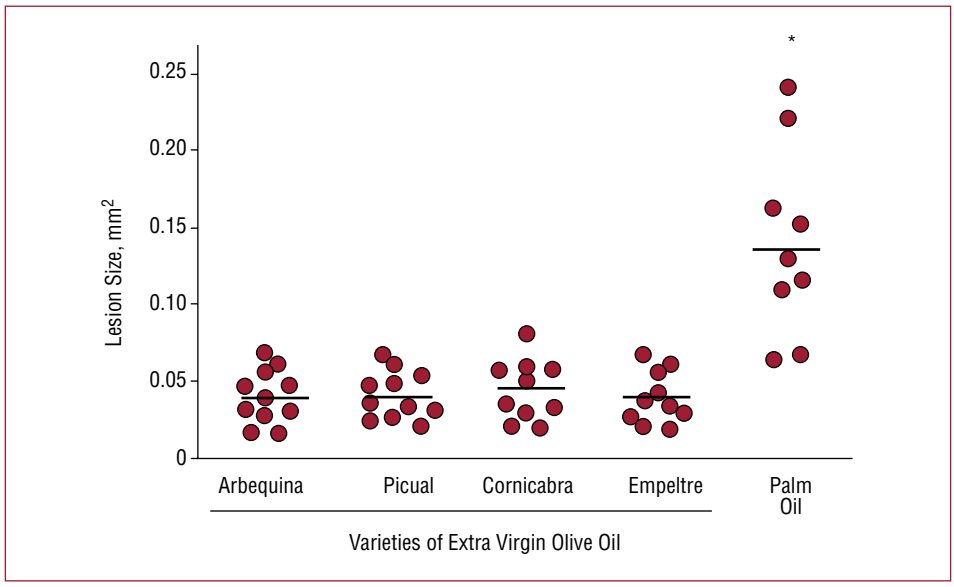

Figure 5. Comparison of western-type diets with 0.15% (w/w) cholesterol enriched with different varieties of extra virgin olive oil (20% [w/w]) compared with a palm oil-enriched diet. Effects on the size of atherosclerotic lesions in apo E-deficient mice. The values for each animal and the group mean are shown. Statistical analysis was conducted using the Mann-Whitney U test. **P*<.001 versus all the groups fed with olive oil. Adapted from Arbonés-Mainar et al,[47] with permission from Elsevier.

compounds play an important role in delaying the development of atherosclerotic lesions.[48]

LIVER FAT AND OLIVE OIL

The role of liver fat in the development of atherosclerosis has been the focus of intense debate. When animals were fed a diet containing 10% olive oil (w/w), liver steatosis was not observed.[52] However, the animals receiving diets containing 20% (w/w) extra virgin olive oil with added cholesterol presented steatosis that, in addition, was greater than that in animals receiving an identical percentage of dietary fat in the form of palm oil and cholesterol. To explain these findings, proteomic analysis was conducted and this showed that the animals fed with virgin olive oil presented stronger antioxidant defenses than those fed with palm oil. In addition, there was a clear association between adipophylin and betaine homocysteine methyl transferase as a modulator of hepatic triglyceride metabolism.[53]

Once again, the experiments demonstrated the beneficial effect of a moderate intake of extra virgin olive oil, but also indicated that the steatosis caused by these types of oil when consumed in higher amounts do not have an impact on the development of atherosclerosis since, as mentioned (Figure 5), the animals that were fed these oils presented greater reductions in atherosclerotic lesions than the animals fed a palm oil-enriched diet.[47]

WHICH LIVER GENES ARE INVOLVED IN THE RESPONSE TO OLIVE OIL CONSUMPTION?

The liver is the metabolic center where all the apolipoproteins which are constituents of plasma lipoproteins and most of the enzymes involved in lipoprotein metabolism are biosynthesized. As this organ undergoes changes in its fatty content when olive oil is included in the diet, the hepatic transcriptome was analyzed in the animals fed with refined olive oil or unsaponifiable fraction-enriched olive oil based on the hypothesis that this fraction could modify the expression of multiple genes. ApoE-deficient mice (hybrid with a C57BL/6J × Ola129 genetic background) were fed refined olive oil or unsaponifiable fraction-enriched olive oil, which formed 10% (w/w) of the diet in both cases. Livers were removed, total RNA extracted and hybridized using the Affymetrix (Murine Genome MOE430A array) chip. To provide an initial search for candidate genes, those which presented greater differential expression (signal $\log_2$ ratio >3 or <–3) were selected and this procedure yielded 11 genes. The changes in these genes were confirmed by real-time polymerase chain reaction and then also studied in the apo E-deficient mice on a pure C57BL/6J genetic background fed with both diets. Two genes coding for inflammatory-type proteins (orosomucoide and serum amyloid A_2 protein) presented increased expression that changed according to the strain of animal and always in the absence of inflammation liver. On the other hand, expression of the metallothionein 2 (Mt2) gene and the fatty acid binding protein 5 (Fabp5) gene were strongly increased regardless of the strain of mouse. These genes can be considered good markers of intake of the unsaponifiable compounds and could play a relevant role in the biological activity of these compounds.[52] The exact biological role of Fabp5 is not known, although it is known that, in addition to its function in fatty acid transportation, it can

bind leukotriene A_4[54] and has an association with the response to insulin.[55] Regarding Mt2, this has an antioxidant effect and is thought to participate in the development of obesity.[56] The potential involvement of these proteins in obesity and insulin sensitivity and their modification by the components of olive oil may make them highly relevant research topics in the near future.

THE SEARCH FOR ACTIVE COMPONENTS AND THEIR EFFECT AFTER THEIR ADMINISTRATION IN ISOLATION

Hydroxytyrosol is the most abundant component of the soluble fraction of extra virgin olive oil and a powerful in vitro antioxidant.[57,58] To investigate its effect on the development of atherosclerosis, this compound was administered to animals at a pharmacological dose of 10 mg/kg per mouse per day, and a reduction was observed in apolipoprotein A-I concentrations and an increase in plasma cholesterol. The results indicated that hydroxytyrosol, at this dose and in low-cholesterol diets, enhances the development of atherosclerosis mediated by the activation of circulating monocytes, since these expressed a greater amount the Mac-1 protein.[59] As a result, this compound has important functions in immune system activation. Thus, if functional foods containing this compound are developed to control atherosclerosis, it has to be included in formulations as similar as possible to the virgin olive oil environment.

The effect of administering another component of olive oil as an isolated compound, squalene, has also been studied. Thus, a pharmacological dose of 1 g/kg per mouse per day of squalene reduced the development of atherosclerosis in males, whereas it had no effect on females. This sex-dependent response is related to the effect of squalene on liver fat which decreases in males but does not change in females.[60]

These sex-dependent differences would have to be taken into account in relation to liver fat accumulation especially when investigating the potential application of this component as an antisteatosis agent.

CONCLUSION

Extra virgin olive oil has proven effective in controlling atherosclerotic lesions, mainly within the framework of a Mediterranean-type diet (low cholesterol), although it retains its beneficial effect in western-type diets compared to palm oil. However, there is a limiting dose beyond which this benefit disappears.

It appears that the properties of olive oil are not only due to its high oleic acid content, but also to the large amounts of different compounds that form the unsaponifiable fraction (known as the minority compounds), among which are terpenes, phenolic compounds, phytosterols, etc.

An animal model that reproduces the processes taking place in the development of human atherosclerosis has been crucial to obtaining these conclusions, and this has been provided by the apoE-deficient mouse. Feeding these mice with various olive oils rich in different components or with these components in isolation has made it possible to assess the contribution of those molecules to the beneficial effect of this food and their potential employment as nutraceutics.

The current situation suggests that research on food technology aimed at generating new nutraceutic compounds and the creation of animal models that reproduce human diseases in which these compounds can be tested, provide an excellent combination to advance knowledge on the influence and mechanism of action of nutrients in the development of atherosclerosis.

ACKNOWLEDGMENTS

The group would like to thank the technical personnel (Ángel Beltrán, Jesús Cazo, Jesús Navarro, Carmen Navarro, and Clara Tapia) of the Mixed Research Unit of Zaragoza University for their invaluable help in the care and maintenance of the animals.

REFERENCES

1. Manis JP. Knock Out, Knock In, Knock Down —Genetically Manipulated Mice and the Nobel Prize. N Engl J Med. 2007;357:2426-9.
2. Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, et al. Initial sequencing and comparative analysis of the mouse genome. Nature. 2002;420:520-62.
3. Meir KS, Leitersdorf E. Atherosclerosis in the apolipoprotein E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol. 2004;24:1006-14.
4. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, et al. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell. 1992;71:343-53.
5. van Ree JH, van den Broek WJ, Dahlmans VE, Groot PH, Vidgeon-Hart M, Frants RR, et al. Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. Atherosclerosis. 1994;111: 25-37.
6. Zhang SH, Reddick RL, Piedrahita JA, Maeda N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science. 1992;258:468-471.
7. Reddick RL, Zhang SH, Maeda N. Atherosclerosis in mice lacking apo E. Evaluation of lesional development and progression. Arterioscler Thromb. 1994;14:141-7.
8. Palinski W, Ord VA, Plump AS, Breslow JL, Steinberg D, Witztum JL. ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. Demonstration of oxidationspecific

epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum. Arterioscler Thromb. 1994;14:605-16.
9. Rosenfeld ME, Polinsky P, Virmani R, Kauser K, Rubanyi G, Schwartz SM. Advanced atherosclerotic lesions in the innominate artery of the ApoE knockout mouse. Arterioscler Thromb Vasc Biol. 2000;20:2587-92.
10. Osada J, Joven J, Maeda N. The value of the apoE-knockout mouse for studying the effects of dietary fat and cholesterol on atherogenesis. Curr Opin Lipidol. 2000;11:25-9.
11. Sarria AJ, Surra JC, Acín S, Carnicer R, Navarro MA, Arbonés-Mainar JM, et al. Understanding the role of dietary components on atherosclerosis using genetic engineered mouse models. Frontiers in Biosciences. 2006;11:955-67.
12. Rubio MA. Ácidos grasos, esteroles y enfermedad cardiovascular. Endocrinol Nutr. 2002;49:35-46.
13. Surra JC, Arnal C, Martínez MV, Osada J. Efecto de la composición de la grasa de la dieta en la aterogénesis. Rev Esp Obes. 2005;3:328-39.
14. Keys A. Coronary heart disease in Seven Countries. Circulation. 1974;41:1-211.
15. Menotti A, Lanti M, Puddu PE, Kromjout D. Coronary heart disease incidence in northern and southern European populations: a reanalysis of the seven countries study for an European coronary risk chart. Heart. 2000;84:238-44.
16. Wahrburg U, Kratz M, Cullen P. Mediterranean diet, olive oil and health. Eur J Lipid Sci Technol. 2002;104:698-705.
17. de Lorgeril M, Renaud S, Mamelle N, Salen P, Martin JL, Monjaud I, et al. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet. 1994;343:1454-9.
18. de Lorgeril M, Salen P, Martin JL, Monjaud I, Delaye J, Mamelle N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. Circulation. 1999;99:779-85.
19. Singh RB, Dubnov G, Niaz MA, Ghosh S, Singh R, Rastogi SS, et al. Effect of an Indo-Mediterranean diet on progression of coronary artery disease in high risk patients (Indo-Mediterranean Diet Heart Study): a randomised single-blind trial. Lancet. 2002;360:1455-61.
20. Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. N Engl J Med. 2003;348:2599-608.
21. Estruch R, Martinez-Gonzalez MA, Corella D, Salas-Salvado J, Ruiz-Gutierrez V, Covas MI, et al. Effects of a Mediterraneanstyle diet on cardiovascular risk factors: a randomized trial. Ann Intern Med. 2006;145:1-11.
22. Pérez-Martínez P, López-Miranda J, Delgado-Lista J, LópezSegura F, Pérez-Jiménez F. Aceite de oliva y prevención cardiovascular: más que una grasa. Clin Invest Arterioscl. 2006; 18:195-205.
23. Serra-Majem L, de la Cruz JN, Ribas L, Salleras L. Mediterranean diet and health: is all the secret in olive oil? Pathophysiol Haemost Thromb. 2003;33:461-5.
24. Alarcon de la Lastra C, Barranco MD, Motilva V, Herrerias JM. Mediterranean diet and health: biological importance of olive oil. Curr Pharm Des. 2001;7:933-50.
25. Keys A. Mediterranean diet and public health: personal reflections. Am J Clin Nutr. 1995;61:S1321-3.
26. Viola P. El aceite de oliva y la salud. Madrid: Consejo Oleícola Internacional; 1997.
27. Galli C, Visioli F. Antioxidant properties of Mediterranean diet. Int J Vitam Nutr Res. 2001;71:185-8.
28. Boskou D. Olive oil, chemistry and technology. Champaign: AOCS Press; 1996.
29. Civantos L, Contreras R, Grana R. Obtención del Aceite de Oliva Virgen. 2nd ed. Madrid: Agrícola Española; 1999.
30. Harwood J, Aparicio R. Handbook of olive oil, analysis and properties. Kluwer Academic Publishers, J.L. Harwood, R. Aparicio; 2000.
31. Montedoro GF, Taticchi A, Esposto S, Selvaggini R, Urbani S, Servilli M. Antioxidants in virgin olive oil. Olea. 2007;26: 5-13.
32. Boskou D. Olive oil. World Rev Nutr Diet. 2000;87:56-77.
33. Dieffenbacher A, Pocklington WD. Standard methods for the analysis of oils, fats and derivates. Oxford: Internacional Union of Pure and Applied Chemistry; 1992.
34. Jiménez J, Rondón D, Martínez L, Mataix J. Composición química de los aceites de oliva. In: Mataix J, editor. Aceite de oliva virgen: nuestro patrimonio alimentario. Granada: Universidad de Granada, Puleva Food; 2001.
35. Perona JS, Cabello-Moruno R, Ruiz-Gutiérrez V. The role of virgin olive oil components in the modulation of endothelial function. J Nutr Biochem. 2006;17:429-45.
36. Calapaj R, Chiricosta S, Saija G, Binova V. [Evaluation of gas chromatographic an spectrophotometric analytical results to check the presence of seed oils in olive samples]. Riv Ital Sost Grasse. 1993;70:575.
37. Galli C, Visioli F. Antioxidant and other activities of phenolics in olives/olive oil, typical components of the Mediterranean diet. Lipids. 1999;34:S23-6.
38. Montedoro GF, Servili M, Baldioli M, Miniati E. Simple and hydrolyzable phenolic compounds in virgin olive oil: Their extraction, separation and quantitative and semiquantitative evaluation by HPLC. J Agric Food Chem. 1992;40:1571-6.
39. Arbonés-Mainar JM, Navarro MA, Lou-Bonafonte JM, Martínez-Gracia MV, Osada J. Olive oil phenolic compounds as potential therapeutical agents. Nueva York: Nova; 2008.
40. Perrin JL. Les composes mineurs et les antioxygenes naturels de l'olive et de son huile. Rev Franc Corps Gras. 1992;39: 25-32.
41. Willett WC. Diet and health: what should we eat? Science. 1994;264:532-7.
42. Calleja L, Trallero MC, Carrizosa C, Mendez MT, Palacios-Alaiz E, Osada J. Effects of dietary fat amount and saturation on the regulation of hepatic mRNA and plasma apolipoprotein A-I in rats. Atherosclerosis. 2000;152:69-78.
43. Demetrius L. Of mice and men. When it comes to studying ageing and the means to slow it down, mice are not just small humans. EMBO reports. 2005;6:S39-44.
44. FDA allows qualified health claim to decrease risk of coronary heart disease. Rockville: US Food and Drug Administration; 2004. Available from: http://www.fda.gov/bbs/topics/ news/2004/ NEW01129.html
45. Calleja L, Paris MA, Paul A, Vilella E, Joven J, Jimenez A, et al. Low-cholesterol and high-fat diets reduce atherosclerotic lesion development in ApoE-knockout mice. Arterioscler Thromb Vasc Biol. 1999;19:2368-75.
46. Acín S, Navarro MA, Carnicer R, Arbonés JM, Guzmán MA, Arnal C, et al. Dietary cholesterol suppresses the ability of olive oil to delay the development of atherosclerotic lesions in apolipoprotein E knockout mice. Atherosclerosis. 2005;182: 17-28.
47. Arbones-Mainar JM, Navarro MA, Carnicer R, Guillen N, Surra JC, Acin S, et al. Accelerated atherosclerosis in apolipoprotein E-deficient mice fed Western diets containing palm oil compared with extra virgin olive oils: a role for small, dense high-density lipoproteins. Atherosclerosis. 2007;194: 372-82.
48. Acin S, Navarro MA, Perona JS, Arbones-Mainar JM, Surra JC, Guzman MA, et al. Olive oil preparation determines the atherosclerotic protection in apolipoprotein E knockout mice. J Nutr Biochem. 2007;18:418-24.
49. Merkel M, Velez-Carrasco W, Hudgins LC, Breslow JL. Compared with saturated fatty acids, dietary monounsaturated fatty acids and carbohydrates increase atherosclerosis and VLDL cholesterol levels in LDL receptor-deficient, but not

apolipoprotein E-deficient, mice. Proc Natl Acad Sci U S A. 2001;98:13294-9.
50. Rudel LL, Kelley K, Sawyer JK, Shah R, Wilson MD. Dietary monounsaturated fatty acids promote aortic atherosclerosis in LDL receptor-null, human ApoB100-overexpressing transgenic mice. Arterioscler Thromb Vasc Biol. 1998;18:1818-27.
51. Aguilera CM, Ramirez-Tortosa MC, Mesa MD, Ramirez-Tortosa CL, Gil A. Sunflower, virgin-olive and fish oils differentially affect the progression of aortic lesions in rabbits with experimental atherosclerosis. Atherosclerosis. 2002;162:335-44.
52. Acín S, Navarro MA, Perona JS, Surra JC, Guillen N, Arnal C, et al. Microarray analysis of hepatic genes differentially expressed in the presence of the unsaponifiable fraction of olive oil in apolipoprotein E-deficient mice. Br J Nutr. 2007;97: 628-38.
53. Arbones-Mainar JM, Ross K, Rucklidge GJ, Reid M, Duncan G, Arthur JR, et al. Extra virgin olive oils increase hepatic fat accumulation and hepatic antioxidant protein levels in APOE–/–mice. J Proteome Res. 2007;6:4041-54.
54. Zimmer JS, Dyckes DF, Bernlohr DA, Murphy RC. Fatty acid binding proteins stabilize leukotriene A4: competition with arachidonic acid but not other lipoxygenase products. J Lipid Res. 2004;45:2138-44.
55. Maeda K, Uysal KT, Makowski L, Gorgun CZ, Atsumi G, Parker RA, et al. Role of the fatty acid binding protein MAL1 in obesity and insulin resistance. Diabetes. 2003;52:300-7.
56. Miura N, Koizumi S. Gene expression profiles in the liver and kidney of metallothionein-null mice. Biochem Biophys Res Commun. 2005;332:949-55.
57. Visioli F, Bellomo G, Galli C. Free radical-scavenging properties of olive oil polyphenols. Biochem Biophys Res Commun. 1998;247:60-4.
58. Visioli F, Galli C, Plasmati E, Viappiani S, Hernandez A, Colombo C, et al. Olive phenol hydroxytyrosol prevents passive smoking-induced oxidative stress. Circulation. 2000;102:2169-71.
59. Acin S, Navarro MA, Arbones-Mainar JM, Guillen N, Sarria AJ, Carnicer R, et al. Hydroxytyrosol administration enhances atherosclerotic lesion development in apo E deficient mice. J Biochem (Tokyo). 2006;140:383-91.
60. Guillen N, Acin S, Navarro MA, Perona JS, Arbones-Mainar JM, Arnal C, et al. Squalene in a sex-dependent manner modulates atherosclerotic lesion which correlates with hepatic fat content in apoE-knockout male mice. Atherosclerosis. 2008;196:558-64.