Molecular mechanisms involved in the protective effect of Mediterranean diet and olive oil consumption in humans

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TESI DOCTORAL UPF / 2010

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To my beloved family,
My dad, my mom and my little brother

ACKNOWLEDGMENTS

If I have seen further,
it is only by standing on the shoulders of Giants.
Isaac Newton (1642-1727)

I was taught that while writing the acknowledgments section of a scientific article, I should thank all the finance entities, people and institutions that have participated in the development of the specific study. When the time came to write the acknowledgments section of my entire thesis project, I realized that I needed lots of time and space in order just to only to mention everything and/or everyone I was acknowledging. Since my hometown and my resident town have not been the same place, during my thesis development, I have been helped, encouraged and supported by people from varying nationalities, ethnicities, educational and social backgrounds. Despite their individual differences, they have all shared the similarity of accompanying me on "my journey" and offering me their best.

First of all, I feel obliged to my family, my dad, my mom and my little brother, for the support and courage they have unconditionally given to me during all these years. Despite the obstacles that have arisen, they have always been and will always be there for me, no matter how far we are from each other. I thank them for accepting me and believing in me and in what I was doing.

My heartfelt thanks go to all the members of the URLEC (currently named as Cardiovascular Risk and Nutrition Research Group, and Cardiovascular Epidemiology and Genetics Group) whose names are too many to mention. Dr. Jaume Marrugat and Dr. Roberto Elosua deserve special thanks for having accepted me in their team and encouraging me since our first collaboration in 2001. Thank you for giving me the chance to develop my skills, knowledge and personality by your valuable influence. Dr. Montserrat Fitó has always been there for me, whenever I needed her, closely collaborating, and cheerfully supporting the process of exploring, and learning new things with me, making me feel at home. Dr. Helmut Schröder helped me understand and dive into the core of the nutritional studies. The encouragement and wisdom of all of them

helped sustain and inspired me throughout. Dr. Maria Grau, Joan, Isaac, Hector, Anna, Yolanda, it was a real pleasure to collaborate with all of you during these years. Natalia, thank you for keeping my spirit up whenever I needed it. I deeply appreciate the valuable help of Sandra Farré in editing the final version of the present dissertation. Special thanks certainly go to Daniel Muñoz Aguayo for his everyday help, and for making my long working hours easygoing and happy. Dani, thank you, for having willingly and cheerfully supported, stood by me and made me laugh even in the most difficult days. I also thank "Dani's angels", Gemma, Saray and Sonia for the funny and relaxed moments we have shared all together. The task would have certainly been much more difficult without those people's calm and generous natures.

Special thanks also deserve Dr. Rafael de la Torre and Olha Khymenets, from the Clinical Research in Human Farmacology and Neurosciences Group, IMIM-Hospital del Mar, for their close and valuable collaboration during all these years.

I deeply thank Dr. Euaggelia Tegou for being the first one to encourage and support me in entering the magnificent scientific world. Her faith in me makes her one of the best tutors and friends one may ever have.

My sincere thanks go to Dr. Evangelos Lazos and Dr. Michalis Bratakos for their help during my first attempts to follow a scientific career.

I am grateful to the Fundación IMIM-Hospital del Mar that has supported the work presented here and the final printing of this dissertation. Special acknowledgments go to the State Scholarship Foundation of Greece, (I.K.Y.) for financing my scholarship as well as to its employees who have helped me throughout this period.

I am grateful to Dr. Christoforos Nikolaou for his thoughtful and creative support from the first day I met him. Thank you, Chris, for being yourself, for your always cheering mood and for your brilliant ideas that have made me a better person.

Rena, Maria, Eleni, Magda, Rita, thank you so much, girls, for having always kept thinking of me in spite of the distance and/or

our everyday problems. Thank you for having never stepped aside and having always been there for me; for my happiness and sadness. Isabel, I can not thank you enough for the support, joy and everyday life that you have shared with me as a best friend and a best flat mate. Without you, I would certainly have not been the same.

During this "journey", I was lucky enough to meet wonderful people with superb listening skills and brilliant personalities who I can now call my Friends. I thank all of them in the UPF-DEXS and CRG groups, and especially Dr. Alberto Moldon, Dr. Sarah Djebali, Blanquita, Nati, Dr. Sylvain Foissac, Julien, Francisco for the countless funny moments we had together and for making my "journey" unforgettable.

I thank all my friends, the new ones and the old ones, the ones who have been there for ever and the ones who have disappeared, the ones who believed in me, and whose names I may not all recall, and the ones who did not, for making me stronger, better and wiser.

Last but definitely not least, I devote a great part of the present dissertation to my beloved tutor and best mentor I have ever had, Dr. Maria Isabel Covas.
Maribel has been more than a tutor to me during my thesis development. Her support, understanding, encouragement, and wisdom have helped and inspired me throughout the time I have been on this journey. With persistence and patience, she challenged me to learn, question, think, experiment, and critically analyze. She encouraged me since the very beginning and she had waited for me, when life did not make it possible. She has never stopped guiding me with intelligence and expertise, shedding more and more light on my dissertation path. She has always welcomed my unformulated ideas by allowing me to think out loud. Throughout the PhD experience, Maribel provided me with immeasurably wise and perceptive advice, and has been a real mentor and coach to me, in the true sense of the words. Her insightful questions were extraordinarily helpful in moving me from the uncertain-fuzzy idea stages to concrete, feasible, realistic stages. A small paragraph can not even get close to the deepest feelings of appreciation and gratefulness, I feel for Dr. Covas.
Thank you, Maribel, for being what you are
and for being there for me.

Siempre y cuando, I have been used to hablar, escribir, leer and think en tres idiomas diferentes, δεν θα μπορούσα να terminar αυτές τις ευχαριστίες διαφορετικά…Para los que sepan, será muy fácil accept and recognize το μοναδικό μου style and ύφος. Para los que no sepan, I just hope να διασκεδάσουν τον epilogue!

Εύχομαι πάντα να βλέπω μακρύτερα, στεκόμενη στις πλάτες των Γιγάντων μου…

I wish always to see further by standing on the shoulders of my Giants…

Espero que siempre consiga ver más lejos, apoyándome en los hombros de mis Gigantes…

Abstract

The scope of the present work was to investigate whether the protective role of the traditional Mediterranean diet (TMD), and virgin olive oil (VOO) rich in phenolic compounds (PC), towards cardiovascular disease can be mediated through gene expression changes. Two trials were performed to assess the *in vivo* nutrigenomic effects of TMD and VOO in healthy volunteers. The results point out: a) significant gene expression changes of those genes related with cardiovascular-risk processes after VOO ingestion; b) a down-regulation in the expression of atherosclerosis-related genes after a 3-month intervention with a TMD; and c) an olive oil PC health-protective nutrigenomic effect within the frame of the TMD. Changes in gene expression were concomitant with decreases in oxidative damage and systemic inflammation markers. Data from our studies provide further evidence to recommend both the TMD and the VOO as a useful tool for the prevention of atherosclerosis.

Resumen

El objetivo de este estudio es investigar si el papel protector de la dieta Mediterránea tradicional (TMD) y del aceite de oliva virgen (VOO), rico en compuestos fenólicos (PC), puede ser mediado a través de cambios en la expresión génica. Se realizaron dos ensayos clínicos para evaluar los efectos nutrigenómicos de la TMD y del VOO, *in vivo,* en voluntarios sanos. Los resultados mostraron a) cambios en la expresión génica de genes relacionados con el riesgo cardiovascular tras la ingestión del aceite virgen de oliva, b) una infra-expresión en la expresión de genes relacionados con el proceso aterosclerótico tras una intervención con TMD de 3 meses y c) que los compuestos fenólicos del aceite de oliva ejercen un efecto nutrigenómico protector en el marco de la TMD. Los cambios en la expresión génica fueron coherentes con los obtenidos en los marcadores de inflamación sistémica y daño oxidativo.

Prologue

In Mediterranean European countries a low incidence rate of cardiovascular disease (CVD) has been reported, in spite of a strong prevalence of classical cardiovascular risk factors. The high degree of adherence to the Mediterranean diet has been postulated as a candidate factor for explaining this paradox. CVD is the leading cause of death in the industrialized countries. Currently, CVD accounts for more than $12x10^6$ annual deaths worldwide, and is the paradigm of multifactor disorders where multiple genetic and modifiable risk factors are combined to monitor the disease outcome. Atherosclerosis, considered as an underlying cause of CVD, is usually quite advanced by the time heart problems are detected. Therefore, there is an increased emphasis on preventing atherosclerosis through modifiable factors, such as diet.

Adherence to the traditional Mediterranean diet (TMD), in which olive oil is the main source of fat, has been associated with a reduced risk of overall and cardiovascular mortality, cancer incidence and mortality, and incidence of Parkinson's and Alzheimer's disease. The most impressive benefits of this diet, however, are related to reductions in cardiovascular morbidity and mortality. Data concerning olive oil consumption and primary end points for CVD are still scarce. However, a large body of knowledge exist providing evidence of the benefits of olive oil consumption on secondary end points for CVD. The recent results of the EUROLIVE study have provided evidence of the antioxidant

in vivo role of phenolic compounds from olive oil in humans and the fact that olive oil is more than a MUFA fat.

The exact mechanisms by which the Mediterranean diet and olive oil exert their health effects are not yet understood. Among these mechanisms, the gene-environment and/or gene-diet interaction could play an important role in the development of and/or protection against chronic degenerative diseases. At present, a lack of data exists on the *in vivo* effect of the virgin olive oil and its phenolic compounds on human gene expression. Also, data on the *in vivo* effect of the Mediterranean diet on human gene expression are scarce.

In the nutrigenomic era, attention has been drawn to the importance of genes in human nutrition and the nutritional field has recently started to focus on molecular changes. The prevention of diet-related diseases, the development of Evidence-Based Medicine, and the contribution to Public Health are some of the goals of the nutrigenomic field of research.

Intervention studies, in which subjects receive a controlled dietary intake, provide the best approach for conducting gene-nutrient-phenotype association studies. The hypothesis driven in the present work is that: 1) the traditional Mediterranean diet; 2) virgin olive oil; and 3) virgin olive oil phenolic compounds can modify the *in vivo* gene expression in peripheral blood mononuclear cells of

healthy volunteers. These effects would be directed towards a protective mode for cardiovascular disease development.

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I. ABBREVIATIONS

8-oxo-dG	8-oxo-deoxy-guanosine
ADAM17	A disintegrin and metalloproteinase domain 17 (TNFα, converting enzyme)
ADRB2	Adrenergic beta-2-receptor
AKAP13	A kinase (PRKA) anchor protein 13
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
ARHGAP15	Rho-GTPase activating protein15
BMI	Body Mass Index
CD36	CD36 (thrombospondin receptor)
cDNA	Complementary deoxynucleic acid
CRP	C - reactive protein
CVD	Cardiovascular Disease
DCLRE1C	DNA cross-link repair 1C
DNA	Deoxynucleic Acid
EUROLIVE	The effect of olive oil consumption on oxidative damage in European populations
GC-MS	Gas Chromatography – Mass Spectrometry
GEO	Gene expression omnibus
HDL	High Density Lipoproteins
HT	Hydroxytyrosol
ICAM-1	Intracellular cell adhesion molecule-1
IFNγ	Interferon gamma
IL10	Interleukin 10
IL6	Interleukin 6
IL7R	Interleukin 7-receptor

LDL	Low Density Lipoproteins
LIAS	Lipoic acid synthetase
mM	Mili Molar
MUFA	Monounsaturated Fatty Acid
NFκB	Nuclear transcription factor kappa beta
OGT	O-UDP-N-acetylglucosamine (polypeptide-N-acetylglucosaminyl transferase)
oxLDL	Oxidized Low Density Lipoprotein
PBMNCs	Peripheral Blood Mononuclear Cells
POLK	Polymerase (DNA directed) κ
PPARBP	Peroxisome proliferator-activated receptor binding protein
PREDIMED	Prevention with Mediterranean Diet
PUFA	Polyunsaturated Fatty Acid
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SFA	Saturated Fatty Acid
T	Tyrosol
T2DM	Type 2 Diabetes Mellitus
TLDA	TagMan® low density arrays
TMD	Traditional Mediterranean Diet
TNFα	Tumor necrosis factor alpha
USP48	Ubiquitin-specific protease 48
VCAM-1	Vascular cell adhesion molecule-1
VOO	Virgin Olive Oil
WHO	World Health Organization
WOO	Washed Olive Oil
μM	Micro Molar

INTRODUCTION

II. INTRODUCTION

Nothing in life is to be feared, it is only to be understood.
Maria Skłodowska-Curie (1867 – 1934)

1. Mediterranean diet and cardiovascular health

The traditional Mediterranean diet refers to dietary patterns found in olive-growing areas of the Mediterranean region since the 1960s (1). It can be considered as a single entity consisting of diet-variants from each region in the Mediterranean basin. All these variants share many characteristics, but olive oil is considered a hallmark of this dietary pattern, resulting in high intakes of monounsaturated fatty acids (MUFA) and lower intakes of saturated fatty acids (SFA). The traditional Cretan Mediterranean diet is considered the archetypal Mediterranean diet and most of the focus on the health benefits of the latter have been centered on the Cretan diet (2). The Mediterranean diet may not be markedly different from other recommended diets worldwide but its basic element, olive oil, makes it unique and contributes an additional value to its healthy benefits (3). The Mediterranean diet is also characterized by a) a high consumption of vegetables, legumes, fruits and cereals; b) a regular but moderate wine intake; c) moderate consumption of fish; d) low consumption of meat; and e) from low to moderate intake of dairy products (Figure 1). Associated habits are a moderate-to-high level of physical activity and a daily, high consumption of water.

Total lipid intake may be high, around or in excess of 40% of total energy intake as in Greece, or moderate, around 30% of total energy intake, as in Italy. The ratio of MUFA to SFA is much higher in the regions where a Mediterranean diet pattern is followed than in other places in the world (3).

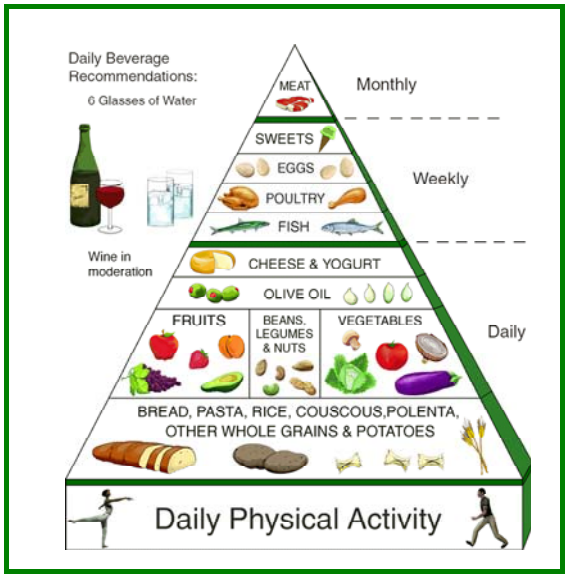

Figure 1. The traditional Mediterranean diet pyramid
(based on Willett *et al* (1995) Am J Clin Nutr 61, 1402S-1406S)

Indirect evidence about the beneficial effects of the Mediterranean diet in human health came firstly from the World Health Organization (WHO) (4) database and its mortality statistics (4;5). It was observed that death rates in the Mediterranean region were generally lower and adult life expectancy generally higher when compared to more developed countries with a superior health care system, like the Northern European countries and United States of America (USA). Nevertheless the prevalence of smoking was higher among the Mediterranean region (6).

The first key epidemiological study to assess the advantages of the Mediterranean diet was launched by Ancel Keys and colleagues in the 1950s. Since then, the Seven Countries Study has proposed the Mediterranean diet as a healthy eating pattern (1). Initially, the benefits of the Mediterranean diet were attributed to the low consumption of SFA associated to this dietary pattern. Since the late 1990s, however, a plethora of basic, clinical, and epidemiological studies have been developed, and a solid body of evidence is growing concerning the beneficial role of the overall Mediterranean dietary pattern on health (7;8).

Adherence to the traditional Mediterranean diet (TMD), in which olive oil is the main source of fat, has been associated with a reduced risk of overall and cardiovascular mortality, cancer incidence and mortality, and incidence of Parkinson's and Alzheimer's disease (7-9). The most impressive benefits of this diet, however, are related to reductions in cardiovascular morbidity and mortality (10).

Cardiovascular disease (CVD) is the leading cause of death in the industrialized countries. Currently, CVD accounts for more than $12x10^6$ annual deaths worldwide (4). CVD is the paradigm of multifactor disorders where multiple genetic and modifiable risk factors are combined for monitoring the disease outcome (Figure 2). Atherosclerosis, considered as the underlying cause of CVD (11), is usually quite advanced by the time heart problems are detected.

Therefore, there is an increased emphasis on preventing atherosclerosis by modifying risk factors, such as diet.

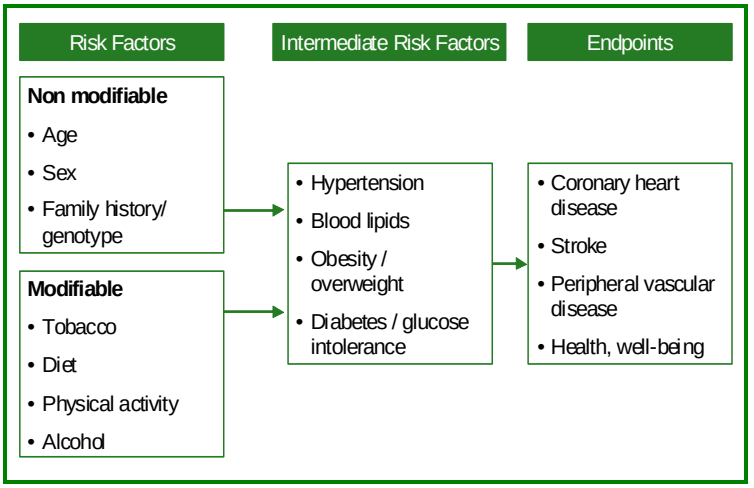

Figure 2. Cardiovascular Disease Risk Factors
(Adapted from Ordovas, J.M. (2006) Am J Clin Nutr 83, 443-446)

In Mediterranean European countries, a low incidence rate of CVD has been reported (6;12) in spite of a high prevalence of classical cardiovascular risk factors (13). The high degree of adherence to the Mediterranean diet, observed in these countries, might contribute for explaining this paradox. The evidence concerning the relationship between the Mediterranean diet and CVD has recently been classified as: a) ecological (independent association), b) analytical (cohort and case-control studies) and c) interventional (nutritional intervention trials). However, on the basis of the precepts of Evidence-Based Medicine, a high level (I or II) of scientific evidence is required before nutritional recommendations, for the general population, can be formulated. Randomized, controlled, double-blind, clinical intervention trials are the ones which can provide the required scientific evidence (level I of

evidence) and, to some extent, large cohort studies (level II of evidence) can also do so (14).

The Lyon Diet Heart Study (15) is the first key work testing a Mediterranean type diet, as a whole dietary pattern, on primary end points for CVD. In this randomized, controlled, parallel, clinical trial 605 patients with CVD participated during 46 months, and the results showed a significant decrease in CVD events in secondary prevention. In 2003, a large cohort study with 22,043 participants from the Greek component of the European Prospective Investigation into Cancer and Nutrition (EPIC) was published (7). Participants were followed-up during 44 months and a greater adherence to the Mediterranean diet was associated with a significant reduction in total mortality, CVD and cancer mortality. Similar results were obtained in the HALE (Health Ageing: a Longitudinal Study in Europe) cohort study (16) in which 2,339 Europeans aged from 70 to 90 years were followed during 6 months. The study showed a more than 50% lower rate of all-causes, and cause-specific mortality, after adherence to the Mediterranean diet. Furthermore, another cohort study with 330 individuals, the Melbourne Study (17), which replicated the design of a previous Greek study (18), demonstrated that the benefits of the Mediterranean diet could be transferred to other elderly population groups, such as Anglo-Celts and Greek-Australians, decreasing their overall mortality.

In October 2003, the Prevention with Mediterranean Diet (PREDIMED) study was launched. This parallel-group, multicenter, controlled, randomized, 5-year follow-up clinical trial is currently ongoing and 7380 high-CVD-risk participants have been recruited. PREDIMED's first results have shown that traditional Mediterranean diets supplemented with olive oil or nuts have beneficial effects on cardiovascular risk factors when compared with a low-fat diet (19). It has been proposed that the Mediterranean diet may be closer to the ancestral foods that were part of human development. Therefore, the human metabolism may have evolved to work optimally on such a diet rather than on other diets, such as those rich in SFA and highly refined and processed foods (20).

The Mediterranean type diet has also shown to be effective in the reduction of secondary end points for CVD. The high antioxidant content of such a diet could prevent the oxidation of low density lipoproteins (LDL) and that of deoxyribonucleic acid (DNA). Current evidence indicates oxidative damage as a promoter of pathophysiological changes occurring in oxidative stress-associated diseases, such as CVD (21). Oxidation of LDL promotes atherosclerosis because is more damaging to the arterial wall than native LDL (21). Elevated concentrations of *in vivo* circulating oxidized LDL (oxLDL) show a positive relationship with the severity of acute coronary events (22). The degree of the *in vivo* LDL oxidation (oxLDL) has been inversely associated with the adherence to a Mediterranean-type diet, in a population-based, cross-sectional study of 2,282 participants (23). Reduced

lipoprotein oxidation has been also observed after 12 weeks of Mediterranean diet in healthy women participating in a linear intervention study (24).

In a subsample of the PREDIMED study, first-level scientific evidence of the beneficial effect of the Mediterranean diet on oxLDL has been recently provided. The subsample consisted of 372 individuals at high-CVD-risk who followed a 3-month intervention of TMD and at the end of which, a significant reduction of the levels of oxLDL was observed (25). In another subsample of the PREDIMED study (1224 participants), it was shown that a traditional Mediterranean diet enriched with nuts could be a useful tool in the management of the metabolic syndrome (26), as it is defined by the National Cholesterol Education Program Adult Treatment Panel III (27). Moreover, in this study (26), the positive effect on metabolic syndrome was achieved by diet alone, in the absence of weight loss or increased energy expenditure in physical activity.

Blood pressure, inflammatory status, endothelial dysfunction, DNA oxidation and prothrombotic profile are also secondary end points for CVD, in which the Mediterranean diet has elicited its protective effects (19;28-31). Moreover, atherosclerosis, the principal cause of CVD, is described, in aggregate, as an inflammatory disease (32). Estruch *et. al.*, in the PREDIMED study (19), have reported a decrease in both systolic blood pressure and the levels of plasma C-reactive protein (CRP) compared with a low-fat diet. The serum

CRP, the prototypic inflammation marker, and the serum cytokine interleukin-6 (IL6), principal messenger of the pro-inflammatory response, have been shown to be predictors of CVD (33;34). In a subsample (n =109) of the PREDIMED study a decrease in the adhesion molecule expression on T lymphocytes and monocytes, 3 months of TMD intervention, but not after the control, low-fat diet, has been reported (35).

An improvement of the inflammation status, measured as serum high-sensitivity CRP, interleukins 6, 7, and 18 (IL6, IL7, and IL18), in patients with metabolic syndrome, after 2 years' adherence to the Mediterranean diet, was also described by Esposito and colleagues (28). In another randomized, controlled, intervention study with 22 hypercholesterolemic patients (29), the endothelial dysfunction profile was better after following a 28-day Mediterranean diet pattern.

An increased risk of CVD and a mortality rate higher than that of the general population have been consistently found in diabetic populations in U.S. and Asia-Pacific regions (36-38). Type 2 Diabetes Mellitus (T2DM) results from the body's inability to respond properly to the action of insulin. Insulin is produced by the pancreas, and T2DM accounts for around 90% of all diabetes cases worldwide (39). Cardiovascular morbidity in patients with T2DM is two to four times greater than that of non-diabetic people (40). The likelihood of developing phenotypic characteristics of T2DM, such as the metabolic syndrome, can be substantially modified by diet.

Despite of this, data on the efficacy of dietary recommendations for the treatment of T2DM are limited (41).

The first randomized, single-blind trial with 180 overweight participants with the metabolic syndrome was published in 2004 (28). Based on these results, a 2 years lifestyle program, focusing mainly on a Mediterranean-style diet, resulted in the net reduction in prevalence of the syndrome by 48%. Giugliano and Esposito have recently suggested that the Mediterranean diet plays a significant role in reducing the risk of developing T2DM (42). The Mediterranean dietary pattern was also shown to increase the level of circulated adiponectin, which has both anti-inflammatory and insulin-sensitizing properties (43). In a recent study with 90 subjects with abdominal obesity, close adherence to a Mediterranean –style diet resulted in an improved endothelial function and in a decrease in diastolic blood pressure(44).

Based on the aforementioned evidence, we could recommend the Mediterranean diet for controlling cardiovascular risk factors, particularly for individuals already being at high risk for CVD. Among all food that Mediterranean diet is comprised of, olive oil is the most well studied of all.

2. Olive oil and cardiovascular health

2.1 Composition and Description

Since the ancient Greek times, in the Mediterranean region, there has always been a strong belief that olive oil is the elixir of youth and health. The Greek epic poet Homer used to call olive oil as "liquid gold". Olive oil occupies a central position in the Mediterranean diet as the main source of fat. Major components of olive oil are the fatty acids (Table 1). The monounsaturated fatty acid (MUFA), named oleic acid, represents from 55% to 83% of the total fatty acids of olive oil. Polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) occupy a range from 4% to 20% and from 8% to 14%, respectively (45). The minor components of olive oil are classified into the unsaponifiable fraction and the soluble fraction. The former is defined as the solvent-extracted fraction after the saponification of the oil, whereas the latter one includes the phenolic compounds (46).

The phenolic compounds can be distinguished as simple or complex. The simple phenolic compounds, named hydroxytyrosol (3,4-dihydroxy-phenyl-ethanol), tyrosol (p-hydroxy-phenyl-ethanol), and their secoiridoid derivatives (e.g. oleuropein) (Figure 3), make up around 90% of the total phenolic content of a virgin olive oil (VOO). The secoiridoids include i) the oleuropein glucoside; ii) SIDs, which are the dialdehydic form of oleuropein (SID-1) and ligstroside (SID-2) lacking a carboxymethyl group; iii)

the aglycone form of oleuropein glucoside (SID-3); and iv) the ligstroside (SID-4). The complex phenols include lignans (e.g. (+)-pinoresinol and (+)-1-acetoxypinoresinol) and flavonols (47).

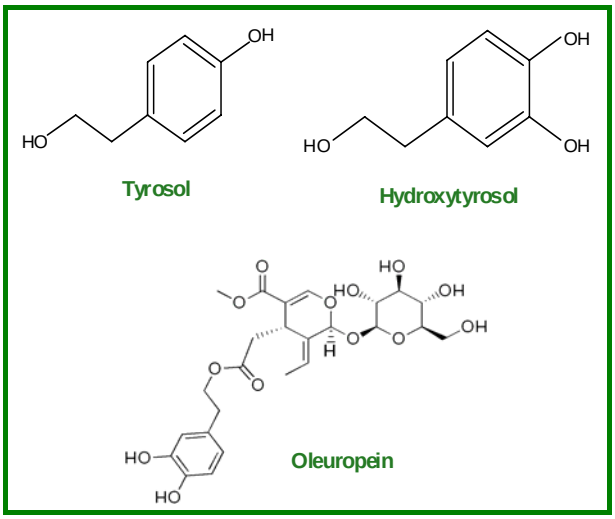

Figure 3. Structures of the major phenolic compounds identified in virgin olive oil

VOO is obtained from olive fruits that are processed only by physical means without any solvent extraction or refining procedures. The content of the minor components of an olive oil depends on the cultivar, climate, ripeness of the olives at harvesting, and the processing system employed. Different processing methods produce virgin, ordinary, or pomace olive oil (48). Extra virgin olive oil (EVOO) is a VOO with a free acidity - expressed as g of oleic acid/100g of olive oil - less than 0.8 g. VOOs with an acidity ≥ 3.3 (International Olive Oil Council Regulation/T.15/NC.n3.Rev2.Nov24, 2006) (≥ 2 in Europe, European Regulation N.1513/01) are submitted to a refining process in which some components, mainly phenolic compounds are lost (49). By mixing virgin and refined olive oil, an ordinary olive oil

(UE, 1991) is produced and marketed. After VOO production the rest of the olive drupe and seed is processed and submitted to a refining process, resulting in pomace olive oil. For commercialization, this pomace olive oil contains a certain quantity of VOO. In addition to the Mediterranean area, where olive oil has always been an essential food, its use has recently expanded to other parts of the world.

Table 1. Chemical composition of olive oil

Saponifiable fraction (98-99%) (Main fatty acid present in triacylglicerols)	Unsaponifiable fraction (about 2%)
MUFA • Oleic acid (18:1n-9) (55-83%) **PUFA** • Linolenic acid (18:3n-3) (0.0-1.5 %) • Palmitoleic acid (18:3n-3) (7.5-20 %) • Linoleic acid (18:2n-6) (3.5-21 %) **SFA** • Palmitic acid (16:0) (7.5-20 %) • Miristic acid (14:0) (0-0.1 %) • Estearic acid (18:0) (0.5-5 %)	• Lipophilic phenolics (tocopherols and tocotrienols) • Hydrophilic phenolics (phenolic acids, phenolic alcohols, secoiridoids, lignans and flavones) • Volatile compounds • Pigments (chlorophylls) • Hydrocarbons (squalene, B-carotene, lycopene) • Sterols (B-sitosterol, campesterol, estigmasterol) • Triterpene alcohols • Aliphatic alcohols • Non-glyceride esters (alcoholic and sterol compounds, waxes)

Adapted from Escrich, E. *et al* (2007) Mol Nutr Food Res 51, 1279-1292

2.2 Cardiovascular healthful properties

2.2.1 Olive oil as a MUFA source of fat

In 2004, the U.S. Food and Drug Administration (FDA) has permitted a qualified health claim for MUFA from olive oil and a

reduced risk of coronary heart disease (CHD) (FDA, Press release P04-100, 2004 http://www.fda.gov/-dms/qhchoice/html). Data concerning olive oil consumption and primary end points for CHD are still scarce (50). In a Spanish case-control study, an 82% relative reduction in the risk of having a first myocardial infarction was negatively associated with olive oil consumption (51). In a 5-year Greek cohort study, with 28,572 participants, a negative association between the MUFA-to-SFA ratio, but not with specific olive oil consumption, and cardiovascular and overall mortality was reported (7). However, a large body of knowledge exist providing evidence of the benefits of olive oil consumption on secondary end points for CVD.

Hypercholesterolemia causes the activation of the endothelium. The infiltration and retention of cholesterol from LDL is responsible for the initiation of an inflammatory response in the artery wall (52). It has been shown that the consumption of MUFA did not affect total cholesterol levels whereas consumption of SFA raised them (53). It has also been established that MUFA consumption maintains the levels of HDL cholesterol (HDL-C) and reduces those of LDL cholesterol (LDL-C) when it is substituted for a source of saturated fatty acids (SFA) (54). There is growing evidence, however, that olive oil consumption increases HDL-C levels. The beneficial effects of olive oil on the lipid profile have been highlighted in the report of the 1st International Conference on Olive Oil and Health, held in Jaen, Spain (50).

The PREDIMED study has recently confirmed an increase in HDL-C after Mediterranean diet consumption (19;25). A high-fat diet (40% of energy), rich in MUFA and low in SFA, and a low-fat carbohydrate-rich diet, had shown to have similar cholesterol-lowering effects. However, a high-MUFA diet did not lower HDL cholesterol or increase triglycerides as did the carbohydrate-rich diet. A meta-analysis of 10 studies provided first level evidence of the benefits of MUFA-rich diets as compared to the carbohydrate-rich diets not only for healthy, but also for diabetic individuals (55). The formed chylomicrons, after olive oil consumption, enter faster into the circulation and are more rapidly cleared than those formed after SFA ingestion (56).

The results of the EUROLIVE (The effect of olive oil consumption on oxidative damage in European populations) study shown a protective role of olive oil consumption on the cardiovascular lipid profile and oxidative damage in humans, at real-life olive oil doses (57). The EUROLIVE study was a large, crossover, multicentre, clinical trial performed in five European countries. Two hundred (200) healthy male participants were randomly assigned to 3 groups receiving 25 mL/day of 3 different olive oils. Olive oils had low (2.7 mg/kg of olive oil), medium (164 mg/kg), or high (366 mg/kg) phenolic content but were otherwise similar. Intervention periods were of 3 weeks preceded by 2-week washout periods. Results of the EUROLIVE study showed an increase in HDL-C and in reduced/ oxidized glutathione as well as a decrease in triglycerides and in oxidative damage after all olive oils administrated.

Oxidative stress, produced by reactive oxygen species (ROS), has been also linked to the development of atherosclerosis (58). Oxidation of the lipid part (59), or directly to the apolipoprotein B (60) of the LDL-C leads to a lipoprotein conformational change. In this way, the modified LDL is better able to enter into the monocyte/macrophage system of the arterial wall, and develop the atherosclerotic process, thus promoting CVD (58). The type of fat ingested is a key factor concerning LDL oxidation because it can modulate the susceptibility of LDL to undergoing these conformational changes. Oleate-rich LDL have been shown to be less susceptible to oxidation than linoleate rich LDL (61). The linoleic acid accounts for 90% of the PUFA present in LDL and is the major substrate for its oxidation. Furthermore, PUFA – rich in double bonds – are more prone to form conjugated dienes than MUFA (62). Another factor that influences LDL oxidability is the LDL particle size. It is also modulated by the dietary fat. Small, dense LDL particles are more prone to oxidation. In a cross-sectional survey, PUFA intake, but not that of MUFA, was negatively associated with the LDL size in diabetic type 2 patients and subjects with impaired glucose metabolism (63).

Oxidative stress does not only affect lipids but also DNA. The most abundant DNA modification is the hydroxylation of guanine, in the 8-position to 8-oxo-deoxy-guanosine (8-oxo-dG) (64). The urinary excretion of 8-oxo-dG is advocated as a biomarker of the whole body DNA oxidation (65). Results of the EUROLIVE study showed that olive oil consumption reduced the DNA oxidation around 13%,

a magnitude comparable to that observed with smoking cessation (66). However, it must be pointed out that the decrease in DNA oxidative damage observed in the EUROLIVE study after olive oil consumption, in spite of the consistency of the results through three randomized intervention periods, was evaluated on a linear basis. This was due to the lack of a placebo group other than the low phenolic olive oil group. In addition, there is an ongoing debate concerning the best method for DNA oxidative damage measurement, the steady-state levels of 8-oxo-dG in lymphocytes considered at present to be the best biomarker (67;68). Studies that have addressed the specific role of olive oil phenolic compounds on DNA oxidation are mentioned below (Table 2).

Table 2. Randomized, crossover, controlled studies on the antioxidant effect of sustained olive oil phenolic compounds consumption on *in vivo* markers of lípid and DNA oxidation. (Adapted from Fitó *et al* (2007) Mol Nutr Food Res 51, 1215-1224)

Duration	Olive oil intervention	Daily olive oil dose	Participants	Washout period	Oxidative markers	Effects	Reference
3 weeks	High- vs Low-phenol	69 g	46 healthy (31 women, 15 men)	2 weeks without olives and olive oil	• MDA • FRAP • LP • PC • LDL-resistance to oxidation [a]	None	Vissiers *et al* (2001) (76)
3 weeks	High- vs Low-phenol	70 g raw	25 healthy (14 women, 11 men)	2 weeks without olives and olive oil	• MDA • FRAP • LP • PC • LDL resistance to oxidation [a]	None	Moschandreas *et al* (2002) (77)
3 weeks	Virgin vs Common vs Refined	22 g (25 mL) raw	30 healthy men	2 weeks with refined olive oil for raw and cooking purposes	• Plasma oxidized LDL • LDL resistance to oxidation [a] • Antibodies against oxLDL • HDL-C	↓ with OO phenolics None ↑ after VOO	Marrugat *et al* (2004) (78)
4 days	High vs Medium vs Low phenol	25 mL raw	12 healthy men	10 days: low phenol olive oil for raw and cooking; very-low antioxidant diet	• Plasma oxidized LDL • MDA in urine • 8oxodG in urine and lymphoctyes • F_2-isoprostanes • GSH-Px • HDL-C	↓ with OO phenolics None ↑ with OO phenolics ↑ with OO phenolics	Weinbrenner *et al* (2004) (79)
7 weeks	Virgin vs refined	40 mL raw	22 lipemic (12 men, 10 women)	4 weeks only with refined olive oil (low-phenol)	• Plasma antioxidant capacity • F_2-isoprostanes	↑ with OO phenolics None	Visioli *et al* (2005) (80)
3 weeks	Virgin vs Refined	50 mL raw	40 coronary heart disease men	2 weeks with refined olive oil for all purposes	• Plasma oxLDL • LP • GSH-Px	↓ with OO phenolics ↑ with OO phenolics	Fitó *et al* (2005) (81)
8 weeks	High- vs Low-phenol	*ad libitum* in substitution of other fats	10 post-menopausal women	2 weeks (usual diet)	• Comet assay for DNA oxidation	↓ with OO	Salvini *et al* (2006) (82)
3 weeks	Virgin vs Common vs Refined	25 mL raw	200 healthy men	2 weeks w/o olives and OO	• Plasma oxLDL • Uninduced dienes • Hydroxy fatty acids • Antibodies against oxLDL • F_2-isoprostans • GSH/GSSG • Antioxidant enzymes	↓ with OO phenolics None ↑ non-related with OO phenolics None	Covas *et al* (2006) (57)
Idem	Idem	Idem	Idem	Idem	• 8-oxo-dG • 8-oxo-guanine • 8-oxo-guanosine	↑ non-related with OO phenolics None	Machowetz *et al* (2006) (30)

[a] *In vitro* test. MDA, malondialdehyde; FRAP, ferric reducing ability of plasma; LP, lipid peroxides; PC, protein carbonyl; 8oxodG, 8-position to 8-oxo-deoxyguanosine; GSH-Px, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; OO, olive oil; ↑, increase; ↓, decrease

Postprandial lipemia is another risk factor for atherosclerosis development and is influenced by the type and amount of the fat administrated in the diet (56;69). Postprandial lipemia and hyperglicemia is also linked with postprandial oxidative stress (70). Data comparing the magnitude of postprandial oxidative stress after olive oil ingestion in comparison with other oils or fats are scarce. Fuhrman *et al* have (71) reported that the ingestion of fish oil, or its major PUFA docosahexaenoic acid, in mice, induced a greater postprandial oxidative stress than that promoted by olive oil. Also, when compared with butter and walnuts, Bellido *et. al.* have shown that olive oil did not elicit postprandial activation of nuclear transcription factor kappa beta (NFκB), in PBMNC from healthy men (72). NFκB is known as a redox-transcription sensitive factor involved in the inflammatory and proliferative response in atherosclerotic areas. A 25 mL single dose of olive oil does not promote postprandial lipemia (73), whereas 40 mL and 50 mL doses of any type of olive oil do (74;75).

Moreover, increased and prolonged postprandial triglyceride concentrations are associated with numerous conditions related to insulin sensitivity. Insulin is the dominant glucoregulatory hormone. In the fasting state, it regulates the plasma glucose concentration, primarily by restraining hepatic glucose production; high concentrations, such as those found after meals, are required to stimulate glucose utilization (83). Insulin plays a central role in determining the triglycerides turnover and clearance, via lipoprotein lipase activation, through the synthesis and secretion of very low

density lipoproteins (VLDL) (84). Insulin secretion can be divided into two different phases: 1) the stimulated (postprandial) state that regulates glucose metabolism when carbohydrate is abundant and must be disposed of, and 2) the basal (post-absorptive) state that prevails during the interprandial phases. Long-term maintenance of serum glucose concentrations is a closely regulated process in mammalian species (85). In the KANWU study – a large, controlled trial with 162 healthy individuals – results showed that substituting SFAs with MUFAs improved insulin sensitivity (86).

Another recent, controlled, crossover study was conducted in insulin-resistant offspring of obese patients with diabetes (87). Similarly as before, the results of the latter study indicated that a MUFA-rich diet improved insulin sensitivity compared with a SFA-rich diet. It is worth mentioning that these effects were observed despite the short treatment period of 28 days, and despite the fact that the total fat intake in the test diets was high (38% of total energy intake). Recently, postprandial insulin sensitivity has been reported to progressively improve as the proportion of MUFA, with respect to SFA, in dietary fat increases (88). Great variations in insulin sensitivity are common even among young healthy individuals (85). Less than one-third of the inter-individual variation in insulin sensitivity is explained by known factors such as obesity. Thus, genetic factors, and gene-environment interactions, deserve consideration to account for other hitherto neglected contributions that can explain this large variation (89).

So far, most of the cardio-protective effects of olive oil, in the context of the Mediterranean diet, have been attributed to its high MUFA content. It is important, however, to emphasize that the most abundant MUFA in olive oil – oleic acid – is also one of the predominant fatty acid in widely consumed animal foods in Western diets, such as poultry and pork (90). Meat intake was positively related to the level of oleic acid in plasma phospholipids in a female population in Granada, Spain, but there were no differences in levels of PUFA (91). It is thus plausible that a high oleic acid intake could not be the primary agent responsible for the healthful properties of olive oil.

2.2.2 The specific role of olive oil's phenolic compounds

Olive oil is a functional food, which besides having a high level of MUFA, also contains multiple bioactive minor components. Among them, the best studied so far are the phenolic compounds. As already mentioned, phenolic compounds are the soluble minor components of VOO that remain after the saponification of the oil. In fact, hydrophilic phenols are components of the unsaponifiable fraction, but, being present as droplets in micro emulsion in the lipidic matrix, they are easily extracted by as simple liquid-liquid procedure with n-hexane and methanol/water (60/40), without a saponification step (92).

One of the prerequisites for assessing the physiological significance of olive oil phenolic compounds in humans is the ability to

determine their bioavailability. Tyrosol (T) and hydroxytyrosol (HT), as measured in urine by GC-MS, are absorbed by humans in a dose-dependent manner with the phenolic content of the administered olive oil (93), even from moderate doses (25mL/day), lower than the traditional daily dietary intake in some Mediterranean countries (94). These observations were made not only after a single dose (73), but also after short (79) and long-term (78;81) consumption of real-life doses of olive oils.

In all the aforementioned studies, around 98% of these phenolic compounds were present in plasma and urine in conjugated forms, mainly as glucurono-conjugates. This fact suggests the existence of an extensive first-pass intestinal/hepatic metabolism of the ingested primary forms (95). The biological activity of olive oil phenolic compounds must, therefore, derive from their metabolites or derivate compounds. Sources of HT from olive oil come from its free form, about 10% of the dose (96), its 4-beta-D-glucoside (97), and oleuropein. Oleuropein is absorbed, metabolized in the body, and recovered in urine, mainly in the form of HT (98).

Both HT and T urinary concentrations are currently in use, in nutritional intervention studies with olive oil, as biomarkers of treatment compliance. However, T may well be a better biomarker of sustained doses of VOO consumption for clinical studies (99). Urinary concentration of tyrosol is dependent on the administrated T dose, whereas urinary concentrations of HT tend to accumulate. One explanation for this could be that HT – also known as DOPET

(3,4-dihydroxy-phenylethanol) – is a well-known metabolite of dopamine. Homovanillic acid is also one of the main metabolites of dopamine, but it has also been reported to be a major metabolite of HT (100). Consecutively, the inter-relationship between HT and dopamine might be a confounding factor in the interpretation of analytical results (101). In this sense, recent data support the hypothesis of an endogenous generation of HT after alcohol consumption via dopaminergic stimulation (102).

Among olive oil minor components, phenolic compounds are those most extensively studied particularly for their antioxidant properties. In experimental studies, olive oil phenolic compounds, like other plant-derived polyphenols (103), counteracted the metal-, radical-, and macrophage-mediated oxidation of lipids and LDL (104-106). Owen *et. al.* showed that the antioxidant properties of olive oil phenolic compounds on lipids exceed those of vitamin E (107). The phenolic compounds exert their antioxidant activity by donating a hydrogen atom to the chain-propagating radicals formed during lipid peroxidation (108). Additionally, olive oil phenolic compounds have shown to, *in vitro*:

1) Decrease the expression of cell adhesion molecules, such as the vascular cell adhesion molecule-1 (VCAM-1), leading to the prevention of endothelial dysfunction (109).
2) Increase nitric oxide (NO) production and inducible NO synthesis probably due to the modulation of the nuclear factor kappa beta (NFκB) activation (110).
3) Inhibit platelet-induced aggregation (111).

4) Enhance mRNA transcription of the antioxidant enzyme glutathione peroxidase, depending on the tissue in which the gene expression was evaluated (106;112).

In animal models, olive oil phenolic compounds have shown to retain their *in vivo* antioxidant properties (113) and to delay the progression of atherosclerosis (114). A recent study with apoE deficient mice, however, has shown that the administration of high doses of HT (10mg/kg/day) led to an enhanced atherosclerotic lesion development (115). These results point out the importance of the concentration matrix and the synergistic effects of all antioxidants present in natural foods such as olive oil.

First level scientific evidence on the *in vivo* antioxidant effect of postprandial and sustained consumption of olive oil phenolic compounds was provided by several randomized, crossover, controlled human studies which are summarized in Table 2. Concerning postprandial olive oil phenolic compound consumption, studies are difficult to compare due to the lack of data on the postprandial lipemia and/or hyperglycemia status of the individuals under study (47). However, in conditions where olive oil ingestion induces oxidative stress, human *in vivo* studies have shown: a) an increase in the serum antioxidant capacity after VOO ingestion, but not after ordinary olive oil, compared with corn oil, suggesting a role for the phenolic compounds (116), and b) a lower lipid oxidative damage after high-phenolic than after low-phenolic olive oil (75;117). Moreover, the phenolic content of LDL directly

correlated with the plasma concentrations of T and HT after the ingestion of a VOO with high phenolic content (366 mg/kg of olive oil) (75).

Concerning sustained doses of olive oil phenolic consumption, results obtained up to year 2004, from human studies, have been controversial (118). The observed extensive differences among studies were due to several factors: experimental design, control of diet, sample population, age of the participants, measurement or not of markers of the intervention compliance, and sensitivity and specificity of the oxidative stress biomarkers evaluated. In 2006, the EUROLIVE study (57) clarified the issue, providing evidence of the *in vivo* protective role of phenolic compounds from olive oil on lipid oxidative damage in humans, at real-life doses. EUROLIVE's results showed that consumption of medium- and high-phenolic content olive oil, besides increasing HDL-C levels, also decreased lipid oxidative damage biomarkers such as plasma oxidized LDL (oxLDL), uninduced conjugated dienes, and hydroxyl fatty acids, without changes in F_2-isoprostanes. The increase in HDL-C and the decrease in the lipid oxidative damage were directly related with the phenolic content of the olive oil consumed. Key conclusion of the EUROLIVE study was that the phenolic content of an olive oil can account for greater benefits on blood lipids and oxidative damages than those provided by the MUFA content of the olive oil. For the first time, these results supported the idea that olive oil is more than a MUFA fat, providing first-level scientific evidence to recommend

phenolic compounds-rich olive oil as a source of fat to achieve additional benefits against cardiovascular risk factors.

Besides a concomitant MUFA increase in the LDL (119), the binding of phenolic compounds to human LDL may be a key factor for explaining VOO's antioxidant activities. Torre-Carbot *et al* have reported that HT and T metabolites, glucuronides and sulfates bind to human LDL after VOO ingestion (120). The susceptibility of LDL to oxidation depends not only on its fatty content, but also on the LDL antioxidant content (*i.e.* vitamin E and phenolic compounds) bound to the LDL particle (121). Phenolic compounds which can bind LDL are likely to perform their peroxyl scavenging activity in the arterial intima, where full LDL oxidation occurs in microdomains sequestered from the richness of antioxidants present in plasma (58).

Concerning DNA oxidation, studies in animal models showed that VOO was more beneficial than sunflower olive oil in preventing the age-associated effects on the antioxidant capacity and on the DNA double-strands breaks (122). Also, in human prostate cells, olive oil phenolic compounds reduced the levels of hydrogen peroxide-induced DNA damage (112). The effect of olive oil phenolic compounds on DNA oxidation in human studies stem controversial results up to now. In postmenopausal women, a daily intake of 50g of olive oil, high in phenolic content, has resulted in about 30% less DNA damage (82). Protective effects of olive oil phenolics on *in vivo* DNA oxidation, measured as 8-oxo-dG in mononuclear cells

and urine of healthy males, was shown in a short-term study (4 consecutive days preceded by 10 days washout periods) (79). However, a 25ml daily consumption of VOO during 3 weeks did not modify the urinary excretion of etheno-DNA adducts in healthy males (123). In the EUROLIVE study, daily consumption of 25mL of olive oil during 3weeks reduced DNA oxidation, but irrespective of the olive oil phenolic content (30). It becomes clear that further studies are required to definitively establish the effect of olive oil phenolic compounds on the DNA oxidative damage in front of other types of fat.

Anti-inflammatory, anti-endothelial activation, and chemo-preventive action are some of the additional activities shown by olive oil phenolic compounds (124;125). The olive oil phenolic compound named oleocanthal has been described as having similar properties to that of the anti-inflammatory molecule ibuprofene (126). However, the human bioavailability of oleocanthal from olive oil ingestion remains to be elucidated. In human studies, olive oil phenolics have been shown to be effective in reducing the eicosanoid inflammatory mediators derived from arachidonic acid, such as thromboxane B2 (TXB2) and 6-keto prostaglandin $F_{1\alpha}$ (6-ket-$PGF_{1\alpha}$) (80;116;127;128), and other inflammatory markers such as hs-CRP or IL-6 (129).

Contradictory results have been obtained concerning the effect of olive oil phenolic compounds on cell adhesion molecules. At postprandial state, an increase in the serum levels of ICAM-1 and

VCAM-1, after VOO consumption, compared with that after refined olive oil ingestion, has been reported in an randomized, crossover trial with 28 individuals (130). However, after sustained virgin or refined olive oil consumption – in a randomized, controlled, crossover study with 28 stable coronary heart disease patients – no differences were reported in ICAM-1 nor in VCAM-1 levels (129), but a decrease in interleukin 6 (IL6) and CRP was observed, after 3 weeks of VOO consumption. The anti-inflammatory effects of olive oil phenolic compounds, in humans, is a promising field, and further studies are required to obtain full evidence on the topic.

To sum up, olive oil is more than a rich, natural source of MUFA. It is a functional food which contains biologically active components able to promote health. It is of great importance to highlight that all actions of olive oil phenolic compounds described, up to now, are exerted without any cytotoxicity. This may account for exploring new agents promoting cardiovascular protection from a natural source that has been used as a whole food since time immemorial. However, the exact mechanisms by which the MUFA and/or the phenolic content of VOO elicit their effects on CVD risk factors are not fully understood. Nutrient-gene interactions could support new knowledge and contribute to clarify this issue.

3. Nutrigenomics Era

3.1 Introduction

The interplay between genes and diet is fundamental to human health. Their interaction is an integral component of evolution, including a cross-talk between a subset of genes and diet that has resulted in adaptations for specific nutrients and dietary patterns (131). Nutrition aims at a fine-tuned balance between many processes and metabolic pathways in order to maintain homeostasis and promote health. Examples of how a food or a food component can affect people's health-state have been known for some time. The haemolysis that may occur after fava beans consumption, in individuals with glucose-6 dehydrogenase deficiency, or the dietary problems among people with genetically determined lactose intolerance or gluten-sensitive enteropathies (132) are some of the most known cases.

In the nutrigenomics era, attention is drawn to the importance of genes in human nutrition and the focus of nutritional field has recently started to change towards more detailed molecular studies of nutrition. There has been a dramatic shift in nutrition research from focusing on preventing nutritional deficiencies to preventing chronic diseases (133). Moreover, the focus is now placed towards complex phenotypes without the "one gene-one disease" approach. The prevention of diet-related diseases, the development of evidence-based nutrition, and the contribution to public health are

only some of the goals of nutrigenomics field of research. An increasing number of large national and international nutrigenomics research clusters are being formed to jointly address the great nutrigenomic challenges (Table 3) (134). Their success is based on a collaborative effort among scientists from different disciplines such as nutrition, molecular biology, medicine, genomics and bioinformatics.

Table 3. Selected international nutrigenomics consortia and networks

Consortium	Country	Focus	Reference
• Center of Excellence for Nutritional Genomics	United States of America	Personalized diet and diet-gene interactions	www.nutrigenomics.ucdavis.edu
• Dutch Nutrgenomics Consortium	The Netherlands	Metabolic syndrome and early biomarkers	www.nutrigenomics.nl/ngc
• Network of Excellence in Nutrigenomics (NUGO)	Europe (EC)	Establishment of a European Nutrigenomics Research Network	www.nugo.org
• Centre of Excellence in Nutrigenomics	New Zealand	Crohn's disease and new food bioactives	www.nutrigenomics.org.nz
• Functional Food Genomics	Japan	Biomarkers and bioactive food ingredients	
• Nutrigenomics Network	Germany	Complex diseases and diet-gene interactions	www.nutrigenomik.de

Adapted from Afman and Müller (2006) J Am Diet Assoc 106, 569-576

3.2 Nutrigenetics vs Nutrigenomics

Nutritional genomics is a research field that may change the prevention and treatment of diseases. It can be divided in two different but collaborating sub-areas: nutrigenetics and nutrigenomics. Although both focus on studying the interactions between nutrition, genetics and health outcomes, there are important conceptual differences in their approaches and aims that need to be clarified (135-137).

Nutrigenetics concentrate on inter-individual differences, in relation to the effects of nutrients or diet, and focus on the characteristics of each individual, which to a certain extent will be determined by their genetic makeup. Thus, nutrigenetics tests the hypothesis that inter-individual differences, in the dietary response, may be associated with the presence or absence of individual-specific biological markers, most commonly genetic polymorphisms (e.g. SNPs), which may allow the prediction of this specific individual response (138). Main goal of nutrigenetics is to point out those SNPs that reveal significant gene-diet interaction, thus providing ways for personalized and more successful dietary recommendations. However, before these recommendations can be directed to the population, they need to be validated by robust scientific evidence. Therefore, it would be useful to apply the principles of Evidence-Based Medicine to nutrigenetics when causality is inferred from the results of association studies (133).

Nutrigenomics, on the other hand, applies to the comprehensive genome-wide assessment of the effects of dietary factors or interventions. Nutrigenomics represents the study of differences among nutrients and/or diet in relation to gene expression response in a single genome (138). Since nutrigenomics is a new field of knowledge, this concept has received different definitions (133), with the most condense being: Nutrigenomics is the study of molecular relationships between nutritional stimuli and the response of the genes (139). Nutrigenomics is an emerging and promising multidisciplinary field that uses new technical and conceptual

developments, derived in part from the human genome project, to study the interactions between nutrition, and its bioactive dietary components, genome and health outcomes.

The understanding of the role of nutrients on gene expression processes has recently become a goal in nutritional sciences. The application of high-throughput genomic tools and the integration of systems biology characterizes this new field of nutrigenomics (137). The focus is placed on differences, among several dietary conditions or nutrients, on quantitative measures of expression and their association with specific phenotypes. Nutrigenomics will thrive in the setting of nutritional research to find the best diettary recommendation from a given series of nutritional alternatives (133). In this work, we focus on nutrigenomics, refered to as the changes in gene expression promoted by nutrients, food, or dietary patterns.

3.3 Nutrigenomics Principles ("know-how")

3.3.1 Introduction to the field

Starting from 2001, where the completion of the full sequence of the human genome took place (140;141), valuable, new bodies of data were provided to scientists of all disciplines, to explore the interactions between all genes in the genome, and environmental factors, such as diet. Nutrition is considered a key environmental factor, involved in the pathogenesis and progression of polygenic

and complex diseases such as CVD, and Type II Diabetes Mellitus (T2DM). The human genome contains approximately 2.9 billion nucleotides or 30.000 genes, part of which is involved in metabolic regulations. Most of the genes that have been identified, so far, do not directly cause those complex diseases (e.g. CVD, T2DM) but rather enhance susceptibility, through a wide range of biochemical, regulatory, and signal transduction pathways (142).

In some ways, the nutrigenomics agenda can be seen as analogous to that of pharmacogenomics (143). However, nutrigenomic effects can not be compared with those from pharmacogenomic studies due to fundamental differences. Firstly, nutrigenomics deals with the complexity and variability of nutrition and not with pure compounds, like drugs. Also, nutrients and dietary patterns can reach high concentrations (μM to mM) without becoming toxic. It has been suggested that the supermarket of today will be the pharmacy of tomorrow (144). Such statements have been derived from recognition of the increasing ability to optimize nutrition, and maintain a state of good health through longer periods of life.

3.3.2 How does nutrigenomics work?

As aforementioned, a well established body of clinical and epidemiological studies has linked dietary habits with degenerative diseases such as CVD, T2DM, and cancer. These complex (multifactorial) diseases require an improved overview (holistic) picture of their early phases to achieve prevention. The complex nature of these diseases includes the interaction of several

mechanisms, at molecular level, which, up to now, were only partly known, primarily because of lack of appropriate research tools. Nutrigenomic approach exploits the multiple, minor and synergistic changes in genomic responses related to nutrition and health, instead of the single "target" response common in drug therapy. It clearly provides new insights into the molecular action of nutrients, without the need for *a priori* knowledge on any mechanisms or physiological end-points.

One nutrigenomics strategy is the traditional hypothesis-driven approach: identification of dietary target genes, by the means of genomics tools, and subsequently identification of their regulatory pathways which influence homeostasis. Another proposed strategy has been the systems biology approach to identify molecular biomarkers of early changes in whole-body homeostatic control (137). Homeostatic mechanisms in organisms are characterized by hierarchical orders and multiple redundancies to maintain a given steady state for as long as possible. Any disturbance in the organism is compensated for in space and time, and even the malfunction of a gene, a protein or even a whole pathway might be overcome by the system's defense, without evident phenotypic alterations (145).

From a nutrigenomics point of view, nutrients act as dietary signals, detected by the cellular sensor, influencing gene and protein expression, and subsequently, metabolite production (137). Nutrigenomics aims to identify genes that influence the risk of diet-related, complex diseases on a genome-wide scale. It is of high

importance to highlight that nutrigenomics examine the whole complexity and variability of nutrition. The molecular structure of each nutrient determines the specific signaling pathways that it will affect. Even a small structural change (e.g. SFA vs MUFA or cholesterol vs plant sterols) have a profound influence on the activation of the subsequent signaling pathway (134). Transcription factors are the main agents through which nutrients influence gene expression. A well studied example is the nuclear receptor super-family of transcription factors, containing 48 members in the human genome. Nuclear receptors include peroxisome proliferation activator receptor-α (PPARα) which binds to fatty acids, liver X receptor-α (LXRα) which binds cholesterol metabolites, or retinoid X receptor (RXR) which binds to specific response elements (specific nucleotide sequences) in the promoter regions of a large number of genes.

Since the potential benefits of harnessing the power of genomics for dietary prevention of disease are enormous, nutrigenomics approach is considered the future of nutritional research (145;146). Although in its infancy, and with relatively few convincing studies in the area, high expectations are already being placed on this promising multidisciplinary field of nutrigenomics.

3.4 Nutrigenomic Tools

The outcome of nutrigenomics studies should be always analyzed in parallel with mechanistic, clinical and/or epidemiological data available for the compound under study. The use of bioinformatics tools to link information between the genome, transcriptome, proteome and metabolome is a major challenge. Bioinformatics tools are necessary for the interpretation of observed expression changes of unsuspected genes with unknown function related to the nutrient of interest (147). Subtle changes in gene expression, even at the single-cell level can be measured by quantitative techniques such as high-density microarrays and real-time PCR (148).

3.4.1 Microarrays

A microarray is a tool used to sift through and analyze the information contained within a genome. Microarrays are high-density arrays and which have been designed and used for quantitative and highly parallel measurements of gene expression (149). Microarrays consist of different nucleic acid probes that are chemically attached (hybridized) to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead. Briefly, the principles of microarray hybridization method are a) deposition or synthesis of no-labeled biomolecules (cold probes or features) as spots, b) hybridization/specific recognition with samples labeled with fluorescence or radioactivity, c) washing of product which is not specifically bound and d) quantitative detection of the bound product. Gene expression microarrays are a sensitive and well

validated nutrigenomics tool. They have become standard tools for gene expression profile and they are used to assess changes in the transcriptome (mRNA levels of a large number of genes) in a single array. Microarray platforms can assess the effect of a specific diet or nutrient on the expression of a large proportion of the whole genome.

A great advantage of microarray technology, in comparison to conventional methods, like Northern plots or reverse transcription polymerase chain reaction (RT-PCR), is that a microarray chip experiment enables large numbers of genes to be screened simultaneously, giving a comprehensive, detailed picture of gene expression changes, and shedding light on complex regulatory interactions like diet-gene interactions. A single microarray can provide information on the expression of tens of thousands of genes. Although, microarrays platforms are a major technological advance, the scale and complexity of the generated data require careful management to warrant correct elaboration and useful results (150). Microarrays have also limitations when applied in nutrition research. The data generated by such experiment is enormous and one must be able to extract from it biological information about the system under study. Since the expected gene expression changes from dietary intervention are subtle and not easy to detect, great care should always be taken in designing and executing microarray studies. A robust research hypothesis is required to ensure that the experimental design (microarray) is appropriate for the question addressed (151).

One of the key solutions is having sufficient biological replicates that could capture and calculate the variances. Normalization and appropriate statistical analysis are also very important steps to assess the differential gene expression profile of the tissue under study. Moreover, all parts of the protocol (i.e. array production, RNA extraction, cDNA labeling and hybridization and data analysis techniques) need to be optimized to reach stable experimental results (151). Commercial arrays come with manufacturers' protocols whose use helps to achieve a degree of standardization. The Microarray Gene Expression Data Society (www.mged.org) has created the Minimum Information About a Microarray Experiment (MIAME) project which gives concrete guidelines about the minimum necessary information. This information ensures that microarray data can be easily interpreted and that results can be independently verified (152;153). Moreover, all microarray data produced worldwide is necessary to be publicly available in on of the main public repositories, under a specific and unique accession number (i.e. Array Express: www.ebi.ac.uk/microarray/ArrayExpress; and National Center for Biotechnology Information Gene Expression Omnibus:www.ncbi.nlm.nih.gov/geo). It becomes clear that this kind of information makes possible the meaningful comparison and integration of data generated in different laboratories, on different platforms, and more importantly avoids errors or undetected misunderstanding.

3.4.2 Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction

The validation of microarray results by means of a Polymerase Chain Reaction (PCR) experiment is a mandatory step when assessing nutrigenomic changes. Real-time PCR is a broadly used tool applied in parallel with microarrays analysis in nutrigenomic studies. Real-time PCR approach follows the general principle of PCR but its key feature is that the amplified product is detected as the reaction progresses, in real time, (i.e. measured at each PCR cycle), whereas in the traditional PCR, the product of the reaction is detected at the end. Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes, consisting of oligonucleotides, that are labeled with a fluorescent reporter and permits detection only after hybridization of the probe with its complementary DNA target.

This latter development of PCR technologies is the basis of reverse transcription quantitative PCR (qRT-PCR) which can determine the presence and abundance of a particular sequence in the samples of interest. The real-time, fluorescence-based, reverse transcription (RT-PCR) is one of the enabling technologies of the genomic age and has become the method of choice for the detection of mRNA (154). This technology can be used in determining how the expression of a particular gene changes over time.

Reverse transcription followed by quantitative PCR (qRT-PCR) is an extremely sensitive, cost-effective method for quantifying gene transcripts from cells. This combines the nucleic acid amplification and detection steps into one homogeneous assay and obviates the need for gel electrophoresis to detect amplification products. Its simplicity, specificity and sensitivity, together with its potential for high throughput analysis have made real-time RT-PCR the benchmark technology for the detection and/or comparison of RNA levels (155). Rules of qRT-PCR have been recently reviewed to ensure reproducible and accurate measurements of transcript abundance in plant and other cells (156).

The generated data can be analyzed by computer software to calculate relative gene expression in several samples. To accurately quantify gene expression, the measured amount of RNA from the gene of interest is divided by the amount of RNA from a housekeeping gene, measured in the same sample, to normalize for possible variation in the amount and quality of RNA between different samples. This normalization permits accurate comparison of the expression of the gene of interest, between different samples, provided that the expression of the used reference (housekeeping) gene is very similar across all the samples. The principle of quantification is straightforward: the more copies of target there are at the beginning of the assay, the fewer cycles of amplification are required to generate the number of amplicons that can be detected reliably (154).

Although Northern blotting is still used to assess gene expression, it requires relatively large amounts of RNA and provides only qualitative or semi-quantitative information of mRNA levels. Consecutively, real time qRT-PCR is the proper tool that can be used in nutrigenomic studies where the objective is to assess the whole genome gene expression after a specific dietary pattern or component consumption.

TagMan® low density arrays (TLDA) are a recent sophisticated application of qRT-PCR which enables the performance of 384 simultaneous real-time PCR reactions without the need for liquid-handling robots or multi-channel pipettors to load samples (Figure 5).

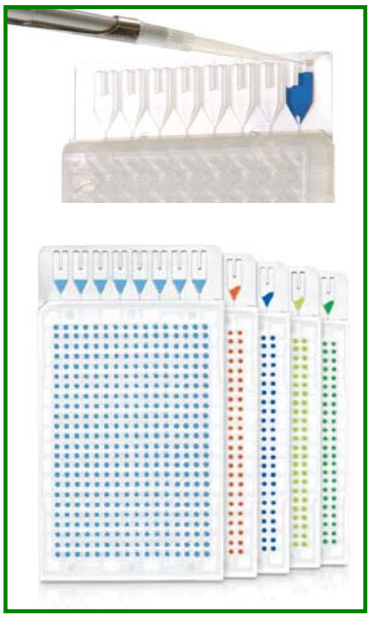

Figure 4. TagMan® low density arrays (TLDA)

TLDAs are the right tool for validating the tens or hundreds of hits that come from microarrays (high density arrays) because they can be customized to include up to 384 of those hits in one easy-to-use array. Up to date, TLDAs have been successfully used to study differential gene expression in human cancer cells, human macrophages (157;158) and other cell models. Custom TLDAs, manufactured by Applied Biosystems (AB, Applied Biosystems, Foster City, CA), have been also used in the present work, in

collaboration with microarrays to evaluate the nutrigenomic effects of the Mediterranean diet and virgin olive oil consumption in healthy humans.

3.4.3 Analyses and Interpretation of the nutrigenomic results

The high-throughput genomic, proteomic, and bioinformatic scanning technologies usually result in a large "interesting" gene list (ranging in size from hundreds to thousands of genes) involved in the biological conditions studied. The analysis of large gene lists is indeed more an exploratory, computational procedure rather than a purely statistical solution. Data analyses of the large gene lists and their variety of biological mechanisms is an important downstream task following high-throughput technologies. It is used to understand the biological meaning of the output gene-list. Special bioinformatic software packages are required for the challenging analysis of these outputs.

One of those packages, specifically used in the present work, is the DAVID Bioinformatics Database (159;160). It is a useful tool to generate a gene-to-gene similarity matrix and to rank overall importance (enrichment) of annotation term groups, including Gene Ontology (GO) terms (161), protein–protein interactions, disease associations, bio-pathways, gene functional summaries, literature etc. Another package is the PANTHER™ Protein Classification System analysis (162), a comprehensive database for classifying protein sequences and making family clustering.

The Gene Ontology Consortium (GO) (161) produces a dynamic, controlled vocabulary that is applied to all eukaryotes. To this end, three independent ontologies accessible on the World-Wide Web (http://www.geneontology.org) have been constructed: biological process, molecular function, and cellular component. GO contains more than 7000 terms to describe molecular function, and almost 5000 terms to describe biological process. KEGG (Kyoto Encyclopedia of Genes and Genomes) is a knowledge base for the systematic analysis of gene funtions (163) working in parallel with GO. KEGG databases are daily updated and freely available (htpp://www.genome.ad.jp/keg/). They enable graphic tools for browsing genome maps, the comparison and manipulation of expression maps, and computational tools.

Grouping genes based on functional similarity can help to enhance the biological interpretation of large lists of genes derived from high throughput studies. It has been shown that disease-related genes tend to interact (164;165) and display significant functional clustering in the analyzed molecular network. Genes associated with similar disorders show both a higher likelihood of physical interactions between their products and a higher expression profiling similarity for their transcripts, supporting the existence of distinct disease-specific functional modules. However, it has been found (164) that the vast majority of disease genes are non-essential and their expression pattern indicates that they are localized in the functional periphery of the network.

4. Olive oil and Mediterranean diet in nutrigenomics studies concerning CVD risk prevention

In the last years, nutrigenomic studies have focused on investigating the molecular mechanisms of action of several foods and nutrients, particularly lipids, on cardiovascular risk factors and other complex traits such as metabolic syndrome, obesity, T2DM and cancer. Dietary fatty acids interact with multiple nutrient-sensitive transcription factors leading to altered dietary fatty acid composition, and explaining the basis of some associated health effects (166).

Regulation of the expression of genes involved in fatty acid metabolism occurs when a dietary fat or metabolite binds to, and activates specific fatty acid transcription factors. The main mechanism described, by which dietary lipids may act stimulating the initiation of human malignancies, is lipid peroxidation and the subsequent oxidative DNA damage. Here, we will focus on the CVD-risk factors and the nutrigenomic effects of Mediterranean diet and olive oil consumption on them.

Intervention studies, in which subjects receive a controlled dietary intake, provide the best approach for conducting gene-nutrient-phenotype association studies. However, the small number of participants, the brief duration of the interventions, and the lack of replication are the main limitations of these studies, conducted up to

now (20). The lack of replication is most likely due to the different characteristics of study subjects (i.e. ethnicity, physical condition, age, life-style differences) and the heterogeneity in the study design.

The gene expression response in human peripheral blood mononuclear cells (PBMNC) after breakfasts rich in butter, walnuts, or olive oil has been compared (72;167). Butter breakfast elicited a higher increase in tumor necrosis factor *TNFα* mRNA expression than olive oil or walnuts in 20 healthy men (167). Also, the increase in the pro-inflammatory *IL6* mRNA expression was greater after butter breakfast than after walnut one (167). Butter- and walnuts-enriched meals, but not olive oil ones, have also elicited a *NFκB* postprandial activation in PBMNC of healthy volunteers (72). These authors concluded that consumption of an olive oil-enriched meal does not activate *NFκB* in monocytes as do butter and walnut-enriched meals and this effect could enhance the cardioprotective effect of olive oil-enriched diets.

A recent randomized, parallel, double-blind intervention tested the differences between diets with conjugated linoleic acids versus olive oil (168). Gene expression changes were assessed in adipose tissue from healthy postmenopausal women. Adipose tissue macrophages are the source of inflammatory pathways and obesity, a well know CVD risk factor, is associated with the progressive infiltration of monocytes and macrophages into adipose tissue (169). In the former study, the mRNA expression of glucose transporter4 (*GLUT4*), leptin, and lipoprotein lipase (*LPL*) was

lower and *TNFα* was higher in the linoleic acid-group versus the olive oil-control group.

A specific effect of MUFA-rich diet versus SFA-rich diets has been also assessed (170). In this parallel, controlled-feeding trial, participated 20 abdominally overweight subjects during 8 weeks. A MUFA diet led to a more anti-inflammatory gene expression profile whereas an SFA diet resulted in a proinflammatory "obesity-linked" profile. Moreover, these results were accompanied by a decrease in serum LDL-C and an increase in plasma and adipose tissue oleic acid content. These results pointed out to a prevention of adipose tissue inflammation when dietary SFA are replaced by MUFA.

Data from the PREDIMED study have shown that a 3-month intervention with VOO-enriched traditional Mediterranean diet (TMD) prevented the increase in cyclooxigenase-2 (*COX-2*) and low density lipoprotein receptor-related protein (*LRP1*) genes, and reduced the expression of monocyte chemoattractant protein (*MCP-1*) gene, compared with a TMD enriched with nuts or with a low-fat diet (171). In this study participated 49 asymptomatic high cardiovascular-risk patients and gene expression changes were assessed in PBMNCs. *COX-2* and *MCP-1* genes are involved in inflammation, whereas *LRP1* is involved in foam cell formation. In this work (171) a decrease in the systolic blood pressure, plasma glucose, total cholesterol and LDL-C was reported. These results support the idea that the benefits associated with olive oil and TMD consumption on cardiovascular-risk patients could be mediated

through changes in the expression of atherosclerosis-related genes. Up to date, however, no data exist concerning the *in vivo* nutrigenomic effects of olive oil phenolic compounds in humans.

The information of genes involved in oxidation processes is extensively broad. Nevertheless the available information of candidate genes involved in atherosclerotic processes in particular, and associated to oxidative stress and cardiovascular disease in general, which can be modulated through dietary patterns, is scarce. Changes in the gene expression associated to dietary patterns, specific foods or particular components of this food could be useful to indentify mechanisms by which nutrients can elicit harmful or beneficial effects in health terms. Also new mechanisms for future therapies can be targeted through nutigenomic information. The field is relatively new, and the body of knowledge is, at the present, building up. Moreover, gene expression must be linked with proteomic studies and furthermore with the functionality of the proteins involved.

HYPOTHESIS and OBJECTIVE

III. HYPOTHESIS

The hypothesis driven in the present work is that the traditional Mediterranean diet, the virgin olive oil, and its phenolic compounds can modify the *in vivo* gene expression in human peripheral blood mononuclear cells towards a protective mode for cardiovascular disease development.

IV. OBJECTIVE

The objective of the present study was to assess the *in vivo* gene expression changes, protective for cardiovascular disease, associated to the consumption of: traditional Mediterranean diet, virgin olive oil, and its phenolic compounds, in healthy volunteers.

METHODS

V. METHODS

The methodology followed in the present thesis project is shown in the flowchart of the Figure 6. Two tasks were performed in order to achieve the objective of this project: i) Task 1, Pilot study-Virgin olive oil (VOO) intervention study and ii) Task 2, traditional Mediterranean diet (TMD) intervention study.

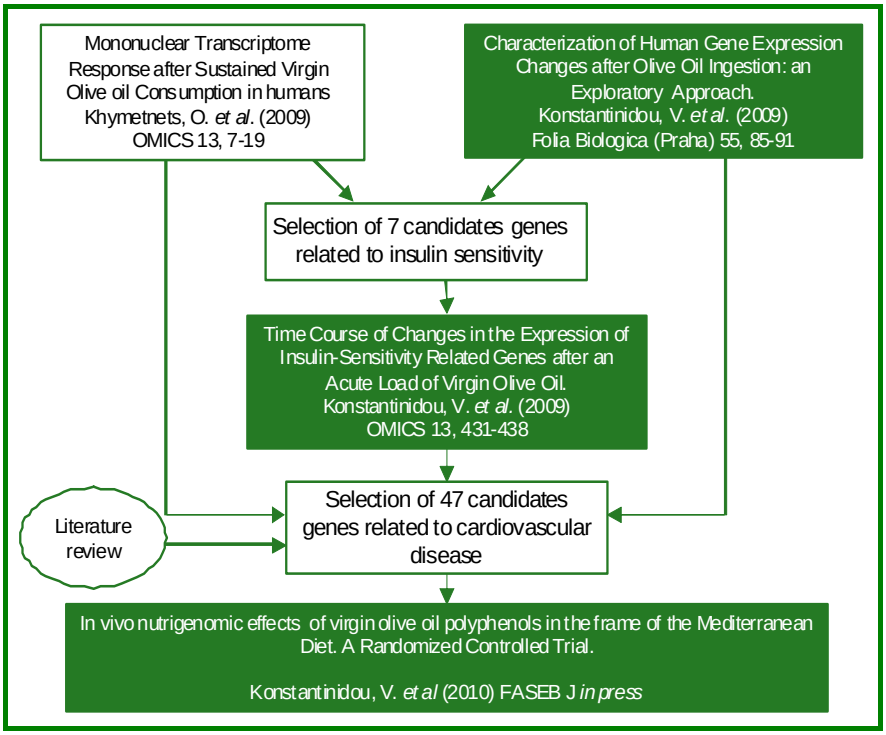

Figure 5. Flowchart of the thesis project
(Publications involved are colored in green)

Task 1: Pilot study-Virgin olive oil (VOO) intervention study

A linear intervention study was performed (Figure 4). Volunteers (n=11), aged 22 to 28, were recruited and considered healthy on the basis of physical examination and routine biochemical and haematological laboratory determinations. The ethical committee (CEIC-IMAS 2002/1512/I) approved the protocol and participants signed an informed consent. Prior to intervention, volunteers followed a one-week washout period, in which sunflower oil was provided as the only source of fat for all purposes, and participants followed an antioxidant-controlled diet. During the last three days of the washout period and on the first day of the intervention, volunteers followed a strict low-phenolic compound diet. A nutritionist gave instructions on excluding several foods rich in phenolic compounds from their diet (vegetables, legumes, fruit, juice, wine, coffee, tea, caffeine-containing soft drinks, beer, cacao, marmalade, olive oil, and olives). Meals were served at the Centre during the intervention day. The olive oil used was a VOO, variety Hojiblanca from Andalucía, Spain.

A single dose of 50 ml of VOO, rich in phenolic compounds (316mg/kg), was administrated at fasting state at 8 a.m on the intervention day. During the first six post-intervention hours, subjects abstained from food and drinks with the exception of caffeine-free, low-energy drinks and water. Blood was collected at

baseline (0 hours, pre-intervention), at 1 hour post-intervention and at 6h post-intervention.

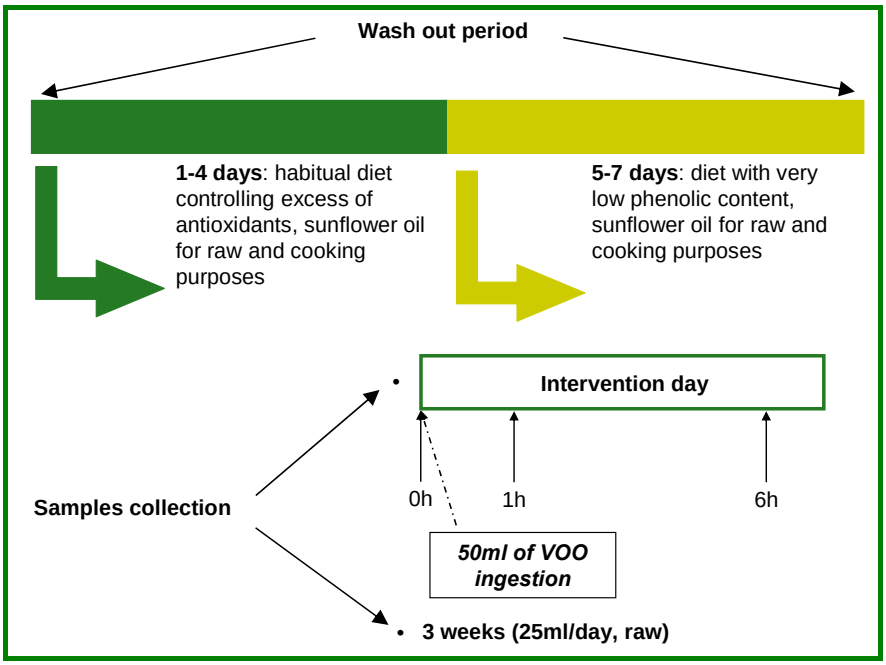

Figure 6. Virgin olive oil intervention study

Peripheral blood mononuclear cells (PBMNCs) were isolated from peripheral blood using the cell preparation tubes (CPT™). Publications No 1 and No 2 of the present dissertation describe in detail all the methods and material used for the analytical experiments. The volunteers continued the daily consumption of 25 mL of the same VOO during 3 weeks. At the end of this period and at fasting state, blood samples were also collected. Total RNA was extracted for PBMNCs and Microarray experiments were performed (Publication No 1). Candidate genes were selected and verified by qRT-PCR. Plasma glucose, lipid profile, insulin, lipid oxidative damage, tyrosol and hydroxytyrosol were measured in all volunteers participated.

Task 2: Traditional Mediterranean Diet intervention study.

A randomized, parallel, controlled, double blind trial with three dietary interventions was performed in order to assess the nutrigenomic effect of the Mediterranean diet and that of the VOO consumption, versus that of ordinary olive oil, in the context of the Mediterranean diet. Volunteers (n =90), aged 20 to 50 years, were recruited. The institutional ethics committee approved the protocol (CEIC-IMAS 2004/1827/I) and the volunteers gave written informed consent before the initiation of the study. This trial has been registered in Current Controlled Trials, London, with the International Standard Randomized Controlled Trial Number (ISRCTN53283428).

Volunteers were considered healthy on the basis of a physical examination and routine biochemical and haematological laboratory determinations. Whole blood and urine samples were collected at 0h (baseline) and after 3 months of intervention. The necessary olive oil was provided to all subjects in a sufficient amount, for the entire family (15 litters per 3 months), and during the whole intervention period, for cooking and row use.

Volunteers were grouped randomly into three groups:
a) Group A (n= 30); Traditional Mediterranean diet with virgin olive oil high in phenolic content (328mg/kg) (TMD+VOO).

b) Group B (n= 30); Traditional Mediterranean diet with olive oil with very low phenolic content (55mg/kg) (TMD+WOO). Washed virgin olive oil (WOO) used in intervention group B was obtained from the virgin olive oil (VOO) used in intervention group A in the Instituto de la Grasa, Sevilla, Spain. The olive oils were similar with the only difference in the amount of phenolic content inWOO which was much lower (55 mg/kg) than that of VOO.
c) Group C; Habitual diet without any recommendation (n= 30).

On the basis of the assessment of an individual 14-points Mediterranean diet score (19), the dietician gave personalized advice during a 30-minute session to each participant in the intervention groups, with recommendations on the desired frequency of intake of specific foods. Instructions were directed at up scaling the TMD score, including i) the use of olive oil for cooking and dressing, ii) the increased consumption of fruit, vegetables, and fish, iii) the consumption of white meat instead of red or processed meat, iv) the preparation of homemade sauce with tomato, garlic, onion, aromatic herbs, and olive oil to dress vegetables, pasta, rice, and other dishes, and v) for alcohol drinkers, moderate consumption of red wine. At the end of the intervention (3 months) all baseline procedures were repeated. Publication No 3 of the present work describes in details all the material and methods used for the analytical experiments of this intervention trial.

Changes in the expression of several cardiovascular disease-related genes were assessed. Plasma glucose, lipid profile, oxidative

damage, tyrosol and hydroxytyrosol, and inflammation markers were also measured in all volunteers participated.

RESULTS

VI. RESULTS

The results of the first task are referred to in Publications No 1 and No 2 of the present dissertation.

Publication No 1

Briefly, in this article, the postprandial gene expression changes, in PBMNCs of healthy individuals, were assessed after an acute 50 mL ingestion of VOO. At baseline (0 h) and at post-ingestion (6 h), total RNA was isolated and gene expression (29,082 genes) was evaluated by microarray. Microarray experiment (GSE19590 Accession Number of GEO Database) was performed and 259 up-regulated and 246 down-regulated genes were indentified. Subjects' baseline data had served as a within-subject control. From microarray data, nutrient-gene interactions were observed in genes related to metabolism, cellular processes, cancer, and atherosclerosis (e.g. ubiquitin-specific protease 48; *USP48*, O-UDP-N-acetylglucosamine*; OGT*) and associated processes such as inflammation (e.g. a kinase anchor protein 13; *AKAP13,* interleukin 10; *IL10*) and DNA damage (e.g. DNA cross-link repair 1C; *DCLRE1C,* polymerase-DNA directed-κ; *POLK*). When results obtained by microarray were verified by qRT-PCR in nine selected genes, full concordance was achieved only in the case of the up-regulated ones (e.g. a disintegrin and metalloproteinase domain 17; *ADAM17, IL10; OGT*; *USP48*; and *AKAP13*).

Characterization of Human Gene Expression Changes alter Olive Oil Ingestion: an Exploratory Approach. V. Konstantinidou, O. Khymenets, M. Fito, R. De La Torre, R. Anglada, A. Dopazo and M. I. Covas. Folia Biol. (Praha) 2009; 55, 77-83.

Original Article

Characterization of Human Gene Expression Changes after Olive Oil Ingestion: an Exploratory Approach

(olive oil / gene expression / microarray / atherosclerosis / cancer)

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Abstract. Olive oil consumption is protective against risk factors for cardiovascular and cancer diseases. A nutrigenomic approach was performed to assess whether changes in gene expression could occur in human peripheral blood mononuclear cells after olive oil ingestion at postprandial state. Six healthy male volunteers ingested, at fasting state, 50 ml of olive oil. Prior to intervention a 1-week washout period with a controlled diet and sunflower oil as the only source of fat was followed. During the 3 days before and on the intervention day, a very low-phenolic compound diet was followed. At baseline (0 h) and at post-ingestion (6 h), total RNA was isolated and gene expression (29,082 genes) was evaluated by microarray. From microarray data, nutrient-gene interactions were observed in genes related to metabolism, cellular processes, cancer, and atherosclerosis (e.g. *USP48* by 2.16; *OGT* by 1.68-fold change) and associated processes such as inflammation (e.g. *AKAP13* by 2.30; *IL–10* by 1.66-fold change) and DNA damage (e.g. *DCLRE1C* by 1.47; *POLK* by 1.44-fold change). When results obtained by microarray were verified by qRT-PCR in nine genes, full concordance was achieved only in the case of up-regulated genes. Changes were observed at a real-life dose of olive oil, as it is daily consumed in some Mediterranean areas. Our results support the hypothesis that postprandial protective changes related to olive oil consumption could be mediated through gene expression changes.

Introduction

There is growing evidence that the Mediterranean diet, in which olive oil is the main source of fat, has a beneficial effect on diseases associated with oxidative damage such as cardiovascular (CVD), cancer, or neurodegenerative diseases, and also on ageing (Covas et al., 2007). Oxidation of low-density lipoproteins (LDL) is a hallmark for atherosclerosis and CVD development (Witztum, 1994), and oxidative DNA damage has been shown to be predictive for cancer development (Poulsen, 2005). In human intervention studies, sustained olive oil consumption has been shown to be able to reduce the *in vivo* lipid and DNA oxidative damage, as well as the inflammatory status (Covas et al., 2007; Fitó et al., 2007).

Nutrients can regulate gene expression at various stages, including transcription, mRNA processing and stability, and trans- and post-translational modifications. In experimental studies, olive oil has been shown to be able to influence: stages of carcinogenesis, cell membrane composition, signal transduction pathways, transcription factors, and tumour suppressor genes (Menendez et al., 2006). In some previous studies the ingestion

Received July 28, 2008. Accepted March 21, 2009.

This study was supported by the SNS contract (CP06/00100) and by the FIS (453304085) from Instituto de Salud Carlos III (ISCIII), Madrid, Spain. CIBER Fisiopatologia de la Obesidad y Nutricion is an initiative of ISCII.

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Abbreviations: BMI – body mass index, GADPH – glyceraldehyde-3-phosphate dehydrogenase, HDL – high-density lipoprotein, IL – interleukin, LDL – low-density lipoprotein, PBMNC – peripheral blood mononuclear cells.

of a 25 ml dose of virgin olive oil did not promote postprandial oxidative stress (Weinbrenner et al., 2004), whereas doses greater than or equal to 40 ml did (Fitó et al., 2007). However, virgin olive oil, rich in phenolic compounds, reduced postprandial oxidative damage to lipids, endothelial dysfunction, and the pro-thrombotic profile both in healthy and hypercholesterolaemic individuals (Covas et al., 2007).

A lack of data exists on the *in vivo* effect of diet on human gene expression. The aim of this work was to explore the changes in gene expression after olive oil ingestion (50 ml) at the end of the postprandial time, particularly the changes related to atherosclerosis and cancer processes, in peripheral blood mononuclear cells (PBMNC) of healthy individuals.

Material and Methods

Subjects, study design and sample collection

Six healthy male volunteers, aged 22 to 28, were recruited. The ethical committee (CEIC-IMAS) approved the protocol and participants signed an informed consent. All volunteers were healthy on the basis of a physical examination and standard biochemical and haematological tests. Subjects had an average weight of 74.1 ± 11.7 kg, and a body mass index (BMI) of 24.5 ± 3.55 kg/m^2.

Prior to intervention, volunteers followed a one-week washout period in which sunflower oil was provided as the only source of fat for all purposes and participants followed an antioxidant-controlled diet. During the last three days of the washout and on the intervention day, volunteers followed a strict low-phenolic compound diet. A nutritionist gave instructions on excluding several foods rich in phenolic compounds from their diet (vegetables, legumes, fruit, juice, wine, coffee, tea, caffeine-containing soft drinks, beer, cacao, marmalade, olive oil, and olives). Meals were served at the Centre during the intervention day. At 8 a.m., at fasting state, 50 ml (44 g) of olive oil were administered to the volunteers in a single dose. During the first six post-intervention hours, subjects abstained from food and drinks with the exception of caffeine-free, low-energy drinks and water. PBMNC were isolated from peripheral blood collected in cell preparation tubes (CPT™ tubes, Beckton Dickinson, Franklin Lakes, NJ) at baseline (0 h, pre-intervention) and at 6 h post-intervention. Whole blood was centrifuged at 1690 *g* for 30 min, and cells were washed with buffer phosphate (AMBION, Foster City, CA), centrifuged at 970 *g* for 15 min, re-suspended in Ultraspec® (Biotecx Laboratories, Houston, TX), and stored at -80 ºC until RNA isolation.

Olive oil characteristics

The olive oil used was virgin olive oil, Hojiblanca variety from Andalucía, Spain. Its fatty acid composition was: 1) monounsaturated fatty acids: 75 %; 2) polyunsaturated fatty acids: 18.6 %; and 3) saturated fatty acids: 6.4 %. Minor components were: α-tocopherol (1.47 mg/kg); β-carotene tocopherol (0.43 mg/kg), sterols (15.6 mg/kg); and phenolic compounds (316 mg/kg). The olive oil was stored in the dark, avoiding exposure to air, light and high room temperature in order to be protected against oxidative stress damage.

RNA extraction and microarray sample preparation

Total RNA was extracted from PBMNC by the Ultraspec® RNA isolation procedure (Khymenets et al., 2005). RNA concentration and purity were measured by a NanoDrop spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, DE). Total RNA integrity was evaluated by an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Samples at 0 h and 6 h were pooled and concentrated using the RNeasy Mini Elute Cleanup system (Qiagen, Barcelona, Spain); checked for total RNA quantity and quality; and concentration adjusted to meet ABI Microarray criteria. Samples were stored in aliquots at -80 ºC prior to use. All reagents, plastic ware, and supplies used were sterile, nuclease free, and of molecular biology grade.

Microarray analysis and q-PCR verification

Gene expression profiles were generated using the Human Genome Survey Microarray v2.0 (Applied Biosystems, Foster City, CA). Each microarray contains 32,878 60-mer oligonucleotide probes representing 29,098 individual human genes. Samples were processed in triplicate. Microarray hybridization, processing, chemiluminescence detection, imaging, auto gridding, and image analysis were performed according to Applied Biosystems protocols and using the 1700 Chemiluminescent Microarray Analyzer Software v.1.0.3. Quantile normalization was applied for inter-array normalization (Bolstad et al., 2003). Genes were excluded when their expression levels were below the detection threshold (signal to noise values < 3 and/or flags > 5,000). The resulting 15,308 genes from the filtering (from the initial 32,878 probe set) were then subjected to further gene selection and typified using PANTHER™ Protein Classification System analysis (Thomas et al., 2003). The microarray dataset is available under GSE 19590 Accession Number of GEO Database.

The identification of genes that were regulated by olive oil ingestion was done by comparing gene expressions in PBMNC at pre-intervention (0 h) with those at post-intervention (6 h). The cut off to consider a gene differentially expressed, on the basis of the pre- and post-intervention variability, was set at a signal $\log_2$ ratio higher than 0.5 (up-regulation, fold-change > 1.41), or lower than -0.5 (down-regulation, fold change < 1.41).

Real-time RT-PCR

The reverse transcription reaction was performed using a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, CA). The expression of nine genes (five up-regulated

and four down-regulated), with an expression range from low to high, was verified by quantitative TaqMan Real-Time PCR (TaqMan® Low Density Array by Design and ABI Prism 7900HT Sequence Detection System, Perkin-Elmer, Applied Biosystems). Human glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) was used as a housekeeping gene. Data obtained were analysed by the SDS 2.1 software.

Results

The general characteristics of the healthy volunteers at baseline are shown in Table 1. Total RNA obtained was of high quality and purity (A_{260}/A_{280} and $A_{260}/A_{230} \geq 1.8$; and RNA integrity number in the range from 8.5 to 9.5). The mean coefficient of variation of the log-signal probe values was lower than 0.1 for the triplicates.

Differential gene expression in PBMNC

From the 15,308 high-quality probes selected, 259 known genes were up-regulated and 246 down-regulated in human PBMNC after 50 ml of olive oil ingestion. The differentially expressed genes belonged to a wide range of gene ontology biological processes including metabolism, signal transduction and signalling, cancer, metabolic disorders, and cellular processes (Fig. 1). The highest up-regulation, from 1.0 to 1.29 units of $\log_2$ ratio, corresponding to a fold-change from 2 to 2.44, was observed in genes related to: 1) cancer, such as the A-kinase anchoring protein 13 (*AKAP13*) and *IKAROS* (*ZNFSF1A*); and 2) cellular processes, such as *CDC14* and ubiquitin protease *USP48*. The highest down-regulation, from -1.79 to -1.02, corresponding to a fold-change from -3.48 to -2.03, respectively, was observed in genes related to: 1) DNA damage, such as the DNA-damage-inducible transcript 4 (*DDIT4*) or the DNA-repair protein *XRCC4*; and 2) carcinogenesis, such as the cyclin-dependent kinase inhibitor 2B (*CDKN2B*), or the v-*akt* murine thymoma viral oncogene (*AKT3*). Due to the fact that phenotypic changes in markers related to atherosclerosis and DNA oxidative damage occur after olive consumption (Poulsen et al., 2005; Covas et al., 2007), genes differentially expressed related to these processes were identified using public databases (Tables 2 and 3). The verification of the microarray gene expression in a set of nine genes by quantitative real-time qRT-PCR showed that, in general, only the features of the gene expression changes for the up-regulated genes were similar (Fig. 2). NIH-DAVID software (version 2.1b) (Dennis et al., 2003) was used to search for Gene Ontology terms (Ashburner et al., 2000) and KEGG pathways (Kanehisa et al., 2000).

Table 1. General characteristics of volunteers at baseline

	Volunteers (N = 6)
Age (years)	24.8 (2.3)
BMI (kg/m^2)	24.5 (3.5)
Glucose (mmol/l)	4.93 (0.3)
Total cholesterol (mmol/l)	4.24 (0.45)
LDL cholesterol (mmol/l)	2.39 (0.64)
HDL cholesterol (mmol/l)	1.48 (0.42)
Triglycerides (mmol/l)	0.66 (0.35–1.20)

Values are presented as mean (SD) with the exception of triglycerides, which are presented as median (25^{th}–75^{th} percentile).

Discussion

The aim of this work was to assess the gene expression changes in PBMNC of healthy volunteers at the end of postprandial time (Axelsen et al., 1999), after 6 h of fat ingestion, virgin olive oil (50 ml). To our knowledge this is the first exploratory report assessing the human *in vivo* gene expression changes after food ingestion. From microarray data, the highest up-regulation was observed in genes related to metabolism, cellular processes, and cancer. The highest down-regulation was observed in genes related to environmental information processing.

Epidemiological studies suggest a protective effect of olive oil consumption on cardiovascular disease and certain types of cancer (Trichopoulou et al., 2000; Covas et al., 2007). After consumption of olive oil a decrease in the urinary concentration of 8-oxo-deoxygyuanosine, considered being a systemic marker of DNA oxidation, has been reported (Fitó et al., 2007). We observed an increase in DNA-repair genes: DNA cross-link repair 1C (*DCLRE1C*) (also known as *ARTEMIS*) and DNA polymerase κ (*POLK*), which were up-regulated at 6 h post-intervention. A recent study provides evidence for a possible protective role for POLK in mammalian nucleotide excision repair (Ogi et al., 2006).

Consumption of olive oil has been reported to increase plasma high-density lipoprotein (HDL) cholesterol levels (Covas et al., 2007; Fitó et al., 2007). In agreement with this, an increase in the *ABCA7* [ATP-binding cassette, sub-family A (ABC1), member 7] gene expression was observed after olive oil ingestion. ABCA7, together with ABCA1, mediates the apolipoprotein-dependent formation of the HDL (Takahashi et al., 2005). Besides increasing the HDL cholesterol, the ingestion of a virgin olive oil-based breakfast has shown to decrease the postprandial glucose and insulin concentrations, and to increase glucagon-like peptide-1 concentrations as compared with a carbohydrate-rich diet (Paniagua et al. 2007). The chain length of the fatty acid is considered to be a key factor for glucagon-like peptide-1 secretion, long chain monounsaturated fatty acids being the most effective ones stimulating Langerghans cells *in vitro* (Rocca et al., 2001). In agreement with this, we observed an up-regulation of some insulin-related genes such as a disintegrin and metalloproteinase domain 17 (*ADAM17*) (Togashi, 2002) and *OGT* (Whelan, 2008) after olive oil ingestion.

Olive oil consumption has also been reported to reduce inflammatory markers (Fitó et al., 2007). We observed an up-regulation of the interleukin 10 (*IL-10*) gene at 6 h after olive oil ingestion. IL-10 is an anti-in-

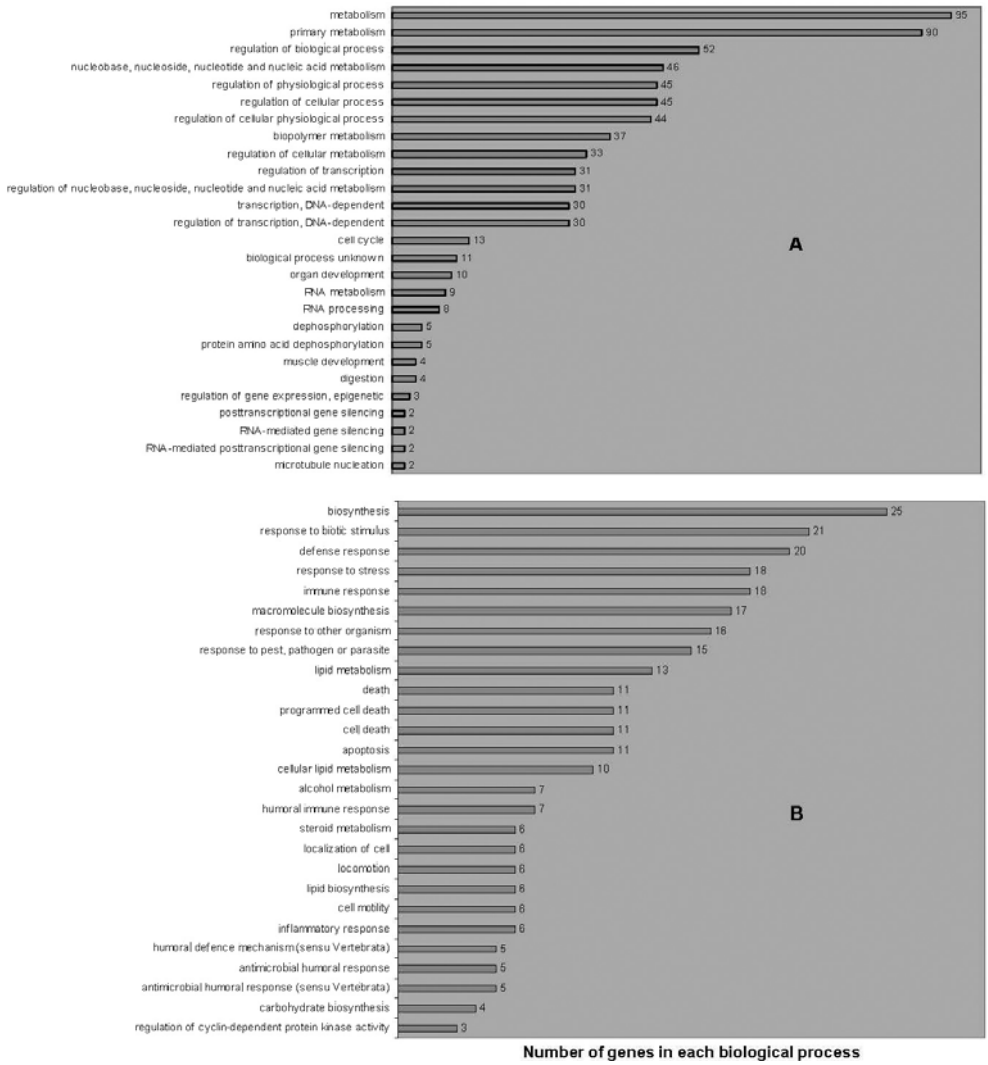

Fig. 1. Functional annotation of differentially expressed genes after olive oil ingestion (50 ml). A: up-regulated genes; B: down-regulated genes.

flammatory cytokine which inhibits production of interleukin 6 (Tedqui et al., 2006), considered as the most important inflammatory mediator. A pro-inflammatory cytokine down-regulated after olive oil ingestion was interferon γ (IFN-γ). IFN-γ is a strong pro-inflammatory cytokine that orchestrates several cellular programmes through transcriptional regulation of immunologically relevant genes, and recent studies suggest that reducing IFN-γ synthesis may lead to new therapies for graft arteriosclerosis (Tellides et al., 2007). USP48, a human ubiquitin-specific protease, is a deubiquitinating enzyme implicated in the regulation of NF-κB activation by members of the tumour necrosis factor receptor superfamily (Tzimas et al., 2006). Also AKAP13 (anchoring protein 13) plays a role in NF-κB activation, mediated by Toll-like receptors 2 (Shibolet et al., 2007).

The comparison of data obtained in pooled samples from microarray and qPCR experiments showed that there was some inconsistency between the results obtained using the different methods. The lack of concordance between methods was observed only in down-regulated genes measured by microarrays.

In this study, an approach was performed to assess whether changes in gene expression in human PBMNC could be detected after olive oil ingestion at postprandial state. Although the subjects' baseline data had served as a within-subject control, a limitation of the study is the lack of a control group for the intervention itself, which does not permit us to specify the contribu-

Table 2. Genes related with atherosclerosis and DNA damage processes up-regulated after olive oil ingestion (50 ml)

Gene ID	Gene Symbol	Gene Name	Change in Log_2 ratio	Fold Change
Oxidative stress				
8473	*OGT*	O-UDP-N-acetylglucosamine (polypeptide-N-acetylglucosaminyl transferase)	0.75	1.68
10539	*TXNL2*	thioredoxin-like 2	0.70	1.62
137872	*ADHFE1*	alcohol dehydrogenase, iron-containing, 1	0.70	1.62
4891	*SLC11A2*	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	0.65	1.57
1850	*DUSP8*	dual-specificity phosphatase 8	0.63	1.55
6095	*RORA*	RAR-related orphan receptor A	0.57	1.48
Inflammation				
3586	*IL-10*	interleukin 10	0.73	1.66
6654	*SOS1*	son of sevenless homologue 1 (Drosophila)	0.72	1.65
1286	*COL4A4*	collagen, type IV, α 4	0.68	1.60
6775	*STAT4*	signal transducer and activator of transcription 4	0.65	1.57
DNA repair				
64421	*DCLRE1C*	DNA cross-link repair 1C (PSO2 homologue, *S. cerevisiae*)	0.56	1.47
51426	*POLK*	polymerase (DNA directed) κ	0.53	1.44
Apoptosis				
11016	*ATF7*	activating transcription factor 7	0.97	1.96
389840	*MAP3K15*	FLJ16518	0.70	1.62
22861	*NALP1*	NACHT, leucine-rich repeat and PYD-containing 1	0.59	1.51
54739	*XAF1*	XIAP-associated factor 1	0.55	1.46
Lipid metabolism				
51422	*PRKAG2*	protein kinase, AMP-activated, γ 2 non-catalytic subunit	1.06	2.09
114881	*OSBPL7*	oxysterol binding protein-like 7	0.82	1.76
84129	*ACAD11*	putative acyl-CoA dehydrogenase	0.66	1.58
22848	*AAK1*	AP2-associated kinase 1	0.63	1.55
8398	*PLA2G6*	phospholipase A2, group VI (cytosolic, calcium-independent)	0.60	1.52
22876	*INPP5F*	inositol polyphosphate-5-phosphatase F	0.58	1.49
10347	*ABCA7*	ATP-binding cassette, sub-family A (ABC1), member 7	0.56	1.47
9517	*SPTLC2*	serine palmitoyl transferase, long-chain base subunit 2	0.54	1.46
5243	*ABCB1*	ATP-binding cassette, sub-family B (MDR/TAP), member 1	0.54	1.46
Tissue remodelling				
11214	*AKAP13*	A kinase (PRKA) anchor protein 13	1.20	2.30
84196	*USP48*	ubiquitin-specific protease 48	1.11	2.16
9924	*USP52*	ubiquitin-specific peptidase 52	0.57	1.48
6868	*ADAM17*	a disintegrin and metalloproteinase domain 17 (TNF, α, converting enzyme)	0.51	1.42
9098	*USP6*	ubiquitin-specific protease 6 (Tre-2 oncogene)	0.51	1.42

tion of the fasting state nor the oil's special characteristics on the observed changes. Due to this, the effects observed on gene expression could be secondary, not only to the virgin olive oil ingestion, but also to a time-course effect on a circadian regulated genes (Khymenets, 2008) and to physiological changes following any fat meal intake. Also, we could not distinguish between the effects promoted by the minor components of olive oil and those promoted by the fat content of the olive oil. However, an advantage of the study was the *in vivo* evaluation of the gene expression in PBMNC after a real-life dose of virgin olive oil, as is used to be consumed in some Mediterranean areas (Helsing, 1995). PBMNC were selected to explore changes in gene expression because they are: 1) critically involved in the atherosclerotic plaque formation; 2) easily available from volunteers considering the feasibility of collection plus deontological reasons; and 3) their collection can be directly done from BD Vacutainer® CPT™ tubes, thus ensuring rapid PBMNC isolation and avoiding *ex vivo* gene activation.

In summary, changes in several genes related with oxidative stress-associated diseases, such as cancer and atherosclerosis, occur in human PBMNC of healthy volunteers at 6 h postprandial after 50 ml olive oil ingestion. Changes were observed at a real-life dose of olive oil, as is daily consumed in some Mediterranean areas. Our results point out that the protective effect observed in primary and secondary markers for CVD or cancer, related to virgin olive oil consumption at postprandial state, could be mediated through gene expression changes.

Acknowledgements

Authors thank Dr. Carmen Lopez-Sabater and her team from the Department of Nutrition and Bromatology, University of Barcelona, Spain, for the olive oil composition analyses. We also thank Ms. Esther Menoyo

Table 3. Genes related to atherosclerosis and DNA damage processes down-regulated after olive oil ingestion

Gene ID	Gene Symbol	Gene Name	Change in Log_2 ratio	Fold Change
Oxidative stress				
1728	*NQO1*	NAD(P)H dehydrogenase, quinone 1	-0.94	-1.92
4698	*NDUFA5*	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 5, 13 kDa	-0.78	-1.72
51167	*NCB5OR*	cytochrome b5 reductase 4	-0.62	-1.54
Inflammation				
969	*CD69*	CD69 molecule	-0.94	-1.92
3576	*IL-8*	interleukin 8	-0.85	-1.80
6361	*CCL17*	chemokine (C-C motif) ligand 17	-0.73	-1.66
7852	*CXCR4*	chemokine (C-X-C motif) receptor 4	-0.62	-1.54
3458	*IFN-γ*	interferon, γ	-0.61	-1.53
1178	*CLC*	Charcot-Leyden crystal protein	-0.54	-1.45
51176	*LEF1*	lymphoid enhancer-binding factor 1	-0.53	-1.44
2357	*FPR1*	formyl peptide receptor 1	-0.51	-1.42
DNA repair				
54541	*DDIT4*	DNA-damage-inducible transcript 4	-1.55	-2.93
7518	*XRCC4*	X-ray repair complementing defective repair in Chinese hamster cells 4	-1.17	-2.25
Apoptosis				
4000	*LMNA*	lamin A/C	-0.82	-1.76
11235	*PDCD10*	programmed cell death 10	-0.78	-1.72
950	*SCARB2*	scavenger receptor class B, member 2	-0.77	-1.71
54205	*CYCS*	cytochrome c, somatic	-0.74	-1.67
27242	*TNFRSF21*	tumour necrosis factor receptor superfamily, member 21	-0.69	-1.61
23421	*ITGB3BP*	integrin β 3 binding protein (β3-endonexin)	-0.54	-1.45
122953	*JDP2*	jun dimerization protein 2	-0.50	-1.41
3553	*IL1B*	interleukin 1, β	-0.50	-1.41
Lipid metabolism				
27284	*SULT1B1*	sulphotransferase family, cytosolic, 1B, member 1	-0.95	-1.93
7941	*PLA2G7*	phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)	-0.71	-1.64
6309	*SC5DL*	sterol-C5-desaturase (ERG3 δ-5-desaturase homologue, *S. cerevisiae*)-like	-0.64	-1.56
3422	*IDI1*	isopentenyl-diphosphate δ isomerase 1	-0.59	-1.51
6342	*SCP2*	sterol carrier protein 2	-0.54	-1.45
56994	*CHPT1*	choline phosphotransferase 1	-0.52	-1.43
8310	*ACOX3*	acyl-coenzyme A oxidase 3, pristanoyl	-0.52	-1.43
Coagulation				
7056	*THBD*	thrombomodulin	-0.86	-1.82

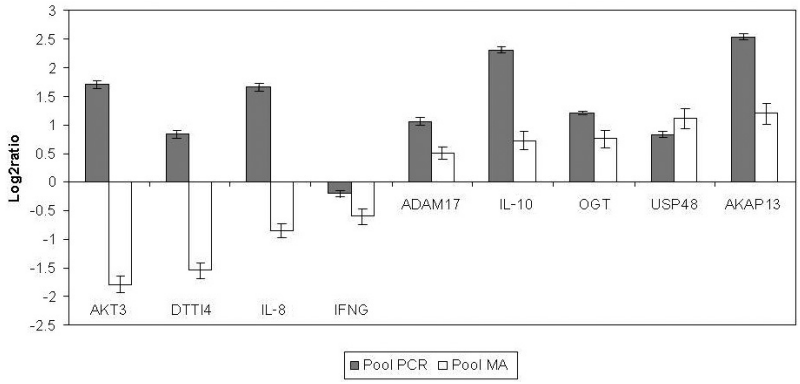

Fig. 2. Assessment of gene expression levels by real-time PCR. Log_2 ratio expresses the gene expression changes in human mononuclear cells according to RT-PCR (black bars) and microarray (white bars). *AKT3*, v-*akt* murine thymoma viral oncogene; *DDIT4*, DNA-damage-inducible transcript 4; *IFNG*, interferon γ; *IL-8*, interleukin 8; *ADAM17*, a disintegrin and metalloproteinase domain 17; *USP48*, ubiquitin specific protease 48; *OGT*, O-linked N-acetylglucosamine transferase; *IL-10*, interleukin 10; *AKAP13*, A-kinase anchoring protein 13.

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Author Disclosure Statement

* These authors contributed equally to this work, no competing financial interests exist.

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Publication No 2

In summary, after an acute load of VOO, we observed changes in the expression of insulin sensitivity-related genes. Plasma glucose, insulin, and hydroxytyrosol increased at 1h and decreased at 6h after VOO load. Lipid oxidative damage increased at 6h. A 1h downregulation was observed in *OGT* (O-UDP-N-acetylglucosamine), and *ALOX5AP* (arachidonate 5-lipoxygenase-activating protein) genes. *OGT* was upregulated at 6h, following a cuadratic trend. *CD36* (CD36 (thrombospondin receptor)) was upregulated at 1h, returning to the basal values at 6h, following also a cuadratic trend. *LIAS* (lipoic acid synthetase), *PPARBP* (peroxisome proliferator-activated receptor binding protein), *ADAM17*, and *ADRB2* (adrenergic beta-2-receptor) genes were upregulated at 6h following an increasing linear trend from baseline to 6h. *ALOX5AP* and *OGT* genes inversely correlated with insulin and glucose levels at 1h. *ADAM17* and *ADRB2* inversely correlated with oxLDL at 6h.

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OMICS A Journal of Integrative Biology
Volume 13, Number 5, 2009

DOI: 10.1089/omi.2008.0085

Original Article

Time Course of Changes in the Expression of Insulin Sensitivity-Related Genes after an Acute Load of Virgin Olive Oil

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Abstract

Our aim was to examine whether an acute fat load could induce changes in the expression of insulin sensitivity-related genes in human peripheral blood mononuclear cells. Selection of candidate genes was based on previous studies with sustained virgin olive oil (VOO) consumption and biological plausibility in relation to insulin sensitivity. Eleven healthy volunteers ingested raw VOO (50 mL). Blood samples were collected at 0, 1 and 6 h. Plasma glucose, insulin and hydroxytyrosol increased at 1 h and decreased at 6 h. Lipid oxidative damage increased at 6 h ($p < 0.05$). Gene expression changes were characterized based on quantification of the samples relative to a reference sample [i.e., relative quantification (RQ) method]. A 1 h downregulation was observed in *O*-linked-N-acetylglucosamine transferase (*OGT*, RQ: 0.62 ± 0.32) and arachidonate-5-lipoxygenase-activating protein (*ALOX5AP*, RQ: 0.64 ± 0.31) genes ($p < 0.005$). *OGT* was upregulated at 6 h (RQ: 1.88 ± 0.28, $p < 0.05$). *CD36* (thrombospondin receptor) was upregulated at 1 h (RQ: 1.6 ± 0.8, $p < 0.05$) returning to the basal values at 6 h. Lipoic acid synthetase (*LIAS*), peroxisome proliferator-activated receptor binding protein (*PPARBP*), a disintegrin and metallopeptidase domain 17 (*ADAM17*), and adrenergic beta-2-receptor (*ADRB2*) genes were upregulated at 6 h (range for the mean RQ: 1.33–1.56) following an increasing linear trend ($p < 0.05$) from baseline to 6 h. *ALOX5AP* and *OGT* genes inversely correlated with insulin and glucose levels at 1 h. *ADAM17* and *ADRB2* inversely correlated with oxLDL at 6 h ($p < 0.05$). Taken together, these observations may inform the future clinical nutrigenomics study designs and indicate that a single dose of VOO can elicit quantifiable and rapid changes in gene expression in targets that are mechanistically relevant for insulin sensitivity and the metabolic syndrome.

Introduction

Postprandial hyperlipidemia, hyperglycaemia, oxidative stress, and insulin resistance may occur after meals with a high fat content (Roche and Gibney, 2000). Postprandial lipidemia has been recognized as a risk factor for atherosclerosis development, as it is associated with oxidative changes (Regnstrom et al., 1992). The impaired ability to eliminate lipids in the postprandial state is an atherogenic trait associated with insulin resistance. Excessive postprandial hyperglycaemia is directly toxic to the endothelium, increasing protein glycation, generating oxidative stress, and causing impaired endothelial function (Ceriello, 1999; 2000; Williams et al., 1998). Hyperinsulinemia itself may also be pathogenic (Pyorala et al., 1998; Stout, 1996). Insulin resistance and compensatory hyperinsulinemia are involved in the development of dyslipidemia, hypertension, impaired fibrinolysis, and other abnormalities that collectively contribute to an increased risk of coronary heart disease (CHD) (Steiner and Lewis, 1996; Zavaroni et al., 1999).

In the present study, seven insulin sensitivity-related genes were selected to analyze their expression changes after an acute oral ingestion of 50-mL raw virgin olive oil (VOO). The selection of the genes was performed on the basis of the

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atherosclerosis-related responsive genes observed in peripheral blood mononuclear cells (PBMNCs) of healthy volunteers after long-term (3 weeks) consumption of 25-mL VOO per day (Khymenets et al., 2009). In that work, 23 responsive genes were identified based on the microarray results and their further screening. Careful and detailed bibliographic research (PubMed database http://pubmed.gov) revealed that seven of them (*ADAM17*, a disintegrin and metallopeptidase domain 17; *ADRB2*, adrenergic beta-2-receptor; *ALOX5AP*, arachidonate 5-lipoxygenase-activating protein; *CD36*, CD36 (thrombospondin receptor); *LIAS*, lipoic acid synthetase; *OGT*, *O*-linked N-acetylglucosamine (*O*-GlcNAc) transferase and *PPARBP*, peroxisome proliferator-activated receptor binding protein) were related to the insulin sensitivity mechanisms (Fujimura et al., 2006; Handberg et al., 2009; Kaaman et al., 2006; Pershadsingh, 2007; Philipson, 2002; Togashi et al., 2002; Whelan et al., 2008). Notewithstanding these results from longer term administration of VOO, it is also of interest to evaluate whether and to what extent these genes respond to acute administration of VOO.

Insulin plays a central role in determining the triglycerides turnover and clearance, via lipoprotein lipase activation, through the synthesis and secretion of very low density lipoproteins (VLDL) (Berge et al., 2005). Insulin secretion can be divided into two different phases, the stimulated (postprandial) state that regulates glucose metabolism when carbohydrate is abundant and must be disposed of, and the basal (postabsorptive) state that prevails during the interprandial phases. Long-term maintenance of serum glucose concentrations is a closely regulated process in mammalian species (Henriksen, 2006). Great variations in insulin sensitivity are common even among young healthy individuals (Pedersen, 1999). Less than one-third of the interindividual variation in insulin sensitivity is explained by known factors such as obesity. Thus, genetic factors along with environmental influences deserve consideration to account for other hitherto neglected contributions that can explain this large variation (Riserus, 2008).

Nutrients can regulate the expressed gene products at transcription, mRNA processing, mRNA stability, translation, and/or posttranslational modification stages (Salati et al., 2004). The ability of an individual to cope with a fatty meal may be a key factor in the development of CHD. Nutrient–gene interactions could be involved in the fat clearance, insulin homeostasis, and insulin sensitivity/resistance changes after fat ingestion. To this end, human nutrigenomics data are scarce. We have previously demonstrated that an oral load of 25 mL of any type of olive oil does not promote postprandial hyperlipidemia and oxidative stress in healthy volunteers (Weinbrenner et al., 2004), whereas doses equal to or greater than 40 mL do (Covas et al., 2006; Fito et al., 2002). The aim of the present study was to examine whether an acute oral ingestion of 50-mL raw VOO results in quantifiable changes in the expression of insulin sensitivity-related genes in human PBMNCs, and to discern their postprandial time course.

Materials and Methods

Subjects

Eleven healthy volunteers (six male and five female), aged 22 to 44, were recruited. The institutional ethics committee (CEIC-IMAS) approved the protocol and the volunteers signed a specific, written and informed consent. All were healthy on the basis of a physical examination and standard biochemical and haematological tests. Subjects had an average weight of 66.28 ± 12.73 kg, and a body mass index of 23.11 ± 3.06 kg/m^2. Ten of them participated in our previous study, which examined gene expression changes after sustained (3 weeks) VOO consumption (Khymenets et al., 2009).

Study design and sample collection

Prior to the ingestion of VOO (intervention), subjects followed a 1-week washout period during which sunflower oil was provided as the only source of fat for consumption (raw and cooked). During the first 4 days of this washout period, participants were asked to control their antioxidant intake. During the 3 days prior to the intervention they followed a strict low-phenolic compound diet. At 8 a.m. of the intervention day, at fasting state, 50 mL (44 g) of raw VOO was administered in a single dose with bread (200 g). During the first postprandial 6 h, subjects abstained from food and drinks with the exception of caffeine-free, low-energy drinks, and water. Blood was collected in 8-mL Cell Preparation Tubes BD Vacutainer® CPT™ (Beckton Dickinson, Franklin Lakes, NJ) at baseline (0 h, predose), at 1 h, and at 6 h after VOO ingestion. To ascertain participants' compliance, the nutritionist verified that they consumed the total amount of VOO administered in the 50-mL containers.

Insulin, hydroxytyrosol, glycaemia, and lipid profile determinations

Insulin levels were measured by an enzyme-linked immunosorbent assay (Mercodia AB, Uppsala, Sweden). Glucose and lipid analyses were performed in a PENTRA-400 autoanalyzer (ABX-Horiba Diagnostics, Montpellier, France). Serum glucose, total cholesterol, and triglyceride levels were measured using standard enzymatic automated methods (ABX-Horiba Diagnostics, Montpellier, France). High-density lipoprotein (HDL) cholesterol was directly determined by an accelerator selective detergent method (ABX-Horiba Diagnostics, Montpellier, France). LDL cholesterol was calculated by the Friedewald (Friedewald et al., 1972) formula whenever triglycerides were <300 mg/dL. Plasma lipid peroxides were assessed by the generation of malondialdehyde equivalents, and measured by the thiobarbituric acid reactive substances method. Oxidized low-density lipoprotein (OxLDL) was determined in plasma by a sandwich ELISA procedure using the murine monoclonal antibody mAB-4E6 as capture antibody, and a peroxidase conjugated antibody against oxidized apolipoprotein B bound to the solid phase (ox-LDL, Mercodia AB, Uppsala, Sweden). OxLDL values were adjusted by low-density lipoprotein (LDL) cholesterol. Concentrations of hydroxytyrosol in plasma samples were determined by gas chromatography-mass spectrometry (GC-MS) (Miro-Casas et al., 2003).

RNA extraction

On the basis of our previous experience (Khymenets et al., 2005), the liquid–liquid RNA isolation method from PBMNCs using the Ultraspec solution (Biotecx Laboratories, Houston, TX) was utilized. In brief, PBMNCs were isolated from peripheral blood using BD Vacutainer® CPT™ cell preparation

tubes (Beckton Dickinson). Whole blood was centrifuged at $1690\times g$ for 30 min; cells were washed with phosphate-buffered saline (PBS), centrifuged at $970\times g$ for 15 min, re-suspended in Ultraspec, and finally stored at -80°C until RNA isolation. Total RNA (tRNA) was extracted from the Ultraspec solution following manufacturers' instructions. Quality and purity of total RNA was obtained. Total RNA concentration and tRNA purity (ratios A260/A280 and A260/A230) were estimated by spectrophotometry (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, DE). tRNA integrity was assessed by micro capillary gel electrophoresis with fluorescent detection (Bioanalyzer, RNA 6000 LabChip kit, Agilent Technologies, Wilmington, DE), and was estimated by the RIN value (RNA integrity number) (Fleige and Pfaffl, 2006). The RIN value was determined by the Agilent 2100 Expert Software algorithm (Agilent Technologies, Palo Alto, CA). All reagents, plastic ware, and supplies used were sterile, nuclease free, and of molecular biology grade.

Reverse-transcription and real-time qPCR

A total of 100 ng of total RNA in a 20-μL reaction was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, Foster City, CA) according to manufacturer's protocols. Quantitative PCR assays (TaqMan® Low-Density Array by Design) were performed using 384-well MicroFluidic cards on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) for seven insulin resistance-related genes: (1) a disintegrin and metallopeptidase domain 17 (also known as tumor necrosis factor-alpha (TNF-OC) converting enzyme, TACE) (*ADAM17*); (2) adrenergic beta-2-receptor (*ADRB2*); (3) arachidonate 5-lipoxygenase-activating protein (*ALOX5AP*); (4) CD36 molecule, thrombospondin receptor, (*CD36*); (5) lipoic acid synthetase (*LIAS*); (6) *O*-linked N-acetylglucosamine (*O-GlcNAc*) transferase (*OGT*); and (7) peroxisome proliferator-activated receptor binding protein (*PPARBP*). The context sequences used in the quantitative real-time PCR (qRT-PCR) are presented in Table 1 based on the manufacturer's indications. The mRNA expressions were normalized by using human glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) as an endogenous control to correct the differences in the amount of total RNA added to each reaction. The data obtained were analyzed and stored in the SDS 2.1 software. Results from each run were analyzed separately using a software-defined baseline and a threshold of 0.20. The change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ relative quantification method (RQ) where data were normalized first to the endogenous reference gene (*GADPH*) and after to the untreated control (baseline) (Livak and Schmittgen, 2001) according to the manufacturer's guidelines (Applied Biosystems).

Statistical analysis

Normality of continuous variables was assessed by normal probability plots. Nonparametric data were normalized by log conversion for further statistical analysis. Correlation analyses were performed by Pearson's correlation test. A general linear model for repeated measurements was used, with multiple paired comparisons corrected by Tukey's method, in order to assess differences among the time points evaluated. The Student's *t*-test for independent samples was performed to assess gender differences. A *p*-value of ≤ 0.05 was considered as statistically significant. All statistical analysis was performed by SPSS 12.3 software (SPSS Inc. Chicago, IL) for Windows XP (Microsoft, Redmond, WA).

Results

The insulin, glucose, and hydroxytyrosol levels increased at 1 h after the olive oil consumption followed by a decrease at 6 h ($p < 0.05$) (Table 2). An increase in oxidized LDL and lipid peroxides were observed at 6 h postprandial ($p < 0.05$). The quality and purity of the total RNA were ascertained using standard measures (A260/A280 and A260/A230 $\geq$1.8 and $8.5 \leq \text{RIN} \leq 9.5$).

Two different patterns were observed concerning the gene expression profile changes. The first (Fig. 1), followed a quadratic trend that was significant in the case of *ALOX5AP* and *OGT* ($p < 0.01$), reaching a borderline significance in the case of *CD36* ($p < 0.1$). A downregulation at 1 h after olive oil ingestion was observed in *OGT* and *ALOX5AP* genes (RQ values, 0.62 ± 0.32 and 0.64 ± 0.31, respectively, $p < 0.005$) followed by an upregulation at 6 h in the case of *OGT* (RQ: 1.88 ± 0.28). CD36 was upregulated at 1 h ($\text{RQ} = 1.6 \pm 0.8$, $p < 0.05$) returning to basal values at 6 h (Fig. 1). The second pattern, followed by *LIAS*, *PPARBP*, *ADAM17*, and *ADRB2* showed an upregulation at 6 h (mean RQ range 1.33–1.56, $p < 0.05$) following a linear trend ($p < 0.05$) from baseline to 6 h (Fig. 2). Changes in *ADRB2* expression at 6 h postprandial were significantly higher than those at 1 h ($p < 0.05$). Several

Table 1. Context Sequences Used in Quantitative Real-Time PCR (qRT-PCR)

Gene symbol	*Gene name*	*Assay ID*	*Context sequence*
ADAM17	ADAM metallopeptidase domain 17 (TACE,Tumour necrosis factor alpha converting enzyme)	Hs00234224_m1	TGTCCAGTGCAGTGACAGGAACAGT
ADRB2	Adrenergic beta-2-receptor	Hs00240532_s1	CCACCCACCAGGAAGCCATCAACTG
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein	Hs00233463_m1	ACTGCCAACCAGAACTGTGTAGATG
CD36	CD36 molecule (thrombospondin receptor)	Hs00169627_m1	ATGATTAATGGTACAGATGCAGCCT
LIAS	Lipoic acid synthetase	Hs00398048_m1	CATATAAAGCAGGTGAATTTTTCCT
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	Hs00269228_m1	ACTTTGAAAAGGCTGTCACCCTTGA
PPARBP	PPARg binding protein	Hs00191130_m1	GGGGAGAATCCTGTGAGCTGTCCGG

Table 2. Insulin, Glucose, Lipid Profile, and Hydroxytyrosol Values at Baseline, 1 Hour and 6 Hours after Olive Oil Ingestion

Parameters	*Baseline (0 hours)*	*1 hour*	*6 hours*	*p value for quadratic trend*
Insulin (mU/L)	11 (4.0)	25 (12)[a]	7.5 (2.3)[b]	0.001
Glucose (mmol/L)	4.83 (3.1)	5.39 (0.9)	4.61 (0.3)[b]	0.045
Total cholesterol (mmol/L)	4.51 (0.80)	4.48 (0.80)	4.46 (0.83)	0.880
LDL cholesterol (mmol/L)	2.64 (0.62)	2.54 (0.62)	2.51 (0.67)	0.368
HDL cholesterol (mmol/L)	1.51 (0.37)	1.53 (0.36)	1.52 (0.34)	0.295
Triglycerides (mmol/L)[a]	0.65 (0.52–0.93)	0.75 (0.59–1.21)	0.76 (0.59–1.11)	0.091[c]
Oxidized LDL (mU/mg LDL cholesterol)	66 (20)	60 (15)	75 (29)	0.042[c]
TBARS (μM/L)	3.5 (2.8–7.2)	2.5 (1.3–5.6)	5.1 (3.3–12.4)	0.044
Hydroxytyrosol (nmol/L)	34 (29–53)	82 (69–136)	49 (34–58)	0.003

Values are expressed as mean (SD) with exception of triglycerides, TBARS, and hydroxytyrosol which are expressed as median (25th–75th percentile).
[a]$p < 0.05$ versus baseline; [b]$p < 0.05$ versus 1 h; [c]linear trend.

correlations among the expression of the seven selected genes at 1 h and 6 h after olive oil ingestion were obtained, which are shown in Tables 3 and 4. No differences were observed between genders.

Correlation analyses showed that the 1-h insulin increase was inversely related with *ALOX5AP* infraexpression ($r = -0.685$, $p = 0.020$) reaching a borderline significance in the case of *OGT* ($r = -0.570$, $p = 0.067$). The 1 h glucose increase was also inversely correlated not only with *ALOX5AP* ($r = -0.786$, $p = 0.004$), but also with *OGT* ($r = -0.744$, $p = 0.009$) (Supplementary Table 1). (See online supplementary material at www.liebertonline.com). At 6 h postprandial, an inverse correlation was observed between oxidized LDL and *ADAM17* and *ADRB2* gene expression ($r = -0.661$, $p = 0.027$ and $r = -0.642$, $p = 0.033$, respectively). *ADRB2* upregulation was also inversely correlated with triglyceride values at 6 h postprandial ($r = -0.684$, $p = 0.027$) (Supplementary Table 2). When the relationship between plasma hydroxytyrosol and gene expression changes was assessed, only at 1 h after olive oil ingestion there was an inverse cor-

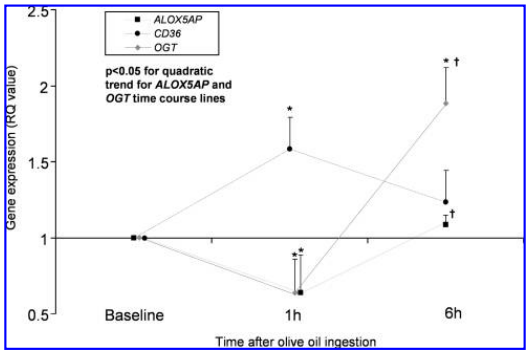

FIG. 1. Time course of changes in the expression of *ALOX5AP*, arachidonate 5-lipoxygenase-activating protein; *CD36*, CD36 molecule (thrombospondin receptor), and *OGT*, *O*-linked N-acetylglucosamine (*O*-GlcNAc) transferase genes after an acute ingestion of 50-mL virgin olive oil. Gene expression is expressed as RQ value (mean ± SEM) calculated using the relative quantification formula ($RQ = 2^{-\Delta\Delta Ct}$) where data were normalized first to the endogenous reference gene (*GADPH*) and after to the untreated control (baseline). $^{*}p < 0.05$ versus baseline; $^{\dagger}p < 0.05$ versus 1 h, according to a general linear model. Tukey's test was used for multiple paired comparisons.

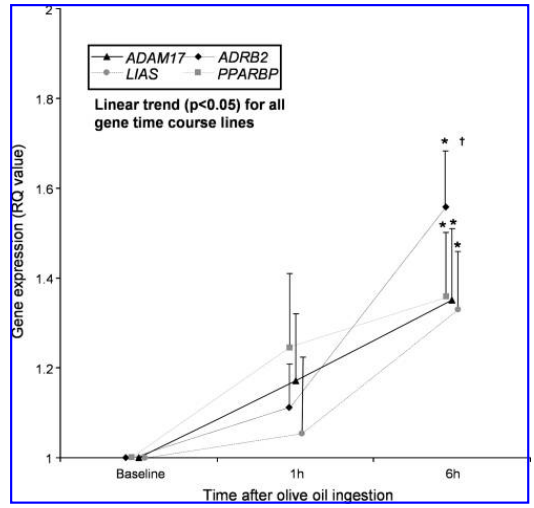

FIG. 2. Time course of changes in the expression of *ADAM17*, a disintegrin and metallopeptidase domain 17; *ADRB2*, adrenergic beta-2-receptor; *LIAS*, lipoic acid synthetase, and *PPARBP*, PPARγ binding protein genes after an acute ingestion of 50-mL virgin olive oil. Gene expression is expressed as RQ value (mean ± SEM) calculated using the relative quantification formula ($RQ = 2^{-\Delta\Delta Ct}$) where data were normalized first to the endogenous reference gene (*GADPH*) and after to the untreated control (baseline). $^{*}p < 0.05$ versus baseline, $^{\dagger}p < 0.05$ versus 1 h, according to a general linear model. Tukey's test was used for multiple paired comparisons.

Table 3. Pearson Correlation Analysis Between the Evaluated Genes at 1 H

	ADAM17	*ADRB2*	*ALOX5AP*	*CD36*	*LIAS*	*OGT*	*PPARBP*
ADAM17	1						
ADRB2	0.748[b]	1					
ALOX5AP	−0.050	0.185	1				
CD36	0.728[a]	0.837[b]	0.139	1			
LIAS	0.410	0.370	−0.217	0.518	1		
OGT	−0.125	−0.094	0.859[b]	−0.024	−0.139	1	
PPARBP	0.798[b]	0.898[c]	0.208	0.918[c]	0.471	0.029	1

Data showed the Pearson correlation coefficient (r), [a]$p < 0.05$, [b]$p < 0.01$ and [c]$p < 0.001$.

relation between hydroxytyrosol values and *ADAM17* expression ($r = -0.727$, $p = 0.027$).

Discussion

In the present study, the time course of changes in the expression of seven insulin sensitivity-related genes in PBMNCs of healthy individuals at baseline, 1 and 6 h after 50 mL VOO ingestion was assessed. In a previous work, we observed changes in the expression of these genes after 3 weeks of sustained VOO consumption in the same volunteers. To date, only few nutrigenomics studies have been performed in humans; an important barrier to such studies has been inaccessibility to tissue samples, which is a crucial requirement for gene expression studies (Muller and Kersten, 2003). In this study, we evaluated gene expression in PBMNCs directly collected from the CPT™ tubes. Importantly, direct sample collection from the CPT™ tubes ensures rapid cell isolation and prevents gene activation *ex vivo*. Changes in the expression of *OGT*, *ALOX5AP*, and *CD36* genes showed a parabolic shape with a different pattern of change at 1 h than at 6 h postprandial (i.e., a quadratic trend) (Fig. 1). A downregulation at 1 h and an upregulation at 6 h after VOO ingestion were observed in *OGT* expression. *ALOX5AP* expression was downregulated and that of *CD36* was upregulated at 1 h, returning to basal values at 6 h. Changes in the expression of *PPARB*, *LIAS*, *ABRB2*, and *ADAM17*, followed a linear trend, increasing from 0 to 6 h postprandial, with the highest gene expression observed at 6 h postingestion (Fig. 2). To the best of our knowledge, these findings have not been previously described.

In agreement with previous reports describing the postprandial time course of glucose and insulin, a peak in these parameters was observed at 1 h after VOO ingestion, which returned to baseline values at 6 h postprandial (Axelsen et al., 1999). This positive quadratic trend was similar to that followed by the changes in the expression of *CD36*. The opposite pattern (negative quadratic trend) was observed for *OGT* and *ALOX5AP* gene expression changes. In agreement with this, at 1 h postprandial changes in *OGT* and *ALOX5AP* were inversely correlated with the peak of insulin and glucose. Also, an oxidative stress status was present at 6 h postprandial, reflected in an increase in the oxidative damage to lipids and LDL. This observation has been previously noted after single-dose VOO ingestion of 40 mL (Covas et al., 2006) and 50 mL (Fito et al., 2002). The linear increase observed in triglycerides and oxidized LDL from 0 to 6 h, at the postprandial state, was similar to that observed for *ADAM17*, *ADRB2*, *LIAS*, and *PPARBP* gene expression changes. However, levels of triglycerides and oxidized LDL at 6 h were inversely related with *ADAM17* and *ADRB2* gene expression changes.

The *OGT* gene encodes the transferase that catalyzes the addition of a single N-acetylglucosamine to serine or threonine residues. The *OGT* gene is activated by the insulin signalling (Whelan et al., 2008). Due to the fact that sustained insulin action would be detrimental to physiological homeostasis, several feedback mechanisms are involved in attenuating the signalling of sustained insulin action (Saltiel and Pessin, 2002; Zick, 2005). Among them, the recruitment of *OGT* from the nucleus to the plasma membrane results in the alteration of key signalling molecules and the attenuation of the insulin signal transduction (Yang et al., 2008). The increase in *OGT* expression at 6 h after VOO ingestion could be related with the above-mentioned feedback mechanisms developed to attenuate the signalling of sustained insulin action (Yang et al., 2008). The expression of *ALOX5AP* has been associated with insulin resistance (Kaaman et al., 2006). In agreement with this, in our study, changes in *ALOX5AP* gene expression were related with the peak of glucose and insulin at 1 h postprandial. The mRNA expression of *ALOX5AP* in the

Table 4. Pearson Correlation Analysis between the Evaluated Genes at 6 H

	ADAM17	*ADRB2*	*ALOX5AP*	*CD36*	*LIAS*	*OGT*	*PPARBP*
ADAM17	1						
ADRB2	0.522	1					
ALOX5AP	0.026	0.057	1				
CD36	0.676[a]	0.116	0.135	1			
LIAS	0.810[b]	0.523	−0.209	0.634[a]	1		
OGT	0.898[c]	0.408	−0.249	0.719[a]	0.941[c]	1	
PPARBP	0.839[c]	0.483	−0.134	0.621[a]	0.947[c]	0.935[c]	1

Data showed the Pearson correlation coefficient (r), [a]$p < 0.05$, [b]$p < 0.01$, and [c]$p < 0.001$.

subcutaneous adipose tissue has shown to be increased in obesity and normalized following weight reduction (Kaaman et al., 2006). A recent study also concluded that genetic variation in the *ALOX5AP* gene contributes to CHD risk in patients with familial hypercholesterolemia (van der Net et al., 2008). Soluble CD36 levels have shown to be closely related with insulin resistance (Handberg et al., 2009). However, CD36 deficiency has also been reported to underlie insulin resistance in spontaneously hypertensive rats (Aitman et al., 1999). Despite a similar quadratic trend observed for insulin and CD36 gene expression changes, we did not observe a relationship between both parameters at 1 h postprandial. At this time, the observed upregulation of *CD36*, a fatty acid transporter (Corpeleijn et al., 2008), could be related with the postprandial increase in plasma fatty acids after the VOO ingestion, as a related mechanism to the fatty acid uptake (Goldberg et al., 2008). The increase in *CD36* could also be associated with the satiety response after olive oil ingestion. Oleic acid, the main fatty acid of olive oil, is the substrate for oleoylethanolamide production. *CD36* mediates the intake of oleic acid. Interestingly, oleoylethanolamide production has been reported to be disrupted in mice lacking the membrane-fatty acid transported *CD36*. The activation of the small-intestinal lipid messenger oleoylethanolamide, enabled by *CD36*-mediated uptake of dietary oleic acid, serves as a molecular sensor linking fat ingestion to satiety (Schwartz et al., 2008). Induction of *CD36* in response to these diverse stimuli, including oxidized LDL, is dependent on the activation of *PPARγ* (Nicholson, 2004). In agreement with this, a direct relationship was observed between *CD36* and *PPARBP* gene expressions at 1 h and 6 h after olive oil ingestion.

Genes presenting a postprandial linear trend pattern of expression changes were comprised of *LIAS*, *PPARBP*, *ABRB2*, and *ADAM17*. This group of genes was significantly upregulated at 6 h after VOO ingestion. Changes in the expression of *LIAS* and *PPARBP* were strongly related at 6 h postprandial. The protein encoded by the *LIAS* gene belongs to the lipoic acid synthetases family. Lipoic acid is a powerful antioxidant that can activate peroxisome proliferator-activated receptors (*PPARα* and *PPARγ*) (Pershadsingh, 2007). *PPARγ* is a nuclear hormone receptor playing a crucial role in adipogenesis and insulin sensitization (Fajas et al., 2001). The improvement of insulin resistance by *PPARγ* agonists is primarily mediated by enhancing the *PPARγ* interaction with *PPAR*-binding protein (*PPARBP*) (Fujimura et al., 2006). Increases in aortic O_2^- production, glucose, and insulin resistance, as well as a decrease in *PPARγ* protein in aorta and heart tissues, were prevented or attenuated in glucose-treated rats fed with lipoic acid (El Midaoui et al., 2006; Midaoui et al., 2003). Thus, it could be hypothesised that the upregulation in the expression of LIAS, the gene which codifies the lipoic acid synthase, and that of *PPARBP*, a *PPARγ* co activator, could be one of the feedback mechanisms for counteracting the postprandial oxidative stress involved in the development of insulin resistance (Giugliano et al., 2008). Activation of *PPARγ* by *PPARBP* may also increase insulin sensitivity by downregulating the expression of *TNFα* (Itoh et al., 1999). TNF-α is the major negative regulator of the insulin receptor pathway. TNF-α is regulated at posttranscriptional level by the TNFα-converting enzyme (*ADAM17*, also known as TACE) (Serino et al., 2007). *ADAM17* is considered to be a significant target for controlling insulin resistance (Togashi et al., 2002). In our study, although a direct relationship was expected (given the increase in both oxidized LDL and *ADAM17* expression), an inversed correlation was observed between both parameters at 6 h after VOO ingestion. The direct relationship observed among changes in the expression of *LIAS*, *PPARB*, and *ADAM17* genes reinforces their involvement in a hypothetical pathway. The inverse relationship observed between the peak in plasma hydroxytyrosol at 1 h after olive oil ingestion and *ADAM17* gene expression is in agreement with previous data showing a decrease of *TNF-α* levels by hydroxytyrosol (Bitler et al., 2005; Gong et al., 2008).

The adrenergic beta-2- receptor (*ADRB2)* gene encodes for a major lipolytic receptor in human fat cells. B2-agonists can affect glucose homeostasis through the modulation of insulin secretion, glucagon secretion, hepatic glucose production, and uptake of glucose into muscle (Philipson, 2002). A functional expression of *β*2 adrenergic receptors is considered to be related to a protection against oxidative stress through the promotion of glutathione synthesis (Takahata et al., 2009). From our results, the *ADRB2* gene expression was inversely correlated with oxidized LDL and triglycerides at 6 h after VOO ingestion, reinforcing the above-mentioned possible protection. Insulin treatment has shown to increase *ADRB2* protein expression in old rats (Paulose and Balakrishnan, 2008). Thus, the *ADRB2* upregulation observed at 6 h VOO ingestion could be promoted by the postprandial insulin peak. This could count for a delayed adrenergic signal for both adipocyte differentiation and oxidative stress protection (Takahata et al., 2009).

Gene expression changes in insulin sensitivity related genes were observed after a single dose of 50 mL (44.5 g) of VOO, a quantity reported (from 30 g to 50 g/day) as usual in the Mediterranean diet (Helsing, 1995). The impact of different doses of olive oil on the gene expression patterns of insulin sensitivity-related genes remains unknown. Lesser doses of olive oil could reduce the expression of genes related with the fatty uptake or postprandial oxidative stress, such as *CD36*, *PPARBP*, *ADRB2*, or *LIAS*. Because the oral fat load used in the present study was VOO, it is still unknown whether the same gene response could be extrapolated after 50 mL of other types of fats. In one of our previous works (Covas et al., 2006), phenolic compounds from olive oil were able to modulate the postprandial oxidative stress. Thus, phenolic compounds present in the VOO administered could account for a differential response in oxidative stress related genes. In our past experience (Khymenets et al., 2009), all the genes referred to in this study increased their expression after 3 weeks of sustained (25 mL/day) olive oil consumption, the strongest responders being *ADAM17*, *CD36*, *LIAS*, *OGT*, and *PPARBP* ($p < 0.001$). The aim of the present study was to compare changes in gene expression associated with olive oil ingestion at different postprandial states. Although the subjects' baseline data had served as a within-subject control, a limitation of the study is, however, the lack of control group for the intervention itself. Due to this, the observed effects on gene expression could be secondary, not only due to the VOO ingestion, but also due to a time course effect on circadian regulated genes (Khymenets et al., 2008) and to physiological changes following any fat meal intake. It remains to be determined in future studies how specific the expression changes of this small set of genes are, compared to other global changes in human gene expression.

In summary, significant changes in the expression of candidate genes related to insulin sensitivity do occur in human PBMNCs after an oral fat load of 50 mL of VOO. Changes in gene expression were modest, as was expected after administration of a real-life dose of a single food, such as raw VOO. In terms of future clinical nutrigenomic study designs, it is useful to know that a single dose of VOO can elicit changes in gene expression in targets that are mechanistically relevant for metabolic syndrome. The exact mechanisms underlying the down- or upregulation of insulin sensitivity-related genes occurring after the oral raw VOO load, their protein expression, and their associations with changes in insulin sensitivity merit further investigation.

Acknowledgments

The CIBEROBN is an initiative of the Instituto de Salud Carlos III, Madrid, Spain. This work has been supported by CICYT (SAF 2004-08173-CO3-00), by SNS contract (CP06/00100) Instituto de Salud Carlos III, by the Greek State Scholarship Foundation (I.KY. Athens, Greece), and partially supported by The Generalitat of Catalunya (2005 SGR 00577).

Author Disclosure Statement

No competing financial interests exist.

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The results of task 2 (Traditional Mediterranean diet intervention study) are included in the publication No 3 of the present dissertation.

Publication No 3

Briefly, consumption of a traditional Mediterranean diet (TMD) consumption, during 3 months, decreased: 1) plasma oxidative and inflammatory status and; 2) the expression of genes related with both inflammation (i.e. interferon *γ*; *IFNγ*, Rho-GTPase activating protein15; *ARHGAP15,* interleukin 7 receptor; *IL7R*) and oxidative stress (i.e. adrenergic-β-2 receptor; *ADRB2*, and polymerase (DNA directed) kappa; *POLK*) in PBMNCs. All effects, with the exception of the decrease in *POLK* expression, were particularly observed when virgin olive oil, rich in phenolic compounds, was present in the TMD dietary pattern. *IL7R* expression was inversely correlated with urinary tyrosol and hydroxytyrosol. Also, differences in urinary levels of tyrosol after interventions were inversely correlated with changes in the expression of *IFNγ*.

In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet. A Randomized Controlled Trial. V. Konstantinidou, M.I. Covas, D. Muñoz-Aguayo, O. Khymenets, R. de la Torre, G. Saez, M. del Carmen Tormos, E. Toledo, A. Marti, V. Ruiz-Gutiérrez, M.V. Ruiz Mendez, and M. Fito. *FASEB J*, 2010 *in press*

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MS ID: FASEBJ/2009/148452
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***In vivo* nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet. A Randomized Controlled Trial.**

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Short title : nutrigenomic effects of olive oil polyphenols

ABSTRACT

The aim of the study was to assess whether benefits associated with the Traditional Mediterranean Diet (TMD) and virgin olive oil consumption could be mediated through changes in the expression of atherosclerosis-related genes. A randomized, parallel, controlled, clinical trial in healthy volunteers (n =90) aged 20 to 50 was performed. Three-month interventions were: 1) TMD with virgin olive oil (TMD+VOO); 2) TMD with washed virgin olive oil (TMD+WOO); and 3) Control group with their habitual diet. WOO was similar to VOO, but with a lower polyphenol content (55mg/kg versus 328mg/kg, respectively). TMD consumption decreased plasma oxidative and inflammatory status and the gene expression related with both inflammation: interferon gamma, Rho-GTPase activating protein15 (*ARHGAP15*), interleukin7 receptor (*IL7R*); and oxidative stress: adrenergic-β-2 receptor (*ADRB2*), and polymerase (DNA directed) kappa (*POLK)* in peripheral blood mononuclear cells. All effects, with the exception of the decrease in *POLK* expression, were particularly observed when VOO, rich in polyphenols, was present in the TMD dietary pattern.Our results indicate a significant role of olive oil polyphenols in the down-regulation of pro-atherogenic genes in the context of a TMD. Also, the benefits associated to a TMD and olive oil polyphenols consumption on cardiovascular risk can be mediated through nutrigenomic effects.

Key words: inflammation, oxidative stress, DNA damage, gene expression, *IFNγ*

Abbreviations: *ADRB2,* adrenergic-β-2 receptor; *ARHGAP15,* Rho-GTPase activating protein 15; DBP, diastolic blood pressure; LDL, low density lipoproteins; HDL, high density lipoproteins; *IFNγ*, interferon-gamma; *IL7R*, interelukin 7-receptor; MUFA, monounsaturated fatty acids; oxLDL, oxidized low density lipoproteins; PBMNCs, peripheral blood mononuclear cells; *POLK*, polymerase (DNA directed) kappa; PUFA, polyunsaturated fatty acids; SBP, systolic blood pressure; SFA, saturated fatty acids; VOO, virgin olive oil;WOO, washed olive oil;

INTRODUCTION

In 1979, Keys et al provided ecological evidence of a reduced risk for coronary heart disease (CHD) associated with the Mediterranean Diet despite its high monounsaturated fat (MUFA) content (1). This diet, when consumed in sufficient amounts, provides all of the known essential micronutrients (ie, vitamins and minerals), fibre, and other plant food substances to promote health (2). A high degree of adherence to the Mediterranean diet has been associated with a reduced risk of overall and cardiovascular mortality, cancer incidence and mortality, and incidence of Parkinson's and Alzheimer's disease (3-4). The most impressive benefits of this diet are, however, related to cardiovascular morbidity and mortality (5).

Olive oil is the main source of fat in the Mediterranean diet. A large body of knowledge provides evidence of the benefits of the Mediterranean diet and the olive oil consumption on risk factors for CHD particularly on the lipid profile, lipid and DNA oxidation, insulin resistance, and inflammation (6-9). In experimental studies, olive oil has also been shown to be able to influence stages of carcinogenesis, cell membrane composition, signal transduction pathways, transcription factors, and tumour suppressor genes (10). The beneficial effects of olive oil on cardiovascular risk factors are now recognized, but often only attributed to the high levels of MUFA present in olive oil (11). Olive oil, however, is a functional food which, besides a high content of MUFA, contains other minor biologically active components (12). Among them, the best studied are the polyphenols. In human studies olive oil polyphenols have been shown to reduce the *in vivo* lipid oxidative damage (13), endothelial dysfunction(14), pro-thrombotic profile (15), as well as the inflammatory status (16-18), in healthy volunteers, and stable CHD or hypercholesterolemic patients.

The exact mechanisms by which the Mediterranean diet and olive oil exert their health effects are not yet understood. Among these mechanisms, the gene-environment and/or gene-diet interaction could play an important role in the development and/or protection of chronic degenerative diseases. At present, few data exists on the *in vivo* effect of the Mediterranean diet on human gene expression (19-20), particularly in healthy volunteers. Gene expression changes in human peripheral blood mononuclear cells (PBMNCs) after virgin olive oil consumption have been reported (21-23). However, no data exist concerning the in vivo nutrigenomic effects of olive oil polyphenols in humans. The aim of the present study was to evaluate whether a Traditional Mediterranean Diet (TMD) and the polyphenols present in olive oil promote changes in atherosclerosis-related genes in healthy volunteers.

MATERIALS AND METHODS

Study design

A randomized, parallel, controlled, clinical trial with three dietary interventions was performed. From October 2007 to October 2008, 99 potential participants were recruited in Primary Care Centres. Nighty eligible participants were community-dwelling men and women aged 20 to 50 years. They were considered healthy on the basis of a physical examination and routine biochemical and haematological laboratory determinations. The institutional ethics committee approved the protocol (CEIC-IMAS 2004/1827/I) and the volunteers gave written informed consent before initiation of the study. This trial has been registered in Current Controlled Trials, London, with the International Standard Randomized Controlled Trial Number (ISRCTN53283428). Volunteers were randomly assigned to three intervention groups (n =30 each one), by means of a computer-generated random-number sequence. They received the following treatments during 3-months: Group 1) Traditional Mediterranean Diet (TMD) with Virgin olive oil (TMD+VOO); Group 2) TMD with washed virgin olive oil (TMD+WOO); and Group 3) Control group with their habitual diet. Volunteers were advised by a dietician to maintain their habitual life style. Exclusion criteria were: 1) intake of antioxidant supplements; 2) intake of acetosalicylic acid or any other drug with established antioxidative properties; 3) athletes with high physical activity (> 3000 kcal per week in leisure-time physical activity); 4) obesity (Body Mass Index [BMI] > 30 kg/m2); 5) hypercholesterolemia (total cholesterol> 8.0 mmol/L or dyslipemia therapy; 6) diabetes (glucose>126mg/dl or diabetes treatment); 7) hypertension (systolic blood pressure (SBP)> 140mmHg and/or diastolic blood pressure (DBP)>90mmHg or anti-hypertensive treatment); 8) multiple allergies; 9) celiac or other intestinal diseases; 10) any condition which could limit the mobility of the subject making study visits impossible; 11) life threatening illnesses or other diseases of conditions that could worsen adherence to the measurements or treatments; 12) vegetarians and people following special diets; and 13) alcoholism or other drug addiction. Fasting blood and first morning spot urine samples were collected between 8-10 a.m. at study entry and after the 3-months intervention.

Randomization and Mediterranean Diet intervention

The baseline examination included the administration of: 1) a previously validated 137-item food frequency questionnaire (24); 2) the Minnesota Leisure Time Physical Activity questionnaire which has been validated for its

use in Spanish men and women (25, 26), and 3) a 47-item general questionnaire assessing life-style, health conditions, socio-demographic variables, history of illness, and medication use. The same dietician carried out the interventions with the 3 randomized groups. On the basis of the assessment of an individual 14-points Mediterranean diet score (8), the dietician gave personalized advice during a 30-minute session to each participant, with recommendations on the desired frequency of intake of specific foods. Instructions were directed at upscaling the TMD score, including the use of olive oil for cooking and dressing; increased consumption of fruit, vegetables, and fish; consumption of white meat instead of red or processed meat; preparation of homemade sauce with tomato, garlic, onion, aromatic herbs, and olive oil to dress vegetables, pasta, rice, and other dishes; and, for alcohol drinkers, moderate consumption of red wine. At the end of the intervention (3 months) all baseline procedures were repeated.

Olive oil characteristics

Washed virgin olive oil (WOO) used in intervention group 2 was obtained from the virgin olive oil (VOO) used in intervention group 1 in the Instituto de la Grasa, Sevilla, Spain. Briefly, VOO was placed in a thermostatic reactor, washed twice with 10% of water at 70 ºC and shaken at 125 rpm. Temperature was maintained at 40 ºC for 20 min at 95 rpm. Oil phase separation was performed by centrifugation, repeating the whole procedure 5 times. This WOO maintained the same characteristics as the VOO with the exception of a lower content of polyphenols (55mg/kg and 328mg/kg respectively). Olive oils were provided to the subjects of both intervention groups 1 and 2 in a sufficient amount for the entire family (15 L/per volunteer) during the intervention periods for both cooking and dressing purposes. The VOO used was of the Hojiblanca variety from Andalucía, Spain. The composition of the olive oils was: MUFA 75%; polyunsaturated fatty acids (PUFA) 18.6%; and saturated fatty acids (SFA) 6.4%. Minor components, other than polyphenols, were α-tocopherol (1.47 mg/kg), β-carotene (0.43 mg/kg), and sterols (15.6 mg/kg). The content of squalene and terpenes were 4346 mg/kg and 4026 mg/kg, and 48.3 mg/kg and 61.3mg/kg for virgin and washed olive oil, respectively. Both olive oils were stored avoiding exposure to air, light, and high temperature in order to prevent oxidation.

Oxidative damage and inflammation biomarkers

Serum glucose, total cholesterol, and triglyceride levels were measured using standard enzymatic methods, and HDL-cholesterol by an accelerator selective detergent method (ABX-Horiba Diagnostics, Montpellier, France), in a

automated PENTRA-400 autoanalyzer (ABX-Horiba Diagnostics, Montpellier, France) . Low density lipoprotein (LDL) cholesterol was calculated by the Friedewald (27) formula whenever triglycerides were <300 mg/dL. Oxidized LDL (OxLDL) was determined in plasma by a sandwich ELISA procedure using the murine monoclonal antibody mAB-4E6 as a capture antibody, and a peroxidase conjugated antibody against oxidized apolipoprotein B bound to the solid phase (ox-LDL, Mercodia AB, Uppsala, Sweden). Urine total F2α-isoprostanes were determined by an immunoassay kit (Cayman Chemical, Ann Arbor, Michigan, USA). The amount of 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) in urine was measured by HPLC with electrochemical detection. Values of isoprostanes and 8-oxo-dG in urine were normalized against creatinine concentration. High sensitivity C-reactive protein (CRP) was measured by immunoturbidometry (ABX-Horiba Diagnostics, Montpellier, France). Plasma levels of IFNγ, monocyte chemoattractant protein 1 (MCP-1), soluble P-selectin (s-Pselectin), and soluble CD40L (sCD40L) were measured by Flow Cytometry (Bender Medsystems Co. Ltd., USA). All analytical determinations were performed in the same batch.

Evaluation of the intervention

After 3 months all baseline procedures were repeated. Biological assessment of the intervention compliance was performed in all participants. Tyrosol and hydroxytyrosol, the major polyphenols present in olive oil, were measured in urine by gas chromatography-mass spectrometry (28).

Gene expression analyses

The selection of candidate genes was performed on the basis of: 1) previous data from our group concerning atherosclerosis-related responsive genes in peripheral blood mononuclear cells (PBMNCs) of healthy volunteers, after long-term (3 weeks) (21) and short term (23, 29) VOO consumption; and 2) their biological plausibility assessed by literature review (http://www.ncbi.nlm.nih.gov/pubmed/). Gene expression analyses were performed in a subsample of 56 participants (20, 16, and 20, in control, TMD+WOO, and TMD+VOO groups respectively). A liquid-liquid method to isolate total RNA from PBMNCs was performed as previously described (21-23). The correct quality, quantity and purity of total RNA were assessed. A total of 100ng of tRNA in a 20µl reaction was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocols. TaqMan® Low Density Array for gene expression

analysis was performed in duplicate using 384-well MicroFluidic cards (TaqMan® Low Density Array by Design) for 48 genes (47+1 control) on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Human glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) gene was used as an endogenous control to correct the differences in the amount of total cDNA added to each reaction. Results from each run were analyzed separately using a software-defined baseline and a C_t threshold of 0.20. Changes in gene expression were calculated using the relative quantification method (RQ) and applying the $2^{-\Delta\Delta Ct}$ formula (30). Each gene expression was firstly normalized to the endogenous reference gene ($\Delta Ct = Ct_x - Ct_{\text{reference gene}}$) and afterwards to its untreated control (baseline) ($\Delta\Delta Ct$). Two genes, *NOX1* (NADPH oxidase 1) and *NOX2* (NADPH oxidase 2), did not amplify. Due to this, they were excluded from the analyses. Data obtained were analyzed using the SDS 2.1 software. We used the Functional Classification Tool of DAVID Bioinformatics Database (32-33) to generate a gene-to-gene similarity matrix.

Statistical Analysis

Normality of continuous variables was assessed by normal probability plots and by means of Shapiro-Wilk test. The relationship between continuous variables was measured by Spearman's rank correlation coefficient. Non-normally distributed variables were log transformed before applying *t* test or general linear modeling statistics. Analysis of variance (ANOVA) was used for assessing differences between control and the two TMD intervention groups at baseline. Comparisons of the 3-month changes were carried out by a covariance model with polynomial content, with age and sex as covariates. Statistical analyses were performed as: 1) two-group analyses: TMD global group (TMD+VOO and TMD+WOO) versus control group, and 2) three-group analyses: considering the three types of intervention separately (TMD+VOO, TMD+WOO, and control). An *a priori* defined p-value<0.05 was considered as statistically significant. All statistical analyses were performed with the SPSS 12.3 software (SPSS Inc. Chicago, IL, USA) for Windows XP (Microsoft, Redmond, WA). Gene Set Enrichment Analysis (GSEA) was applied to the Functional Classification Tool to determine whether an *a priori* defined set of genes showed statistically significant, concordant differences between the two biological states (before and after the intervention). Enrichment score value was used to highlight the most over-represented biological annotation out of thousands of linked terms and contents.

RESULTS

We excluded 1 of the 99 invited participants before randomization for various reasons and 1 participant dropped out of the study after randomization (Figure 1). Table 1 shows the baseline characteristics of the 90 participants (26 men and 64 women) who entered the study. We observed lower levels of plasma IFNγ in the TMD global and the TMD+WOO groups versus control. We did not observe differences in general baseline characteristics among groups (Table 1). Table 2 shows the changes in energy, nutrient intake, and key food items at the end of the intervention period. An increase in vegetables, legumes, and fish consumption was observed in both TMD groups. Participants' compliance with the supplemented olive oil was good, as reflected by both: 1) the increase in VOO consumption and the decrease of olive oil (non-virgin) in the TMD+VOO group, whereas the opposite effect was observed in the TMD+WOO group (Table 2); and 2) the decrease of the urinary tyrosol and hydroxytyrosol concentrations in the TMD+WOO group and the increase in the TMD+VOO group (P=0.007, for quadratic trend) (Figure 2). In the two-groups analyses (TMD-global versus control) plasma glucose levels, HDL-C , $F_{2\alpha}$-isoprostanes, IFN-γ, and C-reactive protein (CRP) decreased after 3 months of TMD intervention (P<0.05) (Table 3). In the three-group analyses (Table 3), the total, HDL, and LDL cholesterol decreased in the TMD+VOO group after 3 months of intervention (P < 0.05), without changes in the total cholesterol/HDL-C or LDL-C/HDL-C ratios. The decrease in the plasma IFNγ, $F_{2\alpha}$-isoprostanes, and s-Pselectin was significant only after the TMD+VOO intervention (p<0.05) (Table 3). Similar trends and results were obtained when the subpopulation involved in gene expression analyses (n = 56) was evaluated. When results were disclosed by sex, in females a decrease in IFNγ in the control group and of CRP in the TMD global and TMD+WOO groups was observed. Also, in the TMD+VOO group we observed an increase in HDL-cholesterol in females and a decrease in LDL-cholesterol in males (Supplemmentary Table 1).

Intra-group comparisons showed no significant differences between pre- and post-treatment values in the evaluated gene expression in any intervention group. Table 4 shows the inter-group comparisons of the gene expression changes in the two group analyses (TMD-global vs control) expressed as $\log_2$ratio of RQ between post-treatment and basal values. Five genes, adrenergic-β-2 receptor (*ADRB2*), Rho-GTPase activating protein 15 (*ARHGAP15*), interferon gamma (*IFNγ*), interleukin 7-receptor (*IL7R*), and polymerase (DNA directed) kappa (*POLK)* were down-regulated compared to the control group (p<0.05) (Table 4). When the three-group analyses were performed, a decreasing linear trend from the control to the TMD+VOO group (P<0.05) was observed (Figure 3) in *ADRB2, ARHGAP15, IL7R*, and *IFNγ* gene expression. The down-regulation was statistically significant in the TMD+VOO group versus control group (p <0.05) for *ADRB2, ARHGAP15,* and *IFNγ* genes, and a borderline significance

(p=0.052) in the case of *IL-7R* gene expression (Figure 3). No differences in the expression of other evaluated genes were observed neither between TMD+VOO and TMD+WOO groups, nor between them and control group. Gene expression changes were observed particularly in the female groups. (Supplemmentary Table 2). Correlation analyses showed that post-intervention IL7R expression values (all volunteers) were inversely correlated with urinary tyrosol (r =-0.273, p = 0.044) and hydroxytyrosol (r = -0.284, p = 0.035) levels. Also, changes in urinary levels of tyrosol after TMD+ VOO intervention were inversely correlated with changes in the expression of *IFNγ* (r = -0.390, p = 0.006).Functional annotation clustering of all 45 genes showed that three of the down-regulated genes, *IFNγ, IL7R* and *ADRB2*, clustered to the same Functional Group (Functional Group 3, GO: 0019219, regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process) (Table 5).

DISCUSSION

In the present study we examined whether the adherence to a traditional Mediterranean diet (TMD) modulates the expression of atherosclerosis-related genes and systemic oxidative stress and inflammation markers, focusing on the impact of olive oil polyphenols. Our results indicate that the TMD decreased the lipid oxidative and inflammatory status. TMD also decreased the expression of genes related to inflammation processes (*IFNγ*, *ARHGAP15,* and *IL7R*), oxidative stress (*ADRB2*), and DNA damage (*POLK*) in PBMNCs. All the above mentioned effects, with the exception of the decrease in the *POLK* expression, were particularly observed when virgin olive oil, rich in polyphenols was present in the TMD pattern. Our work provides, for the first time, evidence of the *in vivo* nutrigenomic effect of olive oil polyphenols down-regulating pro-atherogenic genes in humans. Also, and to the best of our knowledge, the *in vivo* human nutrigenomic effect of the Mediterranean diet in healthy individuals has not been previously reported.

When results were disclosed by sex, the gene expression changes were particularly lower in the female groups. In a previous work, we have reported gender differences in PBMNCs gene expression, with higher expression of *SOD1* and *SOD2* in healthy males (31). In the present work, however, the low number of males in some groups could account for the gender differences observed. Gene expression can be considered as a quantitative trait that is highly heritable. We used the Functional Classification Tool of DAVID Bioinformatics Database (32-33) to generate a gene-to-gene similarity matrix. Grouping genes based on functional similarity can help to enhance the biological interpretation of large lists of genes derived from high throughput studies. It has been shown that disease-

related genes tend to interact (34, 35) and display significant functional clustering in the analyzed molecular network. In our results, after 3 months of TMD+VOO intervention, three of the down-regulated genes, *IFNγ, IL7R,* and *ADRB2*, were clustered to the same functional group. In a previous exploratory approach concerning the human mononuclear cell transcriptome response after acute and sustained VOO consumption we observed gene expression changes in PBMNCs of healthy volunteers (21, 22, 23). In this work (21, 23) the gene ontology analysis of the differentially expressed genes indicated that consumption of VOO could elicit changes in the regulation of transcription and translation activities of human PBMNCs.

The Mediterranean diet, in which the main source of fat is olive oil, is well known to be associated with a low prevalence of CVD (2), cancer (36), and inflammatory diseases (37-38). Inflammation is heavily involved in the onset and development of atherosclerosis (39). Previous data from short- and long-term clinical trials have shown an anti-inflammatory effect of the Mediterranean type diet in cardiovascular risk patients (8, 40). In these patients, a TMD enriched with VOO, prevented the increase in cyclooxigenase-2 (*COX-2*) and low density lipoprotein receptor-related protein (*LRP1*) gene expression, and reduced the monocyte chemoattractant protein (*MCP-1*), compared with a TMD enriched with nuts or with a low-fat diet (19). In experimental models, the anti-inflammatory effects of polyphenols, and other olive oil minor components, have been described (41). Some of the anti-inflammatory effects of olive oil polyphenols could be attributed to oleocanthal, an olive oil polyphenol whith ibuprofen-like activity in *in vitro* models (42). Besides its antioxidant and anti-inflammatory activity, recent data suggest that hydroxytyrosol, a major olive oil phenolic compound, may exert beneficial effects through the stimulation of mitochondrial biogenesis (43). The *in vivo* anti-inflammatory role of olive oil polyphenols in humans is supported by several randomized, controlled, clinical trials (17, 44, 45).

The decrease in systemic inflammatory markers and in the expression of genes related with inflammatory processes observed in the present study is in agreement with the above described previous results concerning the protective effect of Mediterranean diet and olive oil phenolics on inflammation. The decrease in interferon gamma (IFNγ) was observed both at phenotypic and gene expression levels. IFNγ is considered to be a key inflammatory mediator for inducing IL6, a prime regulator of CRP synthesis in the liver (46). We have previously reported a down-regulation of the *IFNγ* expression in PBMNCs of healthy volunteers after a single dose of virgin olive oil (22). *ARHGAP15* encodes for a RHO GTPase-activating protein which regulate GTPases activity (47). Ras superfamily GTPases have been identified as strategic molecular targets in statins-induced T-cell immunosuppression. Statins,

besides being cholesterol-lowering drugs, also harbor strong anti-inflammatory properties (48). Members of the Rho GTPase family have been suggested to be mediators of cardiac hypertrophy (49) but, up to date, little is known about their physiological roles (50).

The protein encoded by the *IL7R* gene is a receptor for interleukin 7 (IL7) which has been related with inflammatory processes (51-52). IL7 has been shown to enhance the expression of chemokines in PBMNCs (53). A recent study has showed an up-regulation of stress-response genes, such as *IL7R* and *POLK*, in the case of induced carbon ion irradiation in murine tumour models (54). *POLK* is a DNA repair gene which copies undamaged DNA templates and is unique among human Y-family DNA polymerases (55). Somatic DNA mutations, promoted by DNA oxidation, are considered to be a crucial step in carcinogenesis as well as to be involved in the atherosclerotic processes (44, 56). We did not observe changes in the levels of 8-oxo-dG after the global TMD interventions, although a decrease was observed after the TMD+VOO intervention. However, the results of the EUROLIVE study, an intervention study performed in 200 healthy males with 3 types of similar olive oils, but with differences in their phenolic content, showed that daily consumption of 25 mL of olive oil during 3 weeks reduced DNA oxidation, irrespective of the olive oil polyphenol content (57). In agreement with the EUROLIVE results, the down-regulation of the *POLK* gene expression observed in our study was associated with the TMD intervention, but not with the olive oil polyphenol content. All theses data suggest a protective role for the MUFA or other minor components of the olive oil on DNA oxidation and damage.

The *ADRB2* gene was also down-regulated after 3 months of TDM intervention particularly in the TDM+VOO intervention group. A recent study has demonstrated that the *ADRB2*-blockade reduces macrophage cytokine production and improves survival after traumatic injury (58). *ADRB2* agonists can affect glucose homeostasis through the modulation of insulin and glucagon secretion, hepatic glucose production, and glucose uptake into muscle (59). In this sense, we have previously reported an up-regulation of the *ADRB2* expression in human PBMNCs at postprandial state after 50 mL VOO ingestion (22). This olive oil ingestion promoted a postprandial peak of insulin, lipid oxidative damage, and triglycerides and the *ADRB2* expression at 6h postprandial was inversely correlated with plasma oxidized low density lipoproteins and triglyceride concentrations (22). Oxidation of the lipids and apoproteins present in LDL leads to a change in the lipoprotein conformation by which LDL is better able to enter the monocyte/macrophage system of the arterial wall, and promote the atherosclerotic process (60). In functional studies the *ADRB2* receptor appears to be protective against oxidative stress (22, 61). In

our present study, after 3 months of TMD+VOO intervention, an improvement in the oxidative status of the volunteers was observed. These data are in agreement with those obtained in the EUROLIVE study in which a dose-dependent decrease of the lipid oxidative damage was observed with the phenol content of the administered olive oil (13).

One of our trial's strengths is that the study design is able to provide first-level scientific evidence (62), reflecting eating habits of community-dwelling individuals. Compliance of the volunteers was good as reflected in the changes of olive oil consumption patterns and urinary tyrosol and hydroxytyrosol. The lack of significance in the increase in urinary olive oil phenolics in TMD+VOO versus control group could be due to:1) the fact that the control group followed their habitual diet, which in Mediterranean countries includes virgin olive oil; 2) the high inter-individual variation in urinary phenolic values, particularly in the case of hydroxytyrosol (28). We worked with whole dietary patterns at real-life doses of food. Administration of isolated antioxidants (i.e. hydroxytyrosol) at high doses has been shown to promote the atherosclerosis lesion, as well an increase in oxidative damage, in ApoE-deficient mice (63). This points out the importance of the matrix and the dose of antioxidants. Changes in gene expression were modest, as was expected in real-life intervention conditions. The lack of a wash-out period at the beginning of the study could also be one responsible factor for the relative low gene response observed.We worked against our own hypothesis, by using current Spanish dietary pattern in our control group, to maintain real-life conditions in all groups. A study's limitation was the inability to assess potential interactions between the olive oil and other diet components that might affect the generalization of the results. However, the effects of food components are subtle and must be considered in the context of chronic exposure. Whether additional or different effects would have been observed over longer periods is unknown. A longer study, however, could have impaired the compliance of the participants.

In summary, a down-regulation in the expression of atherosclerosis-related genes occurs in human PBMNCs after 3 months of TMD. Our results point out a significant role of olive oil polyphenols in the down-regulation of pro-atherogenic genes in the frame of the Mediterranean diet. Changes in gene expression were concomitant with decreases in lipid oxidative damage and systemic inflammation markers. Our results support the idea that the benefits associated with a Mediterranean type diet and olive oil polyphenol consumption on CHD risk can be mediated through changes in the expression of atherosclerosis-related genes. Data from this study provide further evidence to

recommend the TMD and rich-polyphenol olive oils, such as virgin olive oil, as a useful tool for the prevention of atherosclerosis.

ACKNOWLEDGMENTS

The CIBEROBN is an initiative of the Instituto de Salud Carlos III, Madrid, Spain. This work has been supported by FIS-FEDER (PI041308), by the SNS contract Miguel Servet (CP06/00100) Instituto de Salud Carlos III, by the Greek State Scholarship Foundation (I.K.Y. Athens, Greece), by GST RD06/0045/0006 and partially supported by The Generalitat of Catalunya (2005 SGR 00577).

Author Disclosure Statement

No competing financial interests exist.

FIGURE LEGENDS

Figure 1. Study flow diagram. TMD, Traditional Mediterranean Diet; VOO, virgin olive oil; WOO, washed olive oil. Gene expression analyses were performed in a subsample of 56 participants (20, 16, and 20, in control, TMD+WOO, and TMD+VOO groups respectively).

Figure 2. Changes in urinary (A) tyrosol and (B) hydroxytyrosol after the 3-month interventions. * $p < 0.05$ versus control, $^{\dagger}p < 0.05$ versus TMD+WOO.

Figure 3. Gene expression changes in (A) adrenergic-β-2 receptor (*ADRB2*), (B) Rho-GTPase activating protein15 (*ARHGAP15*), (C) interferon gamma (*IFNγ*), and (D) interleukin 7-receptor (*IL7R*) genes after the 3-month interventions. $p<0.05$ for linear trend in all cases, *$p<0.05$ versus control group.

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Figure 1.

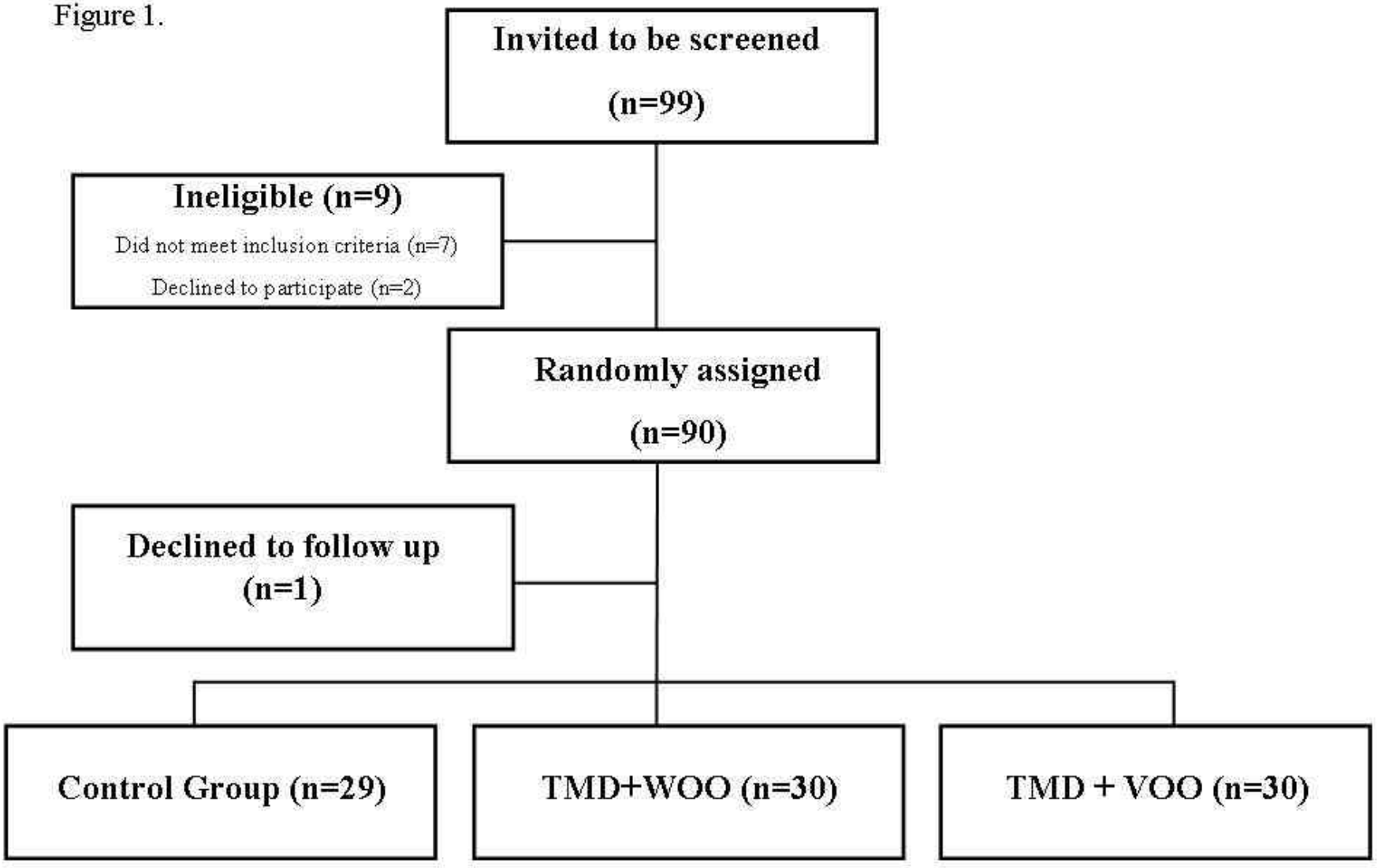
Invited to be screened
(n=99)
Ineligible (n=9)
Did not meet inclusion criteria (n=7)
Declined to participate (n=2)
Randomly assigned
(n=90)
Declined to follow up
(n=1)
Control Group (n=29)
TMD+WOO (n=30)
TMD + VOO (n=30)

Figure 2

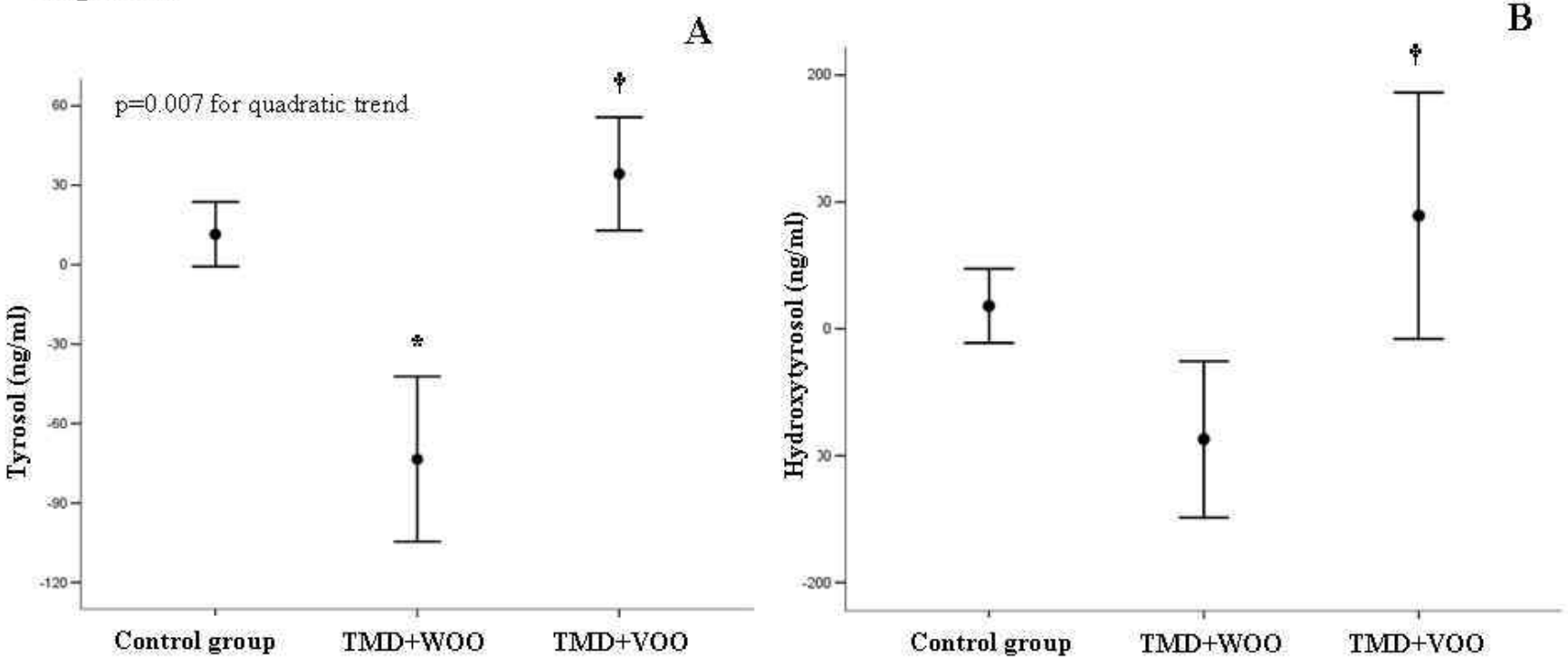
A
p=0.007 for quadratic trend
Tyrosol (ng/ml)
60
30
0
-30
-60
-90
-120
*
†
Control group
TMD+WOO
TMD+VOO
B
Hydroxytyrosol (ng/ml)
200
30
0
30
-200
†
Control group
TMD+WOO
TMD+VOO

Figure 3.

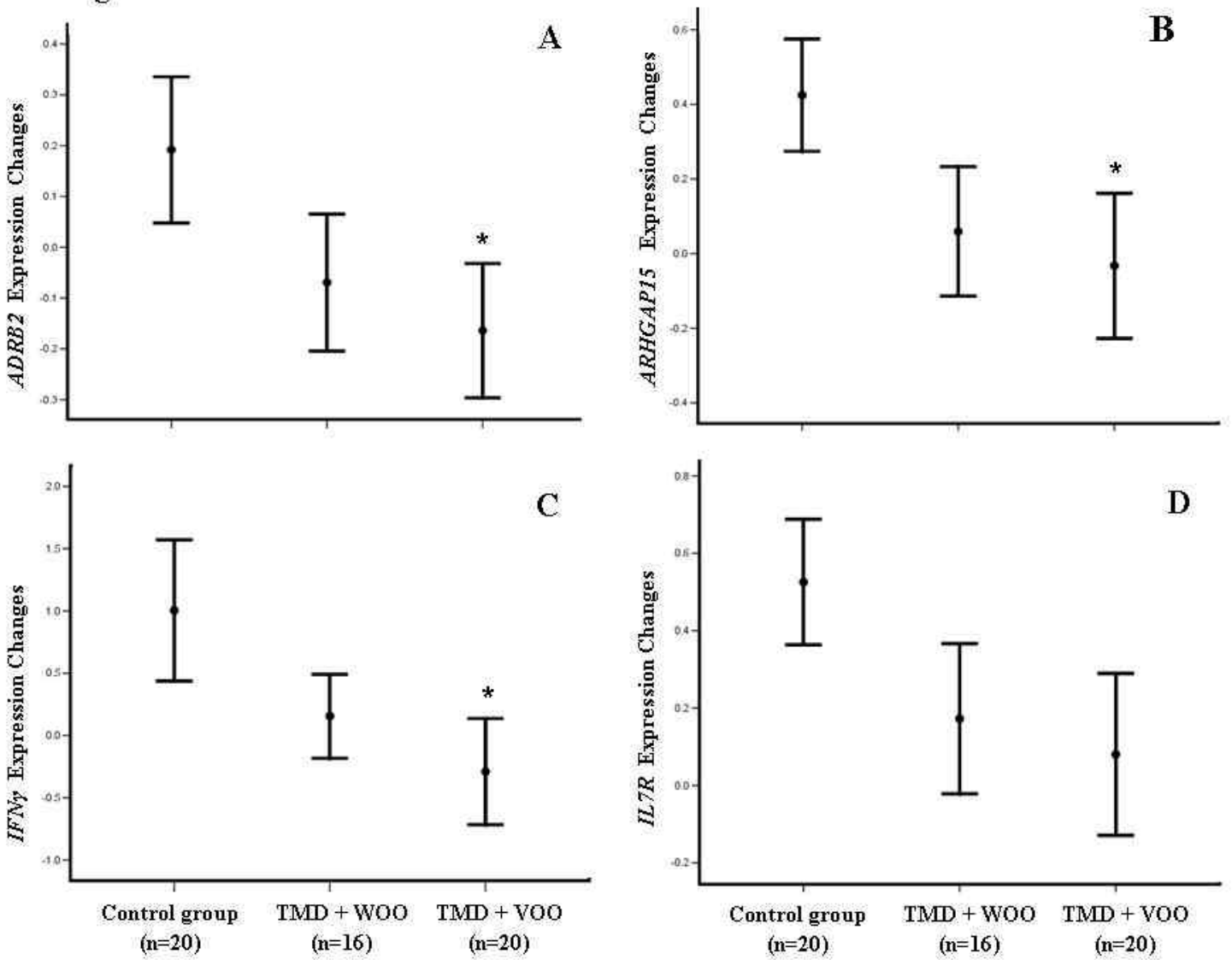
A
ADRB2 Expression Changes
B
ARHGAP15 Expression Changes
C
IFNγ Expression Changes
D
IL7R Expression Changes
*
Control group (n=20)
TMD + WOO (n=16)
TMD + VOO (n=20)

Table 1. Volunteers' baseline characteristics.

Parameter	Control (N=30)	TMD Global (N=60)	TMD+WOO (N=30)	TMD+VOO (N=30)
Age (y)	43 ±13	45 ± 10	44 ± 10	45 ± 10
Men (%)	34.5	25	27	23
Weight (kg)	66 ± 16	68 ± 13	69 ± 13	68 ± 14
BMI (kg/m^2)	25 ± 4	25 ± 4	26 ± 5	25 ± 4
SBP (mm/Hg)	117 ± 12	116 ± 15	117 ± 14	114 ± 16
DBP (mm/Hg)	69 ± 10	72 ± 10	72 ± 10	72 ± 10
Glucose (mg/dL)	85 ±15	84 ±12	84 ± 14	84 ± 9
Total Cholesterol (mg/dL)	202 ± 54	207 ± 50	200 ± 47	214 ± 54
LDL-C (mg/dL)	127 ± 42	131 ± 44	124 ± 37	138 ± 50
HDL-C (mg/dL)	58 ± 13	60 ± 14	58 ± 13	61 ± 15
Total Cholesterol /HDL-C	3.6 ± 1.0	3.6 ± 0.9	3.5 ± 0.6	3.6 ± 1.0
LDL-C / HDL-C	2.2 ± 0.7	2.3 ± 0.8	2.2 ± 0.5	2.3 ± 0.9
Triglycerides (mg/dL)	67 (52, 83)	70 (57, 103)	67 (58, 102)	70 (57, 105)
oxLDL (U/L)	66 ± 29	63 ± 21	62 ± 20	64 ± 22
$F_{2\alpha}$-isoprostanes in urine (pg/mmol creatinine)	42 (39, 79)	67 (39, 83)	54 (41, 79)	72 (39, 85)
IFNγ (ng/mL)	0.086 (0.009, 0.124)	0.018 (0.001, 0.073)*	0.001 (0, 0.068)*	0.027 (0, 0.086)
MCP-1 (pg/mL)	282 (203, 369)	217 (170, 307)	240 (195, 349)	174 (143, 243)
s-Pselectin (ng/mL)	935 ± 741	743 ± 493	768 ± 496	710 ± 498
s-CD40L (pg/mL)	937 (586, 2254)	1217 (602, 2354)	1389 (618, 2306)	1001 (558, 2449)
CRP (mg/dL)	0.02 (0.01, 0.09)	0.07 (0.03, 0.18)	0.11 (0.02, 0.25)	0.07 (0.03, 0.11)
8-oxo-dG in urine (nmol /mmol creatinin)	10.09 ± 4.07	11.32 ± 4.01	11.10 ± 3.89	11.55 ± 4.19
EEPA (kcal/day)	129 (25, 269)	130 (47, 224)	113 (49, 183)	139 (32, 229)

*$p<0.05$ vs control

Values are shown as mean ± SD for the normal variables and median (25^{th}, 75^{th}) for the non parametric variables.

Univariate ANOVA was used to assess differences between groups for the normal variables and Kruskal-Walls test for non parametric variables

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; oxLDL, oxidized low density lipoproteins; IFNγ, interferon gamma; MCP-1, monocyte chemoattractant protein 1; s-Pselectin, soluble P-selectin; s-CD40L, soluble CD40L CRP; C-reactive protein; 8-oxo-dG, 7,8-dihydro-8-oxo-2'-deoxyguanosine; EEPA, energy expenditure in physical activity in leisure time.

Table 2. Changes in the consumption of key food and nutrients (g/d).

	Mean Changes from Baseline at 3 months (95% CI)		
Variables	Control (N=29)	TMD + WOO (N=30)	TMD + VOO (N=30)
Virgin Olive Oil (VOO)	-0.44 (-2.9 to 1.9)	-8.0 (-12.6 to -3.4) * ‡	22.2 (15.1 to 29.2) * ‡ †
Olive Oil [a] (OO)	-0.88 (-5.6 to 3.9)	10.3 (4.7 to 16.0)* ‡	-17.3 (-24.2 to -10.4)* ‡ †
Total olive oil	-13.5 (-42.1 to 15.2)	22.9 (-11.5 to 57.5)	41.3 (11.9 to 70.7) * ‡
Fruits	1.0 (0.1 to 1.2)	-0.76 (-2.84 to 1.33)	-1.31 (-5.06 to 2.44)
Vegetables	1.72 (-2.44 to 5.89)	4.51 (-1.55 to 10.57)	10.31 (4.50 to 16.12) * ‡
Legumes	-0.02 (-0.76 to 0.72)	1.36 (0.47 to 2.25) * ‡	2.25 (1.24 to 3.26) * ‡
Fish	1.93 (-1.14 to 5.00)	3.87 (1.79 to 5.94) *	7.93 (2.89 to 12.98) * ‡
Nuts	1.5 (-1.5 to -4.5)	1.2 (0.2-2.2)*	0.9 (-1.7 to 0)*
Dairy products	6.28 (-6.59 to 19.15)	1.83 (-3.50 to 7.16)	-2.41 (-11.30 to 6.48)
Alcohol	0.18 (-0.14 to 0.49)	0.17 (-0.08 to 0.5)	0.02 (-0.02 to 0.061)
Energy (kcal)	-20.08 (-53.66 to 13.51)	24.88 (-13.15 to 62.90) ‡	51.01 (20.30 to 81.73) * ‡
• Protein (%)	0.17 (-0.15 to 0.49)	-0.08 (-0.45 to 0.28)	0.033 (-0.25 to 0.32)
• Carbohydrate (%)	0.04 (-0.76 to 0.83)	-0.57 (-1.38 to 0.24)	-0.78 (-1.27 to -0.29)*
• Fat (%)	-0.28 (-1.35 to 0.79)	0.67 (-0.52 to 1.86)	0.80 (0.11 to 1.48)*
- MUFA (%)	-0.52 (-1.15 to 0.12)	0.47 (-0.47 to 1.41)	0.96 (0.06 to 1.86)* ‡
- PUFA (%)	0.10 (-0.09 to 0.30)	0.09 (-0.22 to 0.39)	-0.22 (-0.75 to 0.31)
- SFA (%)	0.38 (-0.24 to 1.00)	-0.43 (-1.0 to 0.13) ‡	-0.77 (-1.24 to -0.29)* ‡
α-linolenic acid	0.01 (-0.06 to 0.09)	0.04 (0.01 to 0.07)*	0.05 (0.02 to 0.08)*
Marine n-3 fatty acids	0.01 (-0.004 to 0.03)	0.03 (0.02 to 0.05)*	0.09 (0.02 to 0.17)* ‡

[a] it includes washed olive oil (WOO) *p< 0.05 versus baseline, ‡ p< 0.05 vs control, † p<0.05 versus TMD+WOO. Univariate ANOVA was used to assess differences between groups. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids

Table 3. Changes in biomarkers after 3 months of intervention.

	CONTROL (N=29)		TMD Global (N=60)		TMD + WOO (N=30)		TMD + VOO (N=30)	
	Post-intervention	Change	Post-intervention	Change	Post-intervention	Change	Post-intervention	Change
Weight (kg)	67 ± 16	0.19 (-0.59 to 0.97)	68 ± 14	-0.17 (-0.72 to 0.37)	69 ± 14	-0.25 (-1.03 to 0.53)	67 ± 14	-0.1 (-0.86 to 0.67)
BMI (kg/m^2)	25 ± 4	0.081 (-0.2 to 0.36)	25 ± 4	-0.068 (-0.26 to 0.13)	26 ± 5	-0.1 (-0.38 to 0.18)	25 ± 4	-0.04 (-0.31 to 0.24)
SBP (mmHg)	119 ± 15	1.40 (-2.60 to 5.40)	115 ± 15	-1.03 (-3.76 to 1.7)	116 ± 14	-1.63 (-5.51 to 2.24)	114 ± 15	-0.4 (-4.31 to 3.45)
DBP (mmHg)	71 ± 10	1.67 (-1.23 to 4.58)	72 ± 10	0.17 (-1.81 to 2.15)	71± 9	-0.8 (-3.6 to 2.0)	73 ± 10	1.12 (-1.69 to 3.93)
Glucose (mg/dL)	82 ±12	-2.55 (-5.4 to 0.31)	82 ± 10*	-2.1 (-4.09 to -0.09)	82 ± 11	-1.76 (-4.58 to 1.06)	82 ± 9	-2.43 (-5.3 to 0.44)
Cholesterol (mg/dL)	202 ± 57	-0.12 (-8.56 to 8.33)	202 ± 46	-4.85 (-10.75 to 1.04)	200 ± 48	-0.2 (-8.1 to 7.7)	205 ± 45 *	-10.5 (-19.1 to -1.84)
HDL-C (mg/dL)	57 ± 13	-1.82 (-4.34 to 0.70)	57 ± 13 *	-2.0 (-3.75 to -0.29)	57 ± 12	-1.12 (-3.5 to 1.3)	58 ± 15 *	-3.14 (-5.54 to -0.53)
LDL-C (mg/dL)	129 ± 47	2.1 (-4.35 to 8.56)	128 ± 40	-2.80 (-7.22 to 1.63)	126 ± 40	1.4 (-4.6 to 7.4)	131 ± 41 *	-7.5 (-13.8 to -1.2)†‡
Cholesterol / HDL-C	3.6 ± 1.0	0.09 (-0.05 to 0.24)	3.6 ± 0.8	0.04 (-0.06 to 0.14)	3.5 ± 0.7	0.06 (-0.08 to 0.20)	3.6 ± 0.8	0.02 (-0.13 to 0.17)
LDL-C / HDL-C	2.3 ± 0.8	0.09 (-0.03 to 0.22)	2.3 ± 0.7	0.03 (-0.06 to 0.12)	2.2 ± 0.6	0.06 (-0.06 to 0.18)	2.3 ± 0.8	-0.003 (-0.13 to 0.12)
Triglycerides (mg/dL)	62 (49, 98)	-2.5 (-17, 17.3)	71 (59, 99)	4 (-14, 19)	73 (58, 100)	4.5 (-17.3, 18.5)	68 (60, 97)	4 (-10, 19)
OxLDL (U/L)	70 ± 32	3.38 (-2.36 to 9.16)	64 ± 23	2.3 (-1.69 to 6.19)	65 ± 22	2.4 (-3.04 to 7.9)	63 ± 24	2.1 (-3.68 to 7.83)
Isoprostanes (pg/mmol urine creatine)	39 (34, 65)	-2.8 (-14, 5.1)	49 (41, 66) *	-2.5 (-13.7, 6.6)	52 (43, 66)	-1.6 (-10.5, 7.4)	47 (35, 75)*	-4.3 (-18.2, 6.6)
8-oxo-dG (nmol /mmol urine creatine)	8.9 ± 3.8	-1.1 (-2.5 to 0.26)	10.4 ± 3.9	-0.95 (-1.89 to 0.003)	10.7 ± 3.5	-0.41 (-1.75 to 0.93)	10.1 ± 4.4*	-1.48 (-2.82 to -0.15)
IFNg (pg/mL)	61 (0, 113)	-11 (-52, 5)	0 (0, 46) *	0 (-45, 11)	16 (0, 51)	0 (-47, 33)	0 (0, 39) *	-2.5 (-47, 0)
MCP-1 (pg/mL)	247 (211, 317)	-36 (-119, 27)	202 (176, 305)	0.14 (-37, 35)	253 (175, 328)	8 (-60, 49)	194 (176, 250)	-6 (-25, 29)
s-Pselectin (ng/mL)	696 (493, 1063)	-78 (-286, 323)	578 (346, 808) *	-30 (-383, 122) †	664 (368, 965)	19 (-375, 147)	549 (248, 634) *	-63 (-434, 75)
s-CD40L (pg/mL)	1267 (498, 2013)	-228 (-1109, 789)	943 (587, 2437)	-77 (-1077, 804)	1256 (706, 2773)	123 (-875, 915)	923 (455, 2467)	-81 (-1435, 602)
CPR (mg/dL)	0.04 (0.01, 0.14)	0 (-0.01, 0.06)	0.04 (0.02, 0.11)*	-0.02 (-0.07, 0)†	0.0 (0.02, 0.12)*	-0.03 (-0.1, 0)	0.03 (0.02, 0.11)*	-0.02 (-0.06, 0)†
EEPA (Kcal/day)	129 (52, 226)	6.8 (-23.7 to 37.2)	117 (32, 206)	-1.8 (-23 to 19)	113 (61, 206)	6.7 (-23 to 37)	120 (23, 226)	-10 (-40 to 20)

Post-intervention values are presented as mean ± SD for normal variables and median (25th, 75th) for the non normal ones. Change values are presented as mean (CI 95%) and median (25th, 75th) for the non normal ones. * $p<0.05$ versus baseline, $^{\dagger}p<0.05$ versus control, $^{\ddagger}p<0.05$ versus TMD+WOO. Univariate ANOVA, adjusted by sex and age, was used to assess differences between groups for the normal variables and Kruskal-Walls test for non parametric variables. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; oxLDL, oxidized low density lipoproteins; IFNγ, interferon gamma; MCP-1, monocyte chemoattractant protein 1; s-Pselectin, soluble P-selectin; s-CD40L, soluble CD40L CRP; C-reactive protein; 8-oxo-dG, 7,8-dihydro-8-oxo-2'-deoxyguanosine; EEPA, energy expenditure in physical activity in leisure time.

Table 4. Changes in the expression of atherosclerosis-related genes after 3 months of intervention.

Gene Symbol	Gene Name	Control (N=20)	TMD-Global (N=36)	P between groups
Cholesterol, Lipid Transport, and Metabolism				
ABCA1	ATP-binding cassette, sub-family A,member 1	0.320 ± 0.231	0.051 ± 0.159	0.334
ABCG1	ATP-binding cassette, sub-family G,member 1	0.146 ± 0.127	0.064 ± 0.092	0.608
ANXA1	annexin A1	0.259 ± 0.229	-0.444 ± 0.161	0.160
ARHGAP15	**Rho GTPase activating protein 15**	**0.448 ± 0.175**	**-0.040 ± 0.126**	**0.043**
ARHGAP19	Rho GTPase activating protein 19	0.400 ± 0.151	0.134 ± 0.112	0.166
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor 6	0.460 ± 0.144	0.157 ± 0.106	0.099
CD36	CD36 molecule (thrombospondin receptor)	0.197 ± 0.170	-0.009 ± 0.126	0.342
CETP	cholesteryl ester transfer protein, plasma	-0.262 ± 0.331	-0.058 ± 0.257	0.631
MSR1	macrophage scavenger receptor 1	0.542 ± 0.222	0.253 ± 0.157	0.301
PLA2G4B	phospholipase A2, group IVB	0.148 ± 0.156	0.082 ± 0.109	0.735
SCARB1	scavenger receptor class B, member 1	-0.025 ± 0.078	0.085 ± 0.056	0.261
Inflammation				
IFNg	**interferon, gamma**	**1.048 ± 0.464**	**-0.109 ± 0.330**	**0.049**
IL10	interleukin 10	0.915 ± 0.360	0.609 ± 0.270	0.506
CHUK	conserved helix-loop-helix ubiquitous kinase	0.325 ± 0.192	0.036 ± 0.140	0.236
ADAM17	ADAM metallopeptidase domain 17 (tumor necrosis factor, alpha, converting enzyme)	0.290 ± 0.153	0.008 ± 0.112	0.148
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	0.166 ± 0.208	-0.120 ± 0.150	0.277
IFNA1	interferon, alpha 1	0.726 ± 0.356	0.001 ± 0.258	0.117
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	0.195 ± 0.219	-0.195 ± 0.156	0.157
TNFSF12_13	ttumor necrosis factor (ligand) superfamily, member 12-member 13	-0.021 ± 0.102	0.133 ± 0.075	0.235
IL6	interleukin 6	-0.017 ± 0.588	0.356 ± 0.401	0.612
IL7R	**interleukin 7 receptor**	**0.580 ± 0.182**	**0.095 ± 0.132**	**0.037**
USP48	ubiquitin specific peptidase 48	0.380 ± 0.179	0.203 ± 0.131	0.431
MPO	myeloperoxidase	-0.159 ± 0.121	-0.013 ± 0.090	0.343
RGS2	regulator of G-protein signalling 2, 24kDa	0.439 ± 0.268	0.289 ± 0.196	0.656
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	-0.098 ± 0.082	0.008 ± 0.063	0.315
Nuclear Receptors and Fatty acids Receptors				
NR1H2	nuclear receptor subfamily 1, group H, member 2	-0.081 ± 0.070	-0.003 ± 0.050	0.369
NRIH3	nuclear receptor subfamily 1, group H, member 3	0.166 ± 0.108	0.034 ± 0.077	0.331
PPARA	peroxisome proliferator-activated receptor alpha	0.088 ± 0.123	0.068 ± 0.092	0.897
PPARBP	PPAR binding protein	0.341 ± 0.160	0.022 ± 0.105	0.084
PPARG	peroxisome proliferator-activated receptor gamma	0.002 ± 0.242	0.235 ± 0.175	0.463
PPARD	peroxisome proliferator-activated receptor delta	0.066 ± 0.128	0.010 ± 0.096	0.732
Oxidative Stress				
LIAS	lipoic acid synthetase	0.228 ± 0.197	0.188 ± 0.148	0.874
PTGS1	prostaglandin-endoperoxide synthase 1	-0.176 ± 0.171	-0.170 ± 0.117	0.978
PTGS2	prostaglandin-endoperoxide synthase 2	0.170 ± 0.545	-0.231 ± 0.379	0.557
OLR1	oxidised low density lipoprotein (lectin-like) receptor 1	0.521 ± 0.948	0.113 ± 0.580	0.724
OSBP	oxysterol binding protein	0.219 ± 0.130	0.035 ± 0.093	0.260
ADRB2	**adrenergic, beta-2-, receptor, surface**	**0.225 ± 0.135**	**-0.138 ± 0.098**	**0.036**
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	0.373 ± 0.235	0.014 ± 0.162	0.218
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	-0.101 ± 0.187	-0.116 ± 0.135	0.949
DNA Repair				
CCNG1	cyclin G1	0.396 ± 0.192	0.004 ± 0.139	0.106
POLK	**polymerase (DNA directed) kappa**	**0.595 ± 0.275**	**-0.115 ± 0.204**	**0.045**
TP53	tumor protein p53	-0.071 ± 0.077	-0.048 ± 0.056	0.812
DCLRE1C	DNA cross-link repair 1C	0.406 ± 0.169	0.052 ± 0.123	0.100
ERCC5	excision repair cross-complementing rodent repair deficiency, complementation group 5	0.401 ± 0.227	0.049 ± 0.169	0.221
XRCC5	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80kDa)	0.267 ± 0.152	0.000 ± 0.111	0.166

Gene expression changes, adjusted by age and sex, are presented as mean ± SEM of the relative quantification (RQ) $\log_2$ratio (post-treatment versus basal values).

Table 5. Functional Annotation Clustering (Biological Processes Level 5)

Functional Groups	Enrichment Score	Gene Ontology	Gene Symbol	Adjusted p value
1	3.86	GO:0008203 cholesterol metabolic process	ABCG1, ABCA1, CETP, PPARD, SCARB1	<0.001
		GO:0016125 sterol metabolic process	ABCG1, ABCA1, CETP, PPARD, SCARB1	<0.001
		GO:0008202 steroid metabolic process	OSBP, ABCG1, ABCA1, CETP, PPARD, SCARB1	<0.001
2	2.67	GO:0006915 apoptosis	IL6, **IFNG**, MPO, ERCC5, TP53, IL10, TNFSF10, ARHGEF6, PPARD, ANXA1, SCARB1, **ADRB2**	<0.001
		GO:0042981 regulation of apoptosis	IL6, MPO, ERCC5, TP53, IL10, TNFSF10, ANXA1, **ADRB2**	0.001
		GO:0043066 negative regulation of apoptosis	IL6, MPO, ERCC5, IL10, ANXA1	0.005
		GO:0043065 positive regulation of apoptosis	TP53, TNFSF10, **ADRB2**	0.181
3	2.19	GO:0031325 positive regulation of cellular metabolic process	IL6, **IFNG**, MED1, TP53, IL10, PPARG, ABCA1, PPARA, **ADRB2**	<0.001
		GO:0045935 positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	IL6, **IFNG**, MED1, TP53, PPARG, ABCA1, PPARA, **ADRB2**	<0.001
		GO:0045893 positive regulation of transcription, DNA-dependent	IL6, **IFNG**, MED1, TP53, PPARG, PPARA, **ADRB2**	<0.001
		GO:0031324 negative regulation of cellular metabolic process	IL6, TP53, IL10, PPARG, NR1H2, PPARD	0.006
		GO:0006357 regulation of transcription from RNA polymerase II promoter	MED1, TP53, PPARG,PPARA, PPARD, **ADRB2**	0.014
		GO:0019219 regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	IL6, MED1, TP53, PPARA, XRCC5, NR1H3, IL7R, **IFNG**, PPARG,NFkB2, ABCA1, PPARD, **ADRB2**	0.047
4	1.64	GO:0006631 fatty acid metabolic process	CD36, PTGS1, PTGS2, PPARA, PPARD	0.002
		GO:0032787 monocarboxylic acid metabolic process	CD36, PTGS1, PTGS2, PPARA, PPARD	0.006
		GO:0008544 epidermis development	PTGS2, PPARA, PPARD	0.075
		GO:0009888 tissue development	PTGS2, PPARA, PPARD, ADRB2	0.084
5	1.58	GO:0048534~hemopoietic or lymphoid organ development	IL6, **IL7R**, CHUK, IL10, NFkB2	0.003
		GO:0002521 leukocyte differentiation	IL6, CHUK, IL10	0.045
		GO:0030097 hemopoiesis	IL6, CHUK, IL10	0.116

Supplementary table 1. Changes in biomarkers after 3 months of intervention in males and females.

	CONTROL (N=29)		TMD Global (N=60)		TMD + WOO (N=30)		TMD + VOO (N=30)	
	Males (n=10)	**Females (n=19)**	**Males (n=17)**	**Females (n=43)**	**Males (n=9)**	**Females (n=21)**	**Males (n=8)**	**Females (n=22)**
Weight (kg)	-0.4 (-2.25 to 1.45)	0.28 (-0.56 to 1.12)	0.29 (-1.19 to 1.77)	-0.24 (-0.79 to 0.31)	-0.39 (-2.35 to 1.58)	-0.12 (-0.92 to 0.68)	1.10 (-1.05 to 3.25)	-0.34 (-1.11 to 0.42)
BMI (kg/m^2)	-0.14 (-0.74 to 0.46)	0.12 (-0.2 to 0.45)	0.10 (-0.38 to 0.58)	-0.1 (-0.31 to 0.11)	-0.11 (-0.75 to 0.53)	-0.08 (-0.38 to 0.23)	0.35 (-0.35 to 1.05)	-0.12 (-0.41 to 0.18)
SBP (mmHg)	-0.88 (-9.02 to 7.23)	1.08 (-3.54 to 5.70)	0.1 (-5.19 to 7.18)	-1.12 (-4.12 to 1.88)	-0.5 (-8.94 to 7.9)	-1.59 (-5.91 to 2.73)	2.78 (-6.44 to 12)	-0.68 (-4.9 to 3.55)
DBP (mmHg)	3.63 (-2.5 to 9.75)	0.18 (-3.2 to 3.56)	2.55 (-2.21 to 7.31)	-0.41 (-2.58 to 1.76)	-0.34 (-6.56 to 5.88)	-0.72 (-3.85 to 2.42)	6 (-0.78 to 12.8)	-0.12 (-3.18 to 2.94)
Glucose (mg/dL)	-4.84 (-11.06 to 1.39)	-0.40 (-3.47 to 2.67)	-4.91 (-9.89 to 0.08)	-0.90 (-2.86 to 1.05)	-4.77 (-11.57 to 2.03)	-0.52 (-3.31 to 2.26)	-5.08 (-12.52 to 2.37)	-1.28 (-4.07 to 1.51)
Cholesterol (mg/dL)	2.37 (-11.58 to 16.32)	-2.38 (-13.29 to 8.52)	-7.71 (-19.17 to 3.75)	-3.53 (-10.64 to 3.59)	-4.37 (-19.41 to 10.67)	1.66 (-7.93 to 11.24)	-12.29 (-29.8 to 5.22)	-9.52 (-19.83 to 0.79)
HDL-C (mg/dL)	0.59 (-3.23 to 4.4)	-2.95 (-6.32 to 0.42)	-1.68 (-4.86 to 1.5)	-2.25 (-4.37 to -0.13)	-1.2 (-5.4 to 3.1)	-1.2 (-4.1 to 1.8)	-2.4 (-7.3 to 2.5)	-3.4 (-6.4 to -0.3)
LDL-C (mg/dL)	3.2 (-6.1 to 12.5)	0.63 (-7.9 to 9.2)	-4.7 (-12.8 to 3.4)	-1.8 (-7.2 to 3.6)	0.5 (-9.6 to 10.5)	1.99 (-5.5 to 9.5)	-11.9 (-23.6 to -0.17) *	-5.77 (-13.4 to 1.9)
Cholesterol / HDL-C	0.06 (-0.16 to 0.27)	0.09 (-0.11 to 0.3)	-0.01 (-0.19 to 0.17)	0.07 (-0.06 to 0.19)	-0.004 (-0.24 to 0.24)	0.09 (-0.09 to 0.26)	-0.03 (-0.31 to 0.25)	0.05 (-0.13 to 0.23)
LDL-C / HDL-C	0.08 (-0.11 to 0.26)	0.09 (-0.09 to 0.26)	-0.02 (-0.17 to 0.14)	0.06 (-0.05 to 0.16)	0.05 (-0.15 to 0.25)	0.07 (-0.08 to 0.23)	-0.1 (-0.3 to 0.13)	0.04 (-0.12 to 0.19)
Triglycerides (mg/dL)	-12 (-25, 17)	4 (-14, 22)	-6(-31, 14)	6 (-8, 20)	-20 (-40, 8)	7 (-6, 21)	-20 (-40, 8)	4 (-10, 19)
OxLDL (U/L)	4.3 (-3.6 to 12.3)	2.9 (-5 to 11)	-1.8 (-8.4 to 4.8)	3.6 (-1.4 to 8.5)	0.96 (-7.6 to 9.5)	2.85 (-4.09 to 9.79)	-5.5 (-15 to 5)	4.3 (-2.8 to 11.4)
Isoprostanes (pg/mmol urine creatine)	1.5 (-24, 7)	-4.3 (-13, 2.6)	-10 (-20, 2)	-1.8 (-12, 7)	-8 (-12, 7)	-0.8 (-8, 7)	-14 (-49, -3)	-3 (-16, 7)
8-oxo-dG (nmol /mmol urine creatine)	0.28 (-4.4, 1.3)	-0.78 (-2.46, 0.25)	-1.6 (-3.7, 1.9)	-0.16 (-2.1, 1.6)	-1.5 (-2.3, 1.3)	-0.009 (-1.2, 1.8)	-2.5 (-8.8, 2.3)	-0.42 (-2.8, 1.6)
IFNg (pg/mL)	-6.2 (-34, 21)	-30 (-84, 26)**	-2.1 (-73, 1.3)	0 (-39, 26)	-31 (-109, 0)	0 (-37, 46)	0 (-46, 16)	-14 (-50, 0)
MCP-1 (pg/mL)	-42 (-96, -5)	-4 (-127, 73)	-26 (-54, 26)	8 (-27, 45)	-32 (-108, 34)	17 (-47, 53)	-7 (-42, 18)	-6 (-21, 33)
s-Pselectin (ng/mL)	-121 (-237, 287)	-52 (-326, 328)	-32 (-369, 125)	-28 (-405, 133)	-48(-400, 96)	21 (-251, 227)	-32 (-253, 206)	-72 (-459, 16)
s-CD40L (pg/mL)	-256 (-1073, 1099)	-141 (-1462, 980)	23 (-313, 565)	-184 (-1397, 915)	139 (-230, 468)	-389 (-1135, 1523)	-81 (-1563, 1776)	-101 (-1483, 515)
CPR (mg/dL)	0 (-0.03, 0.01)	0 (-0.005, 0.19)	-0.04 (-0.08, 0.003)	-0.01 (-0.07, 0)*	-0.03 (-0.09, -0.02)	-0.02 (-0.22, 0.013)*	-0.05 (-0.17, 0.39)	-0.01 (-0.05, 0)
EEPA (kcal/day)	6.3 (-18 to 31)	9 (-35 to 52)	16 (-4 to 35)	-8 (-37 to 20)	25 (-1.4 to 50)	-0.25 (-41, 40)	4.7 (-24 to 33)	-16.03 (-56 to 24)

Values for change are presented as mean (CI 95%) for the normal variables and median (25th, 75th) for the non normal ones.**p<0.05 versus baseline

* p<0.05 versus control, † p<0.05 versus TMD+WOO. Univariate ANOVA, was used to assess differences between groups for the normal variables and Kruskal-Walls test for non parametric variables. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; oxLDL, oxidized low density lipoproteins; IFNγ, interferon gamma; MCP-1, monocyte chemoattractant protein 1; s-Pselectin, soluble P-selectin; s-CD40L, soluble CD40L CRP; C-reactive protein; 8-oxo-dG, 7,8-dihydro-8-oxo-2'-deoxyguanosine; EEPA, energy expenditure in physical activity in leisure time.

Supplementary table 2. Changes in the expression of atherosclerosis-related genes after 3 months of intervention

Gene Symbol	Control (N=20)		TMD-Global (N=36)		TMD+WOO(N=16)		TMD+VOO(N=20)	
	Males (n=7)	Females (n=13)	Males (n=10)	Females (n=26)	Males (n=5)	Females (n=11)	Males (n=5)	Females (n=15)
ABCA1	-0.675 (1.097)	0.377 (0.288)	0.098 (0.301)	-0.033 (0.188)	-0.839 (1.076)	-0.052 (0.286)	-0.068 (0.379)	-0.018 (0.255)
ABCG1	0.229 (0.131)	0.098 (0.181)	0.248 (0.112)	-0.010 (0.123)	0.404 (0.147)	-0.042 (0.188)	0.093 (0.147)	0.015 (0.166)
ANXA1	-0.037 (0.375)	0.278 (0.289)	0.430 (0.236)	-0.317 (0.202)	0.427 (0.336)	-0.449 (0.300)	0.432 (0.336)	-0.205 (0.278)
ARHGAP15	0.249 (0.282)	0.521 (0.232)	0.209 (0.223)	-0.067 (0.155) *	0.295 (0.316)	-0.047 (0.242)	0.124 (0.316)	-0.081 (0.207)
ARHGAP19	0.339 (0.194)	0.409 (0,206)	0.422 (0,153)	0.035 (0.145)	0.452 (0.218)	-0.078 (0.224)	0.392 (0.218)	0.117 (0.192)
ARHGEF6	0.297 (0.230)	0.509 (0.188)	0.374 (0.182)	0.297 (0.223)	0.324 (0.258)	-0.065 (0.188)*	0.424 (0.258)	0.237 (0.181)
CD36	-0.130 (0.240)	0.290 (0.228)	0.489 (0.194)	-0.231 (0.157)	0.333 (0.269)	-0.343 (0.237)	0.645 (0.269)	-0.142 (0.212)
CETP	-0.915 (0.515)	-0.170 (0.404)	0.323 (0.314)	-0.194 (0.339)	0.032 (0.388)	-0.251 (0.507)	0.784* (0.488)	-0.144 (0.474)
MSR1	0.311 (0.363)	0.667 (0.295)	0.503 (0.323)	0.187 (0.184)	0.427 (0.410)	0.284 (0.268)	0.636 (0.527)	0.097 (0.258)
PLA2G4B	0.096 (0.201)	0.187 (0.218)	0.293 (0.181)	0.004 (0.136)	0.502 (0.228)	-0.086 (0.198)	0.033 (0.252)	0.087 (0.190)
SCARB1	0.054 (0.190)	-0.078 (0.086)	0.264 (0.137)	0.019 (0.060)	0.208 (0.195)	-0.061 (0.090)	0.319 (0.195)	0.082 (0.080
IFNg	-0.534 (0.793)	1.641 (0.576)	0.012 (0.608)	-0.087 (0.394) *	0.784 (0.794)	-0.067 (0.598) *	-0.759 (0.794)	-0.104 (0.536) *
IL10	0.695 (0.846)	1.002 (0.395)	1.153 (0.669)	0.407 (0.283)	0.903 (0.948)	0.123 (0.427)	1.404 (0.948)	0.631 (0.379)
CHUK	0.189 (0.248)	0.381 (0.265)	0.322 (0.196)	-0.068 (0.182)	0.412 (0.277)	-0.233 (0.288)	0.233 (0.277)	0.043 (0.236)
ADAM17	0.022 (0.193)	0.360 (0.202)	0.410 (0.153)	-0.106 (0.139)	0.480 (0.217)	-0.209 (0.210)	0.341 (0.217)	-0.023 (0.188)
ADAMTS1	0.093 (0.398)	0.303 (0.226)	-0.129 (0.354)	-0.170 (0.160)	0.557 (0.450)	-0.444 (0.236) *	-0.654 (0.399)	0.045 (0.209)
IFNA1	1.368 (0.921)	0.635 (0.441)	-0.160 (0.510)	0.013 (0.333)	0.173 (0.601)	-0.017 (0.663)	-0.700 (0.725)	0.024 (0.398)
TNFSF10	0.118 (0.386)	-0.003 (0.260)	0.151 (0.316)	-0.306 (0.176)	-0.374 (0.386)	-0.488 (0.272)	0.676 (0.386)	-0.172 (0.233)
TNFSF12_13	0.012 (0.139)	-0.159 (0.105)	0.287 (0.117)	0.024 (0.076)	0.142 (0.156)	-0.141 (0.109)	0.432 (0.156)	0.154 (0.097) *
IL6	0.410 (1.318)	-0.109 (0.701)	0.787 (0.959)	0.154 (0.452)	0.142 (1.392)	-0.272 (0.757)	1.337 (1.294)	0.405 (0.578)
IL7R	0.656 (0.306)	0.587 (0.237)	0.237 (0.216)	0.010 (0.169)	0.221 (0.306)	0.073 (0.248)	0.254 (0.306)	-0.048 (0.237)
USP48	0.208 (0.277)	0.299 (0.254)	0.456 (0.219)	0.048 (0.177)	0.544 (0.310)	-0.023 (0.275)	0.368 (0.310)	0.099 (0.235)
MPO	-0.168 (0.165)	-0.096 (0.120)	0.102 (0.154)	0.085 (0.085)	-0.072 (0.171)	0.001 (0.132)	0.419 (0.226)	0.146 (0.111)
RGS2	-0.186 (0.338)	0.692 (0.349)	0.699 (0.267)	0.177 (0.247)	0.779 (0.379)	-0.262 (0.362)	0.619 (0.379)	0.529 (0.324)
NFKB2	0.118 (0.160)	-0.174 (0.100)	0.039 (0.133)	-0.025 (0.073)	0.054 (0.184)	-0.050 (0.114)	0.021 (0.199)	-0.008 (0.096)
NR1H2	0.014 (0.147)	-0.104 (0.086)	0.069 (0.104)	-0.039 (0.059)	0.089 (0.147)	-0.074 (0.089)	0.049 (0.147)	-0.012 (0.080)
NRIH3	0.073 (0.179)	0.202 (0.140)	0.209 (0.127)	-0.030 (0.096)	0.218 (0.179)	-0.108 (0.139)	0.200 (0.179)	0.042 (0.134)
PPARA	0.025 (0.228)	0.095 (0.138)	0.351 (0.191)	0.078 (0.100)	0.449 (0.289)	0.093 (0.157)	0.277 (0.253)	0.068 (0.133)
PPARBP	0.352 (0.257)	0.329 (0.186)	0.339 (0.182)	-0.096 (0.130)	0.343 (0.258)	-0.172 (0.194)	0.334 (0.258)	-0.031 (0.179)
PPARG	0.232 (0.370)	-0.171 (0.350)	0.615 (0.268)	0.033 (0.236)	0.557 (0.492)	-0.425 (0.618)	0.640 (0.333)	0.152 (0.281)
PPARD	0.228 (0.223)	0.005 (0.167)	0.115 (0.178)	-0.046 (0.119)	0.227 (0.250)	0.032 (0.174)	0.004 (0.250)	-0.117 (0.167)
LIAS	0.348 (0.306)	0.158 (0.253)	0.353 (0.241)	0.116 (0.191)	0.350 (0.343)	-0.268 (0.262)	0.355 (0.343)	0.471 (0.252)
PTGS1	-0.364 (0.307)	-0.116 (0.206)	0.025 (0.267)	-0.212 (0.132)	-0.473 (0.348)	-0.343 (0.204)	0.385 (0.301)	-0.115 (0.175)
PTGS2	1.008 (1.097)	-0.283 (0.661)	0.390 (0.871)	-0.505 (0.420)	-0.016 (1.229)	-0.415 (0.625)	0.796 (1.229)	-0.581 (0.578)
OLR1	-0.015 (2.249)	0.666 (1.070)	0.152 (1.499)	0.226 (0.591)	0.614 (1.945)	-0,139 (0.948)	-0.717 (2.652)	0.490 (0.802)
OSBP	0.327 (0.201)	0.171 (0.172)	0.262 (0.143)	-0.050 (0.118)	0.283 (0.202)	-0.122 (0.179)	0.241 (0.202)	0.008 (0.160)
ADRB2	0.172 (0.215)	0.278 (0.174)	-0.120 (0.185)	-0.157 (0.119)*	0.146 (0.241)	-0.195 (0.180)	-0.385 (0.241)	-0.126 (0.162)
OGT	0.422 (0.318)	0.335 (0.317)	0.313 (0.243)	-0.091 (0.209)	0.615 (0.318)	-0.185 (0.316)	0.010 (0.318)	-0.015 (0.283)
ALDH1A1	0.026 (0.330)	-0.192 (0.231)	0.150 (0.265)	-0.202 (0.163)	-0.267 (0.330)	-0.382 (0.239)	0.567 (0.330)	-0.047 (0.222)
CCNG1	0.405 (0.282)	0.371 (0.256)	0.308 (0.201)	-0.103 (0.179)	0.232 (0.282)	-0.203 (0.267)	0.383 (0.282)	-0.016 (0.247)
POLK	0.325 (0.454)	0.653 (0.353)	0.442 (0.346)	-0.282 (0.251) *	0.605 (0.463)	-0.418 (0.384) *	0.241 (0.510)	-0.174 (0.339)
TP53	0.073 (0.141)	-0.116 (0.097)	-0.070 (0.111)	-0.056 (0.067)	-0.046 (0.158)	-0.121 (0.100)	-0.094 (0.158)	-0.004 (0.090)
DCLRE1C	0.434 (0.278)	0.383 (0.219)	0.294 (0.197)	-0.027 (0.151)	0.352 (0.278)	-0.159 (0.228)	0.236 (0.278)	0.079 (0.204)
ERCC5	0.408 (0.326)	0.409 (0.300)	0.425 (0.236)	-0.048 (0.210)	0.470 (0.340)	-0.164 (0.312)	0.379 (0.340)	0.052 (0.289)
XRCC5	0.075 (0.165)	0.323 (0.210)	0.297 (0.130)	-0.088 (0.144)	0.285 (0.185)	-0.160 (0.218)	0.310 (0.185)	-0.029 (0.196)

Gene expression changes, adjusted by age and sex, are presented as mean (SEM) of the relative quantification (RQ) $\log_2$ratio (post-treatment versus basal values). *$p<0.05$ versus control

DISCUSSION

VII. DISCUSSION

The objective of the present work was to assess whether the benefits associated with the consumption of the traditional Mediterranean diet (TMD), virgin olive oil (VOO), and olive oil phenolic compounds, on cardiovascular disease risk could be mediated through a nutrigenomic effect. From our results, several genes related to insulin-resistance, inflammation, and oxidative stress modulated their expression. Figure 7 summarizes the modulated genes from each intervention.

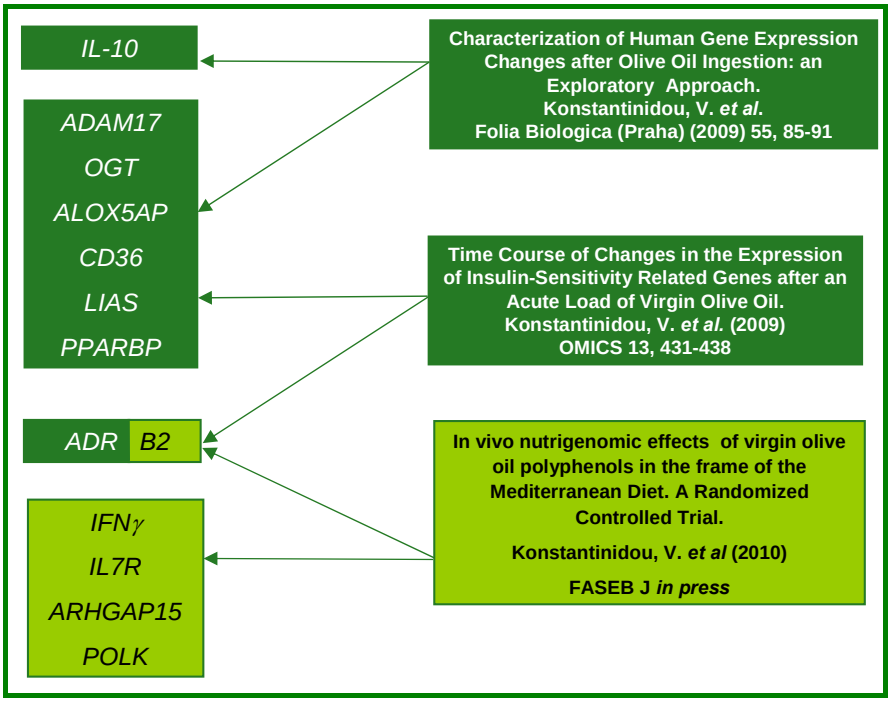

Figure 7. Genes modulated by Mediterranean diet, virgin olive oil and its phenolic compounds

In all the studies presented here, gene expression changes were evaluated in peripheral blood mononuclear cells (PBMNCs) of healthy volunteers. PBMNCs were selected to explore gene expression changes because they are: 1) critically involved in the atherosclerotic plaque formation; 2) easily available from volunteers, considering their feasibility of collection and for deontological reasons; and 3) directly collected from BD Vacutainer CPT™ tubes, thus ensuring rapid PBMNCs isolation and avoiding *ex vivo* gene activation.

To achieve our goal, we first designed a linear postprandial and long-term linear intervention study with VOO, as has been previously described (Methods, page 59). Samples were obtained on the intervention day to study the postprandial response after an acute VOO consumption, and after 3 weeks to study the long-term, sustained, and moderate VOO consumption. The latter task was not an objective of the present work and is described elsewhere (172).

From the samples obtained on the intervention day, after acute VOO ingestion, we assessed a) the postprandial (6h) whole genome gene expression changes (publication No 1) and b) the postprandial time-course of changes in seven insulin-related genes in PBMNCs of healthy individuals (publication No 2).

Microarray data demonstrated that the highest level of up-regulation was observed in genes related to metabolism, cellular processes, and cancer. The highest level of down-regulation was observed in

genes related to DNA damage and carcinogenesis. In both long-term (172) and postprandial state, the gene ontology analyses of the differentially expressed genes indicated that consumption of VOO could elicit changes in the expression of genes related to inflammation, oxidative and DNA damage, apoptosis, and cellular metabolism.

However, when the obtained microarray data were validated by qRT-PCR, some inconsistency between the two different methods was observed. The lack of concordance was observed only in down-regulated genes measured by microarrays, and was in agreement with data from our previous work assessing the long-term gene expression changes after sustained VOO consumption (172). Microarray data also showed that several genes, related with oxidative stress-associated diseases, such as cancer (*AKAP13*, *USP48*) and atherosclerosis (*ADAM17*, *IL10*, *OGT*), up-regulated their postprandial (6h) expression in PBMNCs of healthy volunteers, after a single dose of 50mL (44.5g) VOO ingestion.

Publication No 1 was the first exploratory report assessing the human *in vivo* gene expression changes after VOO ingestion. Changes were observed after a real-life dose of olive oil, as it is daily consumed in some Mediterranean areas (94). These results provided a first insight that the protective effect, related to VOO consumption at postprandial state observed in markers for CVD, could be mediated through gene expression changes.

Data from the microarray experiments also pointed out that some insulin-related genes were able to change their expression after both VOO acute and long-term consumption (172). Insulin resistance and compensatory hyperinsulinemia are involved in the development of dyslipidemia, hypertension, impaired fibrinolysis, and other abnormalities that collectively contribute to an increased risk of CVD (173;174). The ability of an individual to cope with a fatty meal, which is largely a postprandial phenomenon, may be a key factor in the development of CVD (175).

Due to this, we examined the time-course of postprandial changes in the expression of some insulin sensitivity-related genes in PBMNCs of healthy volunteers after an acute, oral, raw VOO ingestion (50 mL) (Publication No 2). The evaluated genes were:
1) *ADAM17*, a disintegrin and metallopeptidase domain 17. *ADAM17* is a *TNFα* converting enzyme (also known as *TACE*) which regulates *TNFα* at post-transcriptional level (176). *TNFα* is the major negative regulator of the insulin receptor pathway and *ADAM17* is considered to be a significant target for controlling insulin resistance (177).
2) *ABRB2*, adrenergic beta-2-receptor. *ADRB2* gene encodes for a major lipolytic receptor in human fat cells. A functional expression of β2-adrenergic receptors is considered to be related to a protection against oxidative stress through the promotion of glutathione synthesis (178). Experiments in old rats have shown that insulin treatment increased ADRB2 protein expression (179).

3) *ALOX5AP*, arachidonate 5-lipoxygenase-activating protein. The expression of *ALOX5AP* has also been associated with insulin resistance (180). The mRNA expression of *ALOX5AP*, in the subcutaneous adipose tissue, increased with obesity and normalized following weight reduction (180). A recent study also concluded that genetic variation in the *ALOX5AP* gene contributes to CHD risk in patients with familial hypercholesterolemia (181).

4) C*D36*, CD36 thrombospondin receptor. *CD36* is a fatty acid transporter (182). Soluble CD36 levels have been shown to be closely related with insulin resistance (183) and its deficiency has been reported to underlie insulin resistance in spontaneously hypertensive rats (184).

5) *LIAS*, lipoic acid synthetase. Lipoic acid is a powerful antioxidant (185) that can activate peroxisome proliferator-activated receptors (*PPARα* and *PPARγ*) (186). *PPARγ* is a nuclear hormone receptor playing a crucial role in adipogenesis and insulin sensitization (187). When glucose-treated rats were fed with lipoic acid, an increase in aortic O_2 production, glucose, and insulin resistance, as well as a decrease in *PPARγ* protein in aorta and heart tissues were prevented or attenuated (188;189).

6) *OGT*, O-linked N-acetylglucosamine (O-ClcNAc) transeferase. The *OGT* gene is activated through the insulin signaling pathway (190). Among the several feedback mechanisms involved in attenuating the signaling of sustained insulin action (191;192), the recruitment of *OGT* from the nucleus to the plasma membrane results in the alteration of key signaling molecules and the attenuation of the insulin signal transduction (193).

7) *PPARBP*, peroxisome proliferator-activated receptor binding protein. Enhancing the *PPARγ* interaction with *PPARBP* primary mediated the improvement of insulin resistance by *PPARγ* agonists (194).We also assessed the postprandial changes in glucose, insulin, and lipid oxidative damage.

In agreement with previous reports describing the postprandial time course of glucose and insulin (195), a peak in these parameters was observed at 1h after VOO ingestion, which returned to baseline values at 6 h postprandial. This positive quadratic trend was similar to that followed by the changes in the expression of *CD36*. The opposite pattern (negative quadratic trend) was observed for *OGT* and *ALOX5AP* gene expression changes. In agreement with this, at 1h postprandial, changes in *OGT* and *ALOX5AP* were inversely correlated with the peak of insulin and glucose. The observed increase in *OGT* expression at 6h after VOO ingestion could be related with feedback mechanisms developed to attenuate the signaling of sustained insulin action (193). As has been refered to previously, the expression of *ALOX5AP* has been associated with insulin resistance (180). In agreement with this, in our study, changes in *ALOX5AP* gene expression were related with the peak of glucose and insulin at 1h postprandial.

In addition, an oxidative stress status was present at 6h postprandial, as reflected by the increase in the oxidative damage to lipids and LDL. This observation has been previously noted after a single dose of VOO ingestion of 40mL (75) and 50mL (74). Despite a similar

quadratic trend observed for insulin and *CD36* gene expression changes, we did not observe a relationship between both parameters at 1h postprandial. At this time, the observed upregulation of *CD36*, a fatty acid transporter (182), could be related with the postprandial increase in plasma fatty acids after the VOO ingestion, as a related mechanism to the fatty acid uptake (196). The increase in *CD36* could also be associated with the satiety response after olive oil ingestion. The activation of the small intestinal lipid messenger oleoylethanolamide, enabled by *CD36*-mediated uptake of dietary oleic acid, serves as a molecular sensor, linking fat ingestion to satiety (197). Induction of *CD36* in response to these diverse stimuli, including oxLDL, is dependent on the activation of *PPARγ* (198). In agreement with this, a direct relationship was observed between *CD36* and *PPARBP* gene expressions at 1h and 6h after olive oil ingestion.

The linear increase observed in triglycerides and oxLDL from baseline to 6h, at the postprandial state, was similar to that observed for *ADAM17*, *ADRB2*, *LIAS*, and *PPARBP* gene expression changes. Changes in the expression of *LIAS* and *PPARBP* were directly related at 6h postprandial. The protein encoded by the *LIAS* gene belongs to the lipoic acid synthetases family. Lipoic acid is a powerful antioxidant that can activate peroxisome proliferator-activated receptors (*PPARa* and *PPARγ*) (186). Thus, it could be hypothesised that the upregulation in the expression of *LIAS*, and that of *PPARBP*, a *PPARγ* co-activator, could be one of the

feedback mechanisms for counteracting the postprandial oxidative stress involved in the development of insulin resistance (83).

Activation of *PPARγ* by *PPARBP* may also increase insulin sensitivity by downregulating the expression of *TNFa* (199). *TNFa* is the major negative regulator of the insulin receptor pathway. *TNFa* is regulated at post-transcriptional level by the *TNFa* converting enzyme (*ADAM17*, also known as *TACE*) (176). A direct relationship was observed among changes in the expression of *LIAS*, *PPARB*, and *ADAM17* genes. This fact reinforces their involvement in a hypothetical pathway. The inverse relationship observed between the peak in plasma hydroxytyrosol at 1h after olive oil ingestion and *ADAM17* gene expression is in agreement with previous data showing a decrease of *TNFa* levels by hydroxytyrosol (200;201).

A functional expression of β_2-adrenergic receptors is considered to be related to a protection against oxidative stress through the promotion of glutathione synthesis (178). From our results, the *ADRB2* gene expression was inversely correlated with oxLDL and triglycerides at 6h after VOO ingestion, reinforcing the above-mentioned possible protection. Thus, the *ADRB2* upregulation, observed at 6h after VOO ingestion, could be promoted by the postprandial insulin peak. This could account for a delayed adrenergic signal for both adipocyte differentiation and oxidative stress protection (178).

In one of our previous works (172), all the above-mentioned genes, with the exception of *ALOX5AP* and *ABRB2,* had also increased their expression after 3 weeks of sustained and moderate (25mL per day) olive oil consumption. The strongest responders were *ADAM17*, *LIAS*, *OGT*, and *PPARBP* ($p<0.001$). A postprandial oxidative stress response occurs after acute 50mL VOO ingestion (74). This postprandial oxidative stress is involved in the development of insulin resistance (83). Thus, it could be hypothesized that several feedback mechanisms, which include the up or down regulation of those genes, exist for counteracting this stress. If so, that could explain the differences observed in the expression of those genes after acute and regular or moderate long-term consumption of VOO.

In summary, significant changes in the expression of candidate genes related to insulin sensitivity occurred in human PBMNCs after an oral fat load of 50mL of VOO. Changes in gene expression were related among them, following physiological inter-relationships, and also with phenotypic markers such as insulin, lipid profile, and lipid oxidative damage.

From the previous reports (Publications 1 and 2), we observed that VOO could elicit changes in the expression of atherosclerosis-related genes. However, it has not been assessed which particular components of olive oil could be responsible for this fact. It is also important to point out that in real-life conditions VOO is never consumed alone, but within a whole dietary pattern, the

Mediterranean diet. In order to assess this issue, we performed a randomized, controlled trial to evaluate the nutrigenomic effects of the phenolic compounds of olive oil in the context of a traditional Mediterranean diet in healthy individuals (Publication No 3).

The aim of this latter study (Publication No 3) was to assess whether benefits associated with the Traditional Mediterranean Diet (TMD) and VOO consumption could be mediated through changes in the expression of atherosclerosis-related genes. Adherence to the traditional Mediterranean diet (TMD) was examined to assess whether it modulates 1) the expression of atherosclerosis-related genes, 2) systemic oxidative stress and 3) inflammation markers, focusing on the impact of olive oil phenolic compounds. Our results indicate that the TMD decreased the lipid oxidative and inflammatory status. TMD also decreased the expression of genes related to inflammation processes (*IFNγ*, *ARHGAP15,* and *IL7R*), oxidative stress (*ADRB2*), and DNA damage (*POLK*) in PBMNCs. All the above-mentioned effects, with the exception of the decrease in the *POLK* expression, were particularly observed when VOO, rich in phenolic compounds, was present in the TMD pattern.

This work provides first-time evidence of the *in vivo* nutrigenomic effect of olive oil phenolic compounds down-regulating pro-atherogenic genes in humans. Furthermore, and to the best of our knowledge, the *in vivo* human nutrigenomic effect of the Mediterranean diet, in healthy individuals, has not been previously reported. When results were disclosed by sex, the decrease in the

gene expression changes, after the interventions, were particularly observed in the female groups. A previous work has reported gender differences in PBMNCs gene expression, with higher expression of *SOD1* and *SOD2* in healthy males (31). In the present work, however, the low number of males in some groups could account for the gender differences observed.

The Mediterranean diet, in which the main source of fat is olive oil, is well known to be associated with a low prevalence of CVD (7), cancer (202), and inflammatory diseases (203;204). Inflammation is heavily involved in the onset and development of atherosclerosis (205). The decrease in systemic inflammatory markers, and in the expression of genes related with inflammatory processes, observed in the present study is in agreement with previous results described above, concerning the protective effect of the Mediterranean diet and olive oil phenolics on inflammation.

The decrease in interferon gamma (IFNγ) was observed both at phenotypic and gene expression levels. IFNγ is considered to be a key inflammatory mediator for inducing IL6, a prime regulator of CRP synthesis in the liver (206). The observed down-regulation of the *IFNγ* expression in PBMNCs is in agreement with that observed in our previous results after a single dose of VOO (Publication No 1). The protein encoded by the *IL7R* gene is a receptor for interleukin 7 (IL7) which has been associated with inflammatory processes (207;208). IL7 has been shown to enhance the expression of chemokines in PBMNCs (209). A recent study has shown an up-

regulation of stress-response genes, such as *IL7R* and *POLK*, after carbon ion irradiation in murine tumour models (210).

POLK is a DNA repair gene which copies undamaged DNA templates and is unique among human Y-family DNA polymerases (211). Somatic DNA mutations, promoted by DNA oxidation, are considered to be a crucial step in carcinogenesis as well as to be involved in the atherosclerotic processes (116;212). In agreement with the EUROLIVE results (30), the down-regulation of the *POLK* gene expression, observed in our study, was associated with the TMD intervention, but not with the olive oil polyphenol content. All these data suggest a protective role for the MUFA or other minor components of the olive oil on DNA oxidation and damage within the frame of the Mediterranean diet.

The *ADRB2* gene was also down-regulated after 3 months of TDM intervention, particularly in the TDM+VOO intervention group. At postprandial state, however, we observed an increase in the *ADRB2* gene expression in human PBMNCs after 50 mL of VOO ingestion (Publication No 2). As was described there, the 6h postprandial *ADRB2* expression was inversely correlated with plasma oxidized low density lipoproteins and triglyceride concentrations, and could be considered as a compensatory mechanism for the increased postprandial oxidative stress.

Summary of the Discussion

We performed two trials to assess, firstly, whether VOO was able to promote changes in the expression of cardiovascular risk-related genes. Results of this work showed that VOO consumption elicited changes in the expression of genes related to cardiovascular-risk processes (*ADAM17*, *ADRB2*, *AKAP13, ALOX5AP, CD36, DCLRE1C*, *IL10*, *LIAS, OGT*, *POLK*, *PPARBP, USP48*,) such as insulin resistance, oxidative stress, and inflammation. The gene expression changes were observed in a protective mode for counteracting these situations.

When the effects of olive oil were examined within the frame of a traditional Mediterranean dietary pattern, a down-regulation in the expression of atherosclerosis-related genes (*ARHGAP15*, *ADRB2, IFNγ*, *IL7R*, and *POLK*) was observed, in human PBMNCs, after a 3-month adherence to this diet. Our results pointed to a down-regulation of pro-atherogenic genes, within the frame of the TMD, particularly when VOO, rich in phenolic compounds, was present in the TMD pattern. Changes in gene expression were concomitant with decreases in lipid oxidative damage and systemic inflammation markers. Figure 8 shows the modulated genes and the biological processes in which they are involved.

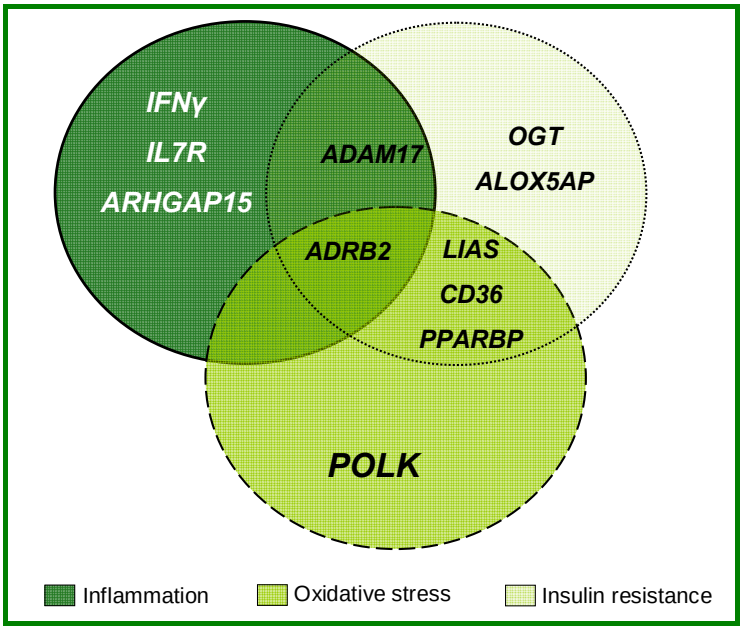

Figure 8. Genes modulated by Mediterranean diet
and/or virgin olive oil and the biological processes to which they belong

Our results support the idea that the benefits associated with the Mediterranean diet, and olive oil phenolic compound consumption, on CVD risk can be mediated through changes in the expression of atherosclerosis-related genes. Data from our studies provide further evidence to recommend both the traditional Mediterranean diet and rich-polyphenol olive oils, such as virgin olive oil, as a useful tool for the prevention of atherosclerosis.

Strengths and limitations

One strength of the approach used in the present dissertation was that real-life conditions applied both in the virgin olive oil (Publications 1 and 2) and in the traditional Mediterranean diet with

virgin and ordinary olive oil (Publication 3) interventions. In the latter study, we worked with a whole dietary pattern. Intervention studies with whole dietary patterns are now claimed as being more representative of a broader picture of food and nutrient consumption, and may thus be more predictive of disease risk than individual foods or nutrients (213). We have used both the dietary pattern and the single component approach in our study (Publication No 3).

Another strength of the approach used was the matrix and the dose of antioxidants. In all studies, changes in gene expression were modest, as was expected in real-life intervention conditions. Virgin olive oil is a crucial component of the traditional Mediterranean dietary pattern and is generally consumed in moderate doses.

A limitation of the virgin olive oil intervention was that, although the subjects' baseline data had served as a within-subject control, there was a lack of control group for the intervention itself. Due to this, the observed effects on gene expression could be secondary, not only to the VOO ingestion, but also to a time course effect on circadian regulated genes (214), and also to physiological changes following any fat meal intake.

Concerning the traditional Mediterranean diet with virgin and ordinary olive oil interventions, one of our trial's strengths is that the study design is able to provide first-level scientific evidence (14) reflecting eating habits of community-dwelling individuals.

Moreover, we were able to assess the compliance of participants to the olive oil assigned through the measurements of olive oil phenolic compounds in urine. We worked against our own hypothesis, by using a current Spanish dietary pattern in our control group, to maintain real-life conditions in all groups.

A limitation of the intervention was the inability to assess potential interactions between the olive oil and other diet components that might affect the generalization of the results. However, the effects of food components are subtle and must be considered in the context of chronic exposure. Whether additional or different effects would have been observed over longer periods is unknown. A longer study, however, could have impaired the compliance of the participants

CONCLUSIONS – FUTURE PLANS

VIII. CONCLUSIONS

1. A 50 mL virgin olive oil dose promote *in vivo* changes in the expression of genes (*ADAM17*, *ADRB2, ALOX5AP*, *CD36*, *IL10, LIAS*, *OGT, PPARBP*,) related with cardiovascular risk processes such as insulin resistance, oxidative stress, and inflammation in peripheral blood mononuclear cells of healthy volunteers.
2. A 3-month intervention with a traditional Mediterranean diet promotes a down-regulation in the expression of cardiovascular risk-related genes (*ADRB2*, *ARHGAP15*, *IFNγ*, *IL7R*, and *POLK*) in peripheral blood mononuclear cells of healthy volunteers.
3. Olive oil phenolic compounds, within the frame of the traditional Mediterranean diet, enhance the health protective nutrigenomic effect concluded previously (Conclusions No 2).
4. Our results support the idea that the benefits associated with a Mediterranean diet, virgin olive oil, and olive oil phenolic compound consumption on cardiovascular risk could be mediated through changes in the expression of cardiovascular risk-related genes.
5. Our results provide further evidence to recommend the traditional Mediterranean diet and olive oils rich in phenolic compounds, such as virgin olive oil, as a useful tool for the prevention of atherosclerosis.

IX. FUTURE PLANS

Life is like riding a bicycle.
To keep your balance you must keep moving.
Albert Einstein (1879–1955)

The present dissertation results provide, for the first time, evidence of a virgin olive oil nutrigenomic health-protective effect within the frame of the traditional Mediterranean diet. These results, however, should be replicated. Due to this, we are performing a similar work in a subsample of the PREDIMED study including cardiovascular-risk individuals.

These results also provide, for the first time, evidence of an olive oil phenolic compound nutrigenomic health-protective effect. These results should also be replicated. We are planning to assess the nutrigenomic effect of olive oils, with differences in their phenolic content, within the frame of the EUROLIVE study.

Changes in gene expression do not necessarily imply that the codified proteins are also modified towards a health-protective mode. Due to this, concomitant proteomic, metabolomic and epigenomic analyses, within the frame of all the above-mentioned studies, will be of interest. Such analyses would permit the establishment of a direct genotypic-phenotypic "link" which could explain, in more detail, the Mediterranean diet and the virgin olive oil protective effects towards CVD prevention.

The results of the present dissertation allow the hypothesis that dietary patterns could affect and modulate the gene expression of a group of genes rather than individual, separated genes. Further investigation concerning clusters of biologically related genes (i.e. pathways) is required. When the state-of-the-art permits it, it would be possible to assess how diet might affect the regulation of related pathways of genes after a dietary intervention.

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X. BIBLIOGRAPHY

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