UNIVERSIDAD DE CÓRDOBA
DEPARTAMENTO DE MEDICINA

INFLUENCE OF A MEDITERRANEAN DIET SUPPLEMENTED IN COENZYME Q_{10} ON METABOLOMIC PROFILE AND ON THE EXPRESSION OF ANTIOXIDANT, PRO-INFLAMMATORY AND ENDOPLASMIC RETICULUM STRESS-RELATED GENES IN AN ELDERLY POPULATION

Trabajo presentado por
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Licenciada en Bioquímica, para optar al grado de
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Dirigido por
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TITULO: *Influence of a mediterranean diet supplemented in coenzyme Q10 on metabolomic profile and on the expression of antioxidant, pro-inflammatory and endoplasmic reticulum stress-related genes in an elderly population*

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TÍTULO DE LA TESIS: INFLUENCE OF A MEDITERRANEAN DIET SUPPLEMENTED IN COENZYME Q_{10} ON METABOLOMIC PROFILE AND ON THE EXPRESSION OF ANTIOXIDANT, PRO-INFLAMMATORY AND ENDOPLASMIC RETICULUM STRESS-RELATED GENES IN AN ELDERLY POPULATION

DOCTORANDA: LORENA GONZÁLEZ DE LA GUARDIA

INFORME RAZONADO DE LOS DIRECTOR/ES DE LA TESIS

El trabajo de tesis realizado por Lorena González de la Guardia, bajo nuestra dirección en la Unidad de Lípidos y Arteriosclerosis del Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC)/Hospital Universitario Reina Sofía de Córdoba/Universidad de Córdoba, se ha basado en el estudio del perfil metabolómico y de los cambios en la expresión de genes relacionados con estrés oxidativo, inflamación y estrés del retículo endoplásmico, en sangre periférica de pacientes mayores de 65 años, tras la ingesta de dietas con diferente composición en ácidos grasos, una de ellas suplementada en Coenzima Q_{10}. Los resultados obtenidos responden a los objetivos planteados inicialmente.
En cuanto a la difusión de los resultados de la tesis, la doctoranda ha participado en 8 congresos científicos, presentando comunicaciones orales y de tipo poster. Asimismo, dichos resultados ya se han publicado o están en vías de publicación, en colaboración con sus tutores de tesis y otros colaboradores de nuestro grupo de investigación: 2 artículos SCI publicados y 2 en vías de publicación.
Las publicaciones derivadas de esta tesis doctoral son las siguientes:

Yubero-Serrano EM, **Gonzalez-Guardia L**, Rangel-Zuñiga O, Delgado-Lista J, Gutierrez-Mariscal FM, Perez-Martinez P, Delgado-Casado N, Cruz-Teno C, Tinahones FJ, Villalba JM, Perez-Jimenez F, Lopez-Miranda J. *Mediterranean diet supplemented with coenzyme Q_{10} modifies the expression of proinflammatory and endoplasmic reticulum stress-related genes in elderly men and women.* J Gerontol A Biol Sci Med Sci. 2012 Jan; 67(1):3-10.

Yubero-Serrano EM, **Gonzalez-Guardia L**, Rangel-Zuñiga O, Delgado-Casado N, Delgado-Lista J, Perez-Martinez P, Garcia-Rios A, Caballero J, Marin C, Gutierrez- Mariscal FM, Tinahones FJ, Villalba JM, Tunez I, Perez-Jimenez F, Lopez-Miranda J. *Postprandial antioxidant gene expression is modified by Mediterranean diet supplemented with coenzyme Q_{10} in elderly men and women*. Age (Dordr). 2013 Feb; 35(1):159-70.

Lorena González-Guardia, Elena María Yubero-Serrano, Javier Delgado-Lista, Pablo Perez-Martinez, Antonio Garcia-Rios, Carmen Marin, Antonio Camargo, Nieves Delgado-Casado, Helen M Roche, Francisco Perez-Jimenez, Lorraine Brennan, José López-Miranda. *Effects of the Mediterranean diet supplemented with coenzyme Q_{10} on metabolomic profiles in elderly men and women*. Under review in Journal Gerontology: Medical Sciences.

Lorena González-Guardia, Elena María Yubero-Serrano, Oriol Rangel-Zuñiga, Carmen Marin, Antonio Camargo, Pablo Pérez-Jiménez, Javier Delgado-Lista, Francisco Gómez-Delgado, Antonio Garcia-Rios, Francisco José Tinahones, Helen M Roche, José López- Miranda. *Influence*

of endothelial dysfunction on telomere length in subjects with metabolic syndrome: LIPGENE study. Under review in Age (Dordr).

A nuestro juicio, el trabajo realizado por la doctoranda Lorena González de la Guardia reúne los méritos suficientes para ser defendido antes el tribunal correspondiente y poder optar al grado de Doctor.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 7 de MAYO de 2014

Firma del/de los director/es

Fdo.: Prof. José López Miranda

Fdo.: Dra. Elena María Yubero Serrano

"Si buscas resultados distintos,
no hagas siempre lo mismo"
Albert Einstein

"I have not failed.
I've just found 10000 ways that won't work"
Thomas Alva Edison

"Vive como si fueras a morir mañana.
Aprende como si fueras a vivir siempre"
Mahatma Gandhi

ABBREVIATIONS

The most used abbreviations throughout the text are outlined below:

^{1}H-NMR: Proton nuclear magnetic resonance spectroscopy

Apo A1: Apolipoprotein A1

Apo B: Apolipoprotein B

ARE: Antioxidant-response element

ATF6: Activating transcription factor 6

BiP/Grp78: Binding immunoglobulin/Glucose-regulated protein 78 kDa

BP: Blood pressure

CAT: Catalase

CoQ: Coenzyme Q_{10}

CRT: Calreticulin

eIF2α: Eukaryotic initiation factor 2

ER: Endoplasmic reticulum

ERAD: ER-associated degradation

GSH: Glutathione

GPx: Glutathione peroxidase

HDL: High-density lipoproteins

IKK-β: Beta subunit of IκB kinase

IL-1β: Interleukin 1-β

IRE1: Inositol-requiring kinase 1

IRH: Ischemic reactive hyperemia

IκB-α: alpha subunit of NF-κB inhibitor

JNK-1: c-Jun N-terminal kinase-1

Keap-1: Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associating protein 1

LDL: Low-density lipoprotein

LPL: Low density lipoproteins

LPO: Lipid peroxidation

Med diet: Mediterranean diet

Med+CoQ diet: Mediterranean diet supplemented with coenzyme Q_{10}

MetS: Metabolic syndrome

MMP-9: Matrix metalloproteinase-9

MUFA: Monounsaturated fatty acids

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor kappa B

NO: Nitric oxide

Nrf2: Nuclear factor erythroid 2 – related factor 2

oxLDL: Oxidized low-density lipoprotein

OxS: Oxidative stress

$p22^{phox}$: $p22^{phox}$ nicotinamide adenine dinucleotide phosphate oxidase subunit

$p47^{phox}$: $p47^{phox}$ nicotinamide adenine dinucleotide phosphate oxidase subunit

PBMC: Peripheral blood mononuclear cells

PC: Protein carbonyl

PCA: Principal component analysis

PERK: Double-stranded RNA-activated protein kinase-like ER kinase

PLS-DA: Partial least squares discriminant analysis

PUFA: Polyunsaturated fatty acids

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RTL: Relative telomere length

SFA diet: Saturated fatty acid-rich diet

SOD: Superoxide dismutase

sXBP-1: X-box binding protein 1

TC: Total cholesterol

TG: Triglycerides

TRL: Triglycerides-rich lipoproteins

TrxR: Thioredoxin reductase

UPR: Unfolded protein response

VLDL: Very low density lipoproteins

VIP: Variable importance in the projection

VOO: Virgin olive oil

I. ABSTRACT

RESUMEN

I. ABSTRACT

Introduction: Accelerated aging population is a global phenomenon that is expanding over the last decades. The aging process is known to be accompanied by an increase in oxidative stress (OxS) and endoplasmic reticulum (ER) stress, which have been associated with the activation of inflammatory pathways. Nutrition and other lifestyle habits have a major impact on the risk of developing chronic diseases that appear more frequently in the elderly. Diet, particularly fat quality, may affect the reactive oxygen species (ROS) production and, in consequence, both OxS and ER stress. Due to the fact that postprandial state is the main physiological condition of the occidental society, it is crucial to study the postprandial effect induced by the long-term consumption of different dietary patterns. In this connection, the consumption of a diet with antioxidant properties such as the Mediterranean diet (Med diet) and supplementation with a natural antioxidant compound such as Coenzyme Q_{10} (CoQ) have been proved to decrease the postprandial OxS by reducing processes of cellular oxidation.

Hypothesis: The consumption of diets with different fat quality modulates the processes related to OxS, ER stress and the inflammatory response in terms of gene expression in an elderly population, during both fasting and postprandial state. Moreover, the supplementation with a natural antioxidant such as CoQ further improves this modulation.

Main objective: To evaluate whether the long-term consumption of three dietary patterns with different fat quality (Med diet with or without supplementation in CoQ and saturated fatty acid-rich diet) modulates OxS in fasting and postprandial state by modifying the gene expression of Nrf2, $p22^{phox}$ and $p47^{phox}$ NADPH oxidase subunits, SOD1, SOD2, Gpx1, TrxR and

the protein levels of cytoplasmic and nuclear Nrf2 and Keap-1 in peripheral blood mononuclear cells (PBMC) from elderly people.

Secondary objectives: 1) To investigate the effect of the long-term consumption in fasting and postprandial state of the three dietary patterns on the expression of genes related to ER stress (sXBP-1, CRT, BiP/Grp78) in PBMC from elderly people. **2)** To study the effect of the long-term consumption in fasting and postprandial state of the three dietary patterns on the expression of genes related to inflammatory response (p65 subunit of NF-κB, IκB-α, IKK-β, MMP-9, IL1-β and JNK-1) in PBMC from elderly people. **3)** To investigate whether diets with different fat quality and supplementation with CoQ affect the metabolomic profile in urine samples from elderly people. **4)** To study whether there is a direct relationship between endothelial function, through OxS mechanisms, and relative telomere length (RTL) in a cross-sectional study involving an elderly population and a subgroup of metabolic syndrome (MetS) participants.

Subjects, design and methodology: 20 elderly participants (≥ 65 years) were randomly assigned to receive, in a crossover design, three isocaloric diets with different fat quality for 4-week period each. The three diets were as follows: **A.** Mediterranean diet supplemented with 200mg of CoQ (Med+CoQ diet), **B.** Mediterranean diet supplemented with placebo (Med diet) and **C.** Western diet rich in saturated fat (SFA diet). At the end of the dietary intervention period, participants were given a fatty breakfast with the same fat composition as they have consumed in each intervention dietary period. Subsequent blood samples were drawn at 0 hours (before the test meal), at 2 and 4 hours. We determined the CoQ plasma levels, Nrf2 and Keap-1 protein levels, and the expression of genes related to OxS (Nrf2, $p22^{phox}$, $p47^{phox}$, SOD1, SOD2, Gpx1, TrxR), ER stress (sXBP-1, CRT, BiP/Grp78) and inflammatory response (p65, IκB-α,

IKK-β, MMP-9, IL1-β, JNK-1) in PBMC. In urine samples, we analyzed the metabolomic profile at baseline (pre-intervention) and after a 12 h fast (time 0, post-intervention) by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR).

Aditionally, a cross-sectional study was conducted with the involvement of the 20 elderly participants and 66 MetS participants from the LIPGENE cohort, who were divided into four groups by quartiles of relative telomere length (RTL). We measured ischemic reactive hyperemia (IRH), total nitrite (NO) and protein carbonyl (PC) levels, superoxide dismutase (SOD) and gluthatione peroxidase (GPx) plasma activities.

Results: The long-term consumption of Med diet, with or without CoQ supplementation, reduced the fasting gene expression of $p22^{phox}$, SOD2 and Gpx1. Moreover, the supplementation of CoQ had an additive effect on the Med diet resulting in a greater fasting increase in cytoplasmic Nrf2 and Keap-1 compared to the SFA diet. Regarding to the postprandial state, the Med diet with or without CoQ supplementation, reduced the gene expression of Nrf2, SOD (SOD1 and SOD2), TrxR and NADPH-oxidase subunits ($p22^{phox}$ and $p47^{phox}$). Additionally, the Med+CoQ diet decreased postprandial Nrf2 and Gpx1 gene expression and also increased the postprandial cytoplasmic Nrf2 and Keap-1 protein levels, in comparison with the SFA diet. We found that the long-term intake of the Med diet and the Med+CoQ diet, reduced the postprandial gene expression of sXBP-1 and BiP/Grp78, and the fasting gene expression of CRT when comparing to the SFA diet. Furthermore, we found lower gene expression of MMP-9, IL-1β and JNK-1 at fasting and postprandial state after Med diet intake, with or without CoQ supplementation. Notably, CoQ supplementation produced and additive effect on the Med diet by decreasing the postprandial gene expression of p65 (RelA) and IKK-β compared to SFA diet in PBMC from elderly people. As a result of the

metabolomic analysis, we found differences in the urinary profile of elderly women when comparing the Med+CoQ diet and the SFA diet, with greater excretion of hippurate levels after Med+CoQ diet and higher excretion of phenylacetylglycine levels after SFA diet consumption. Interestingly, hippurate excretion was positively correlated with CoQ and β-carotene plasma levels and inversely related to Nrf2, thioredoxin, SOD1 and $gp91^{phox}$ subunit of NADPH oxidase gene expression, following Med+CoQ diet consumption. By contrast, phenylacetylglycine excretion was inversely related to CoQ plasma levels and positively correlated with isoprostanes urinary levels following SFA diet intake.

Regarding to the cross-sectional study involving the elderly population and the MetS participants, we found that IRH and NO plasma levels were higher in participants with longer RTL (quartile 3 and 4), while PC plasma levels, GPx and SOD plasma activities were lower in quartile 4 subjects (longest RTL). Additionally, participants with longer RTL had greater homeostatic model assessment-β (HOMA-β) level and lower triglycerides plasma levels.

Main conclusion: The long-term consumption of Med diet contributes to the redox homeostasis of elderly people at fasting and postprandial state. Supplementation with exogenous CoQ enhances this contribution by modifying antioxidant protein levels and reducing the expression of genes related to OxS processes.

Secondary conclusions: 1) The long-term consumption of Med diet, with or without CoQ supplementation, modulates ER stress by reducing the postprandial expression of genes associated with the unfolded protein response. **2)** The intake of Med diet, with an additive effect provided by CoQ supplementation, modifies the inflammatory response by decreasing the fasting and postprandial expression of pro-inflammatory genes in an elderly

population. **3)** The consumption of Med+CoQ diet modifies the metabolomic urinary profile of elderly women through greater excretion of metabolites associated with antioxidant properties, while metabolites excreted after the intake of a SFA diet are related to increased biomarkers of oxidative damage. **4)** There is a direct relationship between endothelial function, OxS and RTL in elderly people and MetS subjects, where high levels of oxidative damage may induce endothelial dysfunction and may increase telomere attrition.

I. RESUMEN

Introducción: El envejecimiento acelerado de la población es un fenómeno mundial que se está produciendo progresivamente a lo largo de las últimas décadas. Este proceso se asocia con un incremento del estrés oxidativo (EO) y de estrés del retículo endoplásmico (RE), los cuales a su vez están relacionados con la activación de la respuesta inflamatoria. La nutrición y otros hábitos de vida ejercen un gran impacto sobre el riesgo de desarrollar enfermedades crónicas que se presentan con mayor frecuencia en personas de edad avanzada. Se ha demostrado que la dieta, particularmente la calidad de la grasa de la misma, afecta a la producción de especies reactivas de oxígeno y, en consecuencia, a los procesos de EO y de estrés del RE. Debido al hecho de que el estado postprandial constituye la situación fisiológica habitual en la que se encuentra el ser humano a lo largo del día en las sociedades occidentales, es necesario estudiar el efecto postprandial inducido por el consumo de diferentes modelos dietéticos. En este sentido, el consumo de una dieta Mediterránea (dieta Med) con propiedades antioxidantes y la suplementación con un compuesto natural antioxidante como la Coenzima Q_{10} (CoQ) contribuyen a reducir los procesos de oxidación celular, disminuyendo a su vez el EO postprandial.

Hipótesis: El consumo de dietas con distinta calidad grasa puede modular la expresión génica de parámetros asociados con mecanismos de EO, estrés del RE y de respuesta inflamatoria en ayunas y durante el período postprandial en una población de edad avanzada. Además, la suplementación con un antioxidante natural como la CoQ podría aportar un beneficio adicional en estos parámetros.

Objetivo principal: Evaluar si el consumo de tres modelos dietéticos con distinto tipo de grasa (dieta Med con o sin suplementación en CoQ y una dieta rica en ácidos grasos saturados) modula el EO a largo plazo en ayunas y durante el estado postprandial modificando la expresión génica de Nrf2, subunidades $p22^{phox}$ and $p47^{phox}$ de la NADPH oxidasa, SOD1, SOD2, Gpx1, TrxR; así como los niveles citoplasmáticos y nucleares de Nrf2 y Keap-1 en células mononucleares de sangre periférica (PBMC) de personas de edad avanzada.

Objetivos secundarios: 1) Investigar el efecto a largo plazo en ayunas y en estado postprandial del consumo de tres modelos dietéticos con distinto tipo de grasa sobre la expresión de genes relacionados con el estrés del RE (sXBP-1, CRT, BiP/Grp78) en PBMC de personas de edad avanzada. **2)** Estudiar el efecto a largo plazo en ayunas y en estado postprandial del consumo de tres modelos dietéticos con distinto tipo de grasa sobre la expresión de genes relacionados con la respuesta inflamatoria (subunidad p65 de NF-κB, IκB-α, IKK-β, MMP-9, IL1-β y JNK-1) en PBMC de personas de edad avanzada. **3)** Investigar si el consumo de modelos dietéticos con distinto tipo de grasa y suplementación en CoQ afecta al perfil metabolómico en muestras de orina de una población de edad avanzada. **4)** Estudiar si existe asociación directa entre la función endotelial, mediante mechanismos de EO, y la longitud relativa del telómero (RTL) en una población de edad avanzada y un subgrupo de participantes con síndrome metabólico (SMet).

Sujetos, diseño y metodología: 20 voluntarios con edad superior a 65 años fueron sometidos de forma randomizada y cruzada, a tres períodos de intervención dietética de cuatro semanas de duración cada uno. Las tres dietas fueron las siguientes: **A.** Dieta Mediterránea suplementada con 200mg de CoQ_{10} (dieta Med+CoQ), **B.** Dieta Mediterránea suplementada con placebo

(dieta Med), **C.** Dieta rica en grasa saturada (dieta SFA). Tras cada periodo de intervención, los voluntarios consumieron una sobrecarga grasa de similar composición que la dieta a la que habían sido asignados, y se les realizó extracciones sanguíneas a las 0 horas (antes de tomar la sobrecarga), a las 2 y a las 4 horas. Se determinaron las concentraciones plasmáticas de CoQ, los niveles proteicos de Nrf2 y Keap-1, así como los niveles de expresión de genes relacionados con el EO (Nrf2, $p22^{phox}$, $p47^{phox}$, SOD1, SOD2, Gpx1, TrxR), estrés del RE (sXBP-1, CRT, BiP/Grp78) y la respuesta inflamatoria (p65, IκB-α, IKK-β, MMP-9, IL1-β, JNK-1) en PBMC. En muestras de orina, se analizó el perfil metabolómico en el tiempo basal (pre-intervención) y en ayuno de 12 horas (tiempo 0h, post-intervención) mediante espectroscopía de resonancia magnética nuclear (^{1}H-NMR).

Además, se llevó a cabo un estudio comparativo transversal en la población de 20 personas de edad avanzada y en 66 participantes con SMet de la cohorte LIPGENE, los cuales fueron agrupados en cuartiles según su longitud relativa telomérica. Se determinó la hiperemia reactiva post-isquémica (IRH), los niveles de nitritos totales (NO), los niveles de proteínas carboniladas (PC) y las actividades plasmáticas de las enzimas SOD y GPx.

Resultados: El consumo a largo plazo de una dieta Med, con o sin suplementación con CoQ, disminuyó la expresión génica de $p22^{phox}$, SOD2 y Gpx1 en el estado de ayuno. Además, la suplementación con CoQ ejerció un efecto aditivo sobre la dieta Med, resultando en un incremento de la fracción citoplasmática de Nrf2 y Keap-1 en comparación con la dieta SFA. Con respecto al estado postprandial, la dieta Med con o sin suplemento de CoQ, disminuyó la expresión génica de Nrf2, SOD (SOD1 y SOD2), TrxR y de las unidades de la NADPH-oxidasa ($p22^{phox}$ y $p47^{phox}$). El consumo de la dieta Med+CoQ indujo un descenso postprandial en los niveles de expresión génica de Nrf2 y Gpx1, así como un incremento de los niveles proteicos de Keap-1 y

Nrf2 citoplasmático en comparación con la dieta SFA. Adicionalmente, observamos que la ingesta a largo plazo de la dieta Med y de la dieta Med+CoQ, disminuyó los niveles de expresión génica postprandial de sXBP-1 y BiP/Grp78, así como los niveles de CRT en ayunas en comparación con la dieta SFA. La expresión génica de MMP-9, IL-1β y JNK-1 disminuyó en ayunas y en estado postprandial tras la ingesta de la dieta Med, con o sin suplementación en CoQ. Notablemente, la CoQ produjo un efecto aditivo sobre la dieta Med reduciendo la expresión génica de p65 (RelA) e IKK-β en comparación con la dieta SFA en PBMC de personas de edad avanzada. El análisis metabolómico mostró diferencias en el perfil de mujeres de edad avanzada al comparar los efectos producidos por el consumo de la dieta Med+CoQ y la dieta SFA. Se observaron mayores niveles en la excreción del metabolito hipurato tras la dieta Med+CoQ y mayores niveles en la excreción de fenilacetilglicina tras el consumo de la dieta SFA. Los niveles de hipurato excretados se correlacionaron de manera positiva con los niveles plasmáticos de CoQ y β-caroteno y de manera inversa con la expresión génica de Nrf2, tioredoxina, SOD1 y la subunidad $gp91^{phox}$ de la NADPH oxidasa, tras la ingesta de la dieta Med+CoQ. Por el contrario, la excreción de fenilacetilglicina se correlacionó de manera inversa con los niveles plasmáticos de CoQ y de manera directa con los niveles de isoprostanos en orina, tras el consumo de la dieta SFA.

Con respecto al estudio comparativo transversal, encontramos que el IRH y los niveles en plasma de NO eran más elevados en los participantes con mayor RTL (cuartil 3 y 4), mientras que los niveles plasmáticos de PC, y las actividades plasmáticas de GPx y SOD son menores en dichos sujetos. Además, los participantes con RTL más elevado presentaron mayor índice HOMA-β y menores niveles de triglicéridos en plasma.

Conclusión principal: El consumo a largo plazo de una dieta Med contribuye a la homeostasis redox de personas de edad avanzada en ayuno y en el estado postprandial. La suplementación con CoQ mejora esta contribución mediante la modificación de los niveles de proteínas antioxidantes y la disminución en la expresión de genes relacionados con procesos de EO.

Conclusiones secundarias: 1) El consumo a largo plazo de una dieta Med, con o sin suplementación con CoQ, modula el estrés del RE mediante la reducción en la expresión postprandial de genes asociados con el mecanismo de respuesta a proteínas mal plegadas. **2)** La ingesta de la dieta Med, con un efecto aditivo proporcionado por el suplemento en CoQ, modifica la respuesta inflamatoria mediante la reducción en ayuno y en estado postprandial de genes pro-inflamatorios en personas de edad avanzada. **3)** El consumo de la dieta Med+CoQ modifica el perfil metabolómico en orina de mujeres de edad avanzada mediante el aumento en la excreción de metabolitos asociados con propiedades antioxidantes, mientras que los excretados tras la dieta SFA se asocian con un aumento en los biomarcadores de daño oxidativo. **4)** Existe una relación directa entre la función endotelial, el EO y el RTL en personas de edad avanzada y con SMet, donde altos niveles de daño oxidativo podría inducir un aumento en la disfunción endotelial y podría acelerar el acortamiento de los telómeros.

II. INTRODUCTION

II. INTRODUCTION

1. AGING AND OXIDATIVE STRESS

1.1. Aging

Aging is generally defined as the progressive loss of function of an organism accompanied by decreasing fertility and increasing mortality (*1*). The effects of this unavoidable and complex process are manifested at genetic, molecular, cellular, organ and system levels, resulting in a decreased resistance to stress in its different forms, as well as an increased susceptibility to diverse diseases (*2*). For many years, the questioning of why aging occurs has been an intriguing mystery and numerous theories have been postulated in order to provide a meaningful explanation.

One of the most widespread theories is the "free-radical" or oxidative stress (OxS) theory, initially postulated by Harman in 1956. Basically, it proposed that the constant generation of free radicals – or reactive oxygen species (ROS) – as by-products of metabolic reactions, induce damage to cellular components such as lipids, proteins and nucleic acids (*3*). The accumulation of such damage over time is suggested to contribute to the age-related deterioration of the organism (**Figure 1**). In this way, factors that increase ROS production are expected to accelerate aging, whereas factors that defend against ROS, should delay it (*4*).

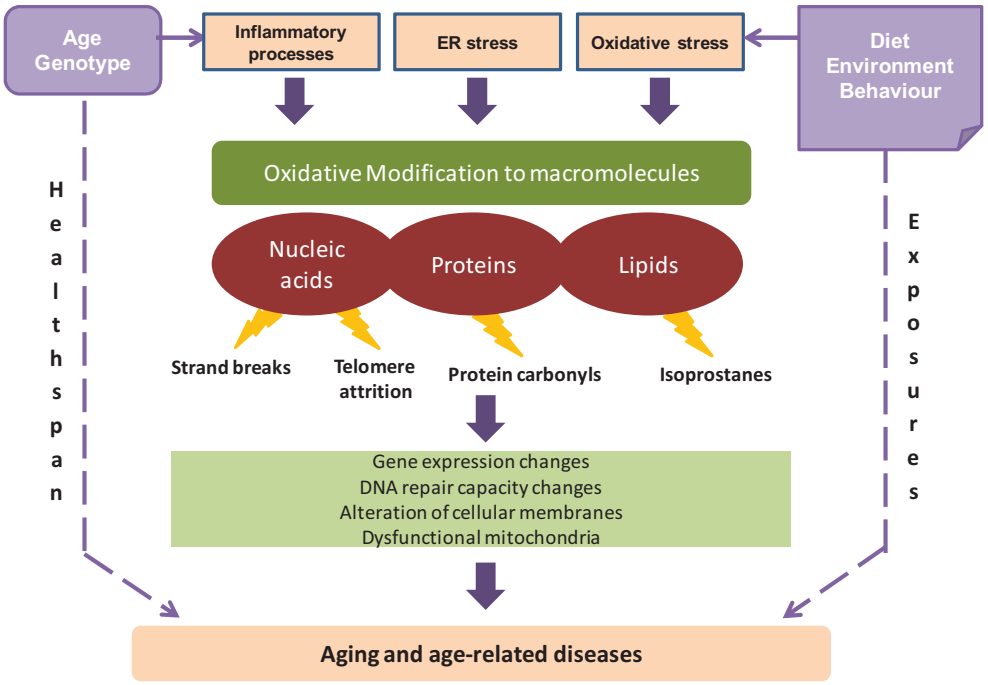

Figure 1. The role of oxidants in aging and age-related diseases. *Both non-modifiable risk factors (age, genotype) and modifiable risk factors (diet, environment) have the propensity to interact with, affect and be affected by inflammatory processes and OxS. The interaction of all of these parameters results in oxidative modifications of cellular macromolecules. These oxidative modifications alone or in association with other biologic factors may lead to changes in both gene expression and DNA repair capacity, or mitochondrial and membrane dysfunction. Adapted from Mechanisms of Ageing and Development 134 (2013) 139–157.*

According to the World Health Organization, the proportion of the world's population over 60 years will double from about 11% to 22% between 2000 and 2050 (**Figure 2**) (*5*). The number of people aged over 60 years is expected to increase from 605 million to 2 billion over the same period. Indeed, in almost every country (especially in low- and middle- income countries) people over 60 years is growing faster than any other age group, as a result of both longer life expectancy and declining fertility rates.

This population aging can be interpreted as a successful fact for public health

policies and for socioeconomic development. On the other hand, it also presents a social and economic challenge, since the need for long-term care is exponentially rising. In fact, the number of elderly people who are no longer able to look after themselves due to physical or mental health problems is forecast to quadruple by 2050 (*6*).

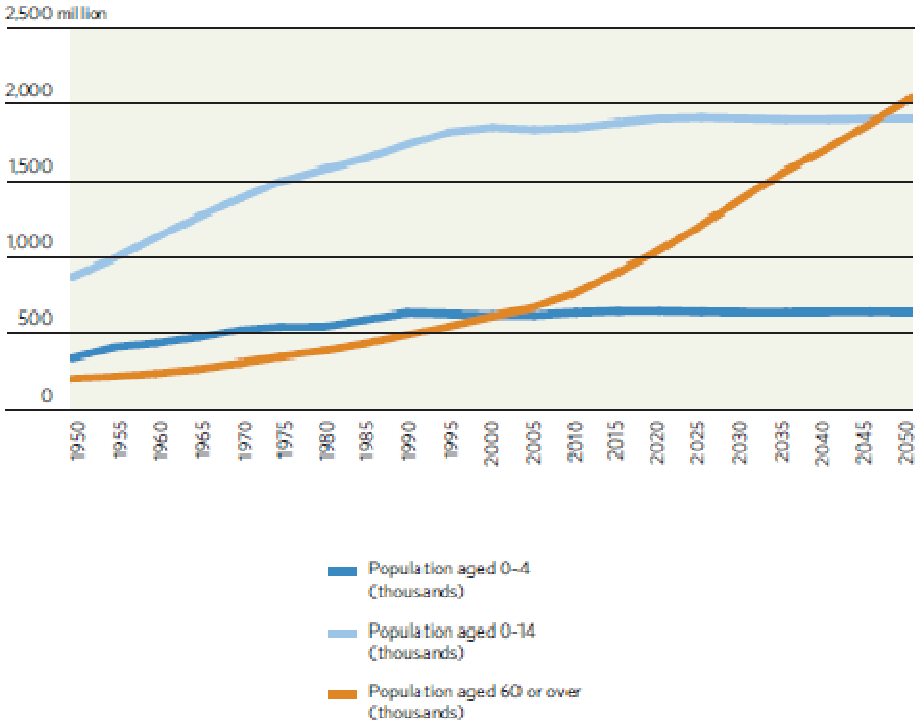

Figure 2. World population aged 0-4, 0-14 and 60 or over, 1950-2050.
Source: UNDESA, Population Division, World Population Prospects: The 2010 Revision (New York, 2011).

Because of this accelerated aging issue, numerous research studies have focused on extending "healthspan" more than "lifespan". The term healthspan emphasizes the goal of a healthy and longer life. It could be defined as the length of time an individual is able to maintain good health, which is recognized by the ability for a system to retain or return to homeostasis in response to challenges (*7*). However, the term lifespan does not explicitly

include the quality of life, it only refers to the period of time for which an organism lives or is expected to live. That is the reason why the question of how to extend lifespan and, at the same time, to maintain the quality of life (healthspan) has been attracting significant attention over the past decade (*8*).

One parameter highly related to aging is the telomere length and due to the universal fact that telomeres shorten with age, the study of its regulation and attrition in connection with the development of aging-related diseases is crucial (*9*). Telomeres are DNA-protein complexes that cap and preserve the integrity of the chromosomal ends. They consist of several thousands of repetitive sequences of TTAGGG that are naturally shortened with each cell division due to the end replication problem (*10, 11*). Progressive attrition of telomeres leads to a critical length that triggers the cell arrest phenomenon known as senescence (*12*), which has been widely associated with numerous health disorders. Notably, it has been reported that telomere attrition and oxidative DNA damage in endothelial progenitor cells is higher in coronary artery disease patients with metabolic syndrome (MetS) than in those without MetS (*13*). In addition, endothelial damage and atherosclerosis have been shown to be associated with telomere shortening in white blood cells more tightly than chronological aging (*10*).

Since the mechanisms of the onset of metabolic diseases and aging-related diseases are very similar, they share the declining of biological functions, understanding the basis and pathways involved in the aging process could be helpful for a better comprehension of the overall disease process (*14*).

1.2. Oxidative Stress

OxS basically defines a condition in which the cellular balance between pro-oxidant and antioxidant species is disturbed in such a way that cellular bio-molecules undergo severe oxidative damage, compromising cell viability (*15*).

There is extensive evidence that ROS are generated continuously within eukaryotic cells as by-products of metabolic reactions under physiological state (*4*). This production is strictly controlled in order to maintain the reduction/oxidation (redox) homeostasis required for the proper functioning of the organism. Two connected situations may compromise this redox balance leading to the development of OxS: when the generation of ROS exceeds the ability of the system to neutralize and eliminate them or when there is a lack of antioxidant capacity caused by a disturbance in its production or distribution. As a result, this scenario leads to modification of lipids, proteins and/or DNA (*16*). Such modifications are called oxidative damage and for this reason OxS has been implicated in a growing list of human diseases as well as in the aging process.

1.2.1. Reactive oxygen species production

It is a well-known fact that the majority of living organisms depend on oxygen for survival. However, organisms have had to protect themselves from oxygen toxicity due to its chemical propensity to produce free radicals such as superoxide ($\cdot O_2^-$) or hydroxyl radical (·OH). Those radicals can be involved in subsequent chemical reactions producing additional molecules that can also elicit cellular damage, although they are not necessarily free radicals (*17, 18*). Thus, ROS and reactive nitrogen species (RNS) are collective terms for radical and non-radical agents derived from oxygen and nitrogen metabolism respectively, which have an extremely short half-life (*19*).

In terms of reactivity with biological molecules, ·OH is the most reactive with a lifetime of 10^{-9}s and practically no selectivity in order to react with most compounds (*20*). By contrast, $\cdot O_2^-$ is less reactive and more selective. In biological systems it is able to form hydrogen peroxide (H_2O_2) through a self-

dismutation reaction in a few seconds. $\cdot O_2^-$ also takes part in a diffusion-controlled reaction with nitric oxide (·NO) to produce a really potent and reactive RNS: peroxynitrite ($ONOO^-$) (*21*), which oxidizes unsaturated fatty acids in biological membranes and also reacts easily with DNA, oxidizing purine and pyrimidine bases (*20, 22*). Most of the cytotoxicity attributed to ·NO is rather due to $ONOO^-$, since nitric oxide is an essential free radical gas that acts as an intracellular messenger molecule in smooth muscle relaxation process and plays a fundamental role on endothelial function (*23*). In fact, it has been established that an increase in OxS levels, coupled with an impairment in nitric oxide (NO) availability, may cause alteration of endothelial cells causing endothelial dysfunction (*24*). These findings are usually reflected in decreased ischemic reactive hyperemia (IRH), which is a method for measuring changes in acute endothelial reactivity (*25*).

It has been described that the majority of the ROS generated endogenously proceed from the mitochondria, where most of the oxygen consumed by aerobic organisms is reduced to water. However, a small proportion of the oxygen is converted to $\cdot O_2^-$ in two concrete sites of the electron transport chain: Complex I (NADH deshydrogenase) and Complex III (ubiquinone-cytochrome c reductase) (*26*). During the respiratory process the free radical semiquinone ($\cdot Q^-$), which acts as a mediatory in the regeneration of coenzyme Q, transfers an electron to molecular oxygen non-enzimatically, generating $\cdot O_2^-$. In this way, the flux of $\cdot O_2^-$ is related to the concentration of potential electron donors, the local concentration of O_2 and the second-order rate constants for the reactions between them (*27*).

1.2.2. NADPH oxidase

The cytosolic enzymatic systems that may contribute to OxS include the

nicotinamide adenine dinucleotide phosphate (NADPH) oxidases family. These enzymes were firstly described in phagocytes, participating in the defense against pathogens through generation of antimicrobial oxidants such as $\cdot O_2^-$ or $\cdot O_2^-$-derived oxidants (*28*) and they are likely to be the predominant source of ROS in the human vasculature. Activation of the NADPH oxidase enzyme complex requires the assembly of the cytosolic regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and rac) with the membrane-bound cytochrome b558 (subunits $gp91^{phox}$ $p22^{phox}$) (*29*).

1.2.3. Pathologies associated with oxidative stress

The existence of diverse mechanisms of ROS production and their complexity highlights the fact that these species have a crucial role in human physiological and pathophysiological processes. Indeed, a large body of evidence supports the importance of ROS at low and regulated levels (*30*). As we discussed above, a normal immune function requires specific oxidative states, in fact, a lack of the ROS-generating NADPH oxidase NOX2 leads to immunodeficiency associated with recurrent infections in chronic granulomatous disease patients (*31*). Besides, ROS have a role in various signaling cascades regarding to growth factor stimulation and control of inflammatory responses (*32*) and they also participate in the regulation of apoptosis, differentiation, proliferation and migration (*30*).

On the other hand, excessive ROS production contributes widely to the onset and development of diverse range of pathologies, being many of them a leading cause of death (**Figure 3**).

The damage caused by ROS to the DNA is probably their main implication to cancer with changes in the genetic material such as base modification, rearrangement of DNA sequences, miscoding of DNA lesion, gene duplication

and oncogenes activation (*33*).

Similarly, the oxidative damage has been studied extensively in cardiovascular disease. According to the theory of OxS, atherosclerosis is the result of oxidative modifications of low density lipoproteins (LDL) in the arterial wall by ROS (*34*). Furthermore, pathological processes such as ischemia/reperfusion injury and hypertension have been related to changes in the expression of NOX enzymes, and consequently ROS production, in vascular cells (*35, 36*). Other pathologies largely associated with aging and OxS are Parkinson's and Alzheimer's diseases, where the amyloid soluble forms can lead to microglia activation and long-term ROS generation, contributing to neuronal damage and finally dementia (*37*). Regarding to MetS, it refers to the clustering of three or more characteristics such as hyperglycemia, hypertriglyceridemia, low level of high-density lipoprotein cholesterol (HDL-C), hypertension and abdominal obesity (*40*) according to the criteria defined by the Third Report of the National Cholesterol Education Program Adult Treatment Panel III (*39*). One of the mechanisms underlying this pathology is OxS (*38*) and it has been recently published that the degree of OxS in MetS pacients is directly influenced by the number of the components of the syndrome (*41*).

Interestingly, those pathological processes have in common that when OxS cellular levels increases, the survival of the cell depends on its ability to adapt itself to the situation or withstand the oxidative damage as well as its capacity to repair and replace the damaged molecules. If the cell cannot afford the OxS, it undergoes apoptosis or programmed cell death.

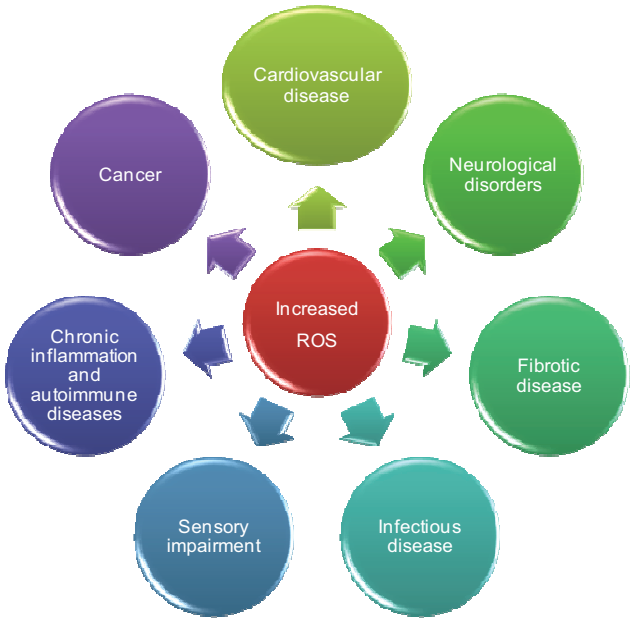

Figure 3. ROS overproduction and its relation with disease.

1.3. Antioxidant defense systems

In order to decrease the intrinsically and extrinsically generated oxidative insults that may threaten the cellular redox status, cells possess highly regulated antioxidant defense systems that contribute to maintain an optimal functioning of the organism. Those mechanisms are divided into enzymatic and non-enzymatic systems. The primary antioxidant defense is formed by several enzymatic complex proteins that often incorporate minerals such as selenium or zinc in their structures. Inside this group, the main antioxidant enzymes include glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD), which serve as the most potent defense against free radicals and ensuing inflammatory reactions of the organism. On the other hand, the non-enzymatic antioxidant group includes vitamin C (ascorbic acid), vitamin E (α-tocopherol), β-carotene, glutathione (GSH), thioredoxin (Trx), polyphenols

and numerous phytochemical agents. Cells must preserve the levels of all these antioxidant species, also defined as antioxidant potential, through endogenous synthesis or diet consumption (*42, 43*).

1.3.1. Nrf2 regulation

The efficiency of the whole set of antioxidant mechanisms lies on its capacity to adjust to increasing levels of stress through the up-regulated expression of its subcomponents. This adaptive response is driven by a group of transcription factors where the nuclear factor erythroid 2 – related factor 2 (Nrf2) acts as orchestra conductor by regulating the inducible cell defense (*44*) through the Keap-1/Nrf2/ARE pathway (**Figure 4**).

Nrf2 normally resides in the cytoplasm of unstressed cells, where interacts with Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associating protein 1 (Keap-1). In the absence of cellular stress, Keap-1 limits the activity of Nrf2 by promoting its degradation through the ubiquitin-proteasome pathway (*45*), as a result, Nrf2 undergoes a constant cycle of production and decomposition that determines its relatively short half-life (10-30 min) (*46*). In response to an oxidative insult, Nrf2 evades Keap-1 mediated repression and accumulates within the nucleus, where it dimerizes with proteins of the Maf family (*47*) and binds with specific affinity to the antioxidant-response element (ARE) located within the promoter regions of genes encoding many antioxidant proteins and phase-II detoxifying enzymes implicated in cell defense (*48*). In fact, AREs have been identified in numerous Nrf2 target genes such as those involved in regulating glutathione metabolism, antioxidant enzymes specialized in detoxification of ROS, xenobiotic transporters and many other stress response proteins (*49, 50*). Thus, Nrf2 widely increases the range of cell defense processes by inducing the expression of a large battery of genes, which in turn enhances the overall

capacity of an organism to detoxify and remove potentially harmful entities.

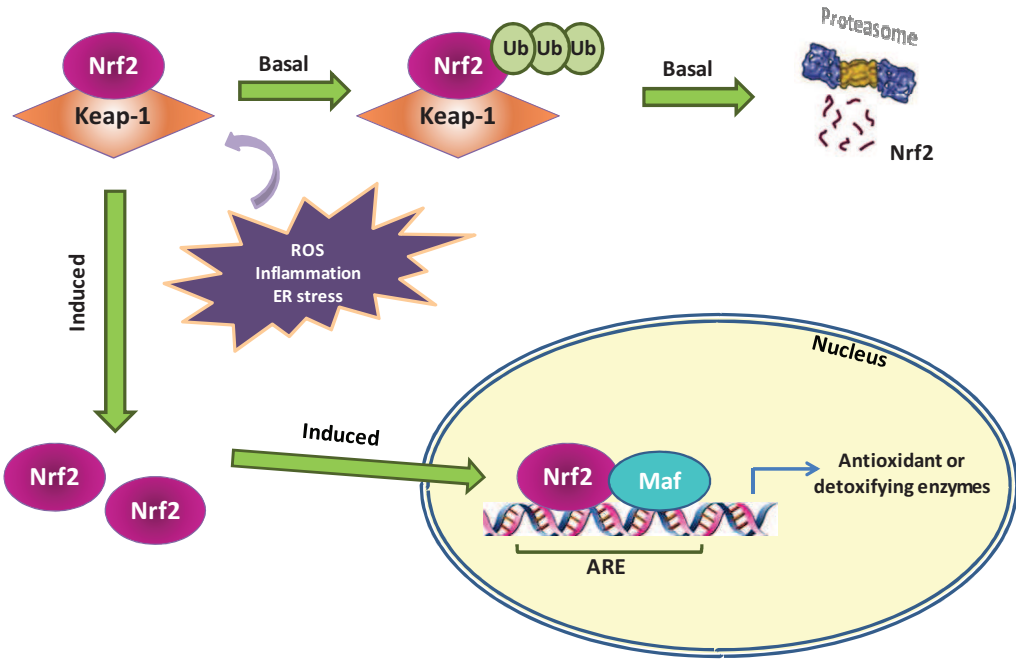

Figure 4. Schematic model of Nrf2 regulation by Keap1. *Keap-1 is a key regulator of the Nrf2-signaling pathway and serves as a molecular switch to turn on and off the Nrf2-mediated antioxidant response. (i) Under basal conditions, Keap-1 constantly targets Nrf2 for ubiquitination and degradation. (ii) OxS and ROS increased production inhibit activity of Keap-1, resulting in increased levels of Nrf2 and activation of its downstream target genes. Adapted from Pharmacological Research 58 (2008) 262–270.*

1.3.2. Antioxidant enzymes: SOD, GPx and TrxR

In this connection, one of the main enzymes of the first line of the antioxidant defense is SOD, which consists of three isoforms in mammals: the cytoplasmic isoform that employs copper (Cu) and zinc (Zn), Cu/ZnSOD (SOD1); the mitochondrial isoform that employs manganese (Mn), MnSOD (SOD2); and the extracellular isoform that requires Cu and Zn, Cu/ZnSOD

(SOD3). In each subcellular fraction, SODs catalyze the dismutation reaction of $\cdot O_2^-$ to O_2 and H_2O_2 and it involves successive reductions and oxidations of a transition metal as cofactors for their activation (*51*). The distinct subcellular location highlights the importance for compartmentalization in redox signaling.

$$2\cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

In contrast to $\cdot O_2^-$, which normally remains close to the place where it was produced, H_2O_2 is able to diffuse through cellular membranes (*52*) and to generate the critically reactive radical ·OH in the presence of reduced transition metals such as iron, as a result of the Fenton reaction (*53*). Since H_2O_2 is a powerful oxidant, several mechanisms are present in the cell in order to neutralize it. The universal enzyme CAT is a tetramer that catalyzes the decomposition of H_2O_2 to water and oxygen (*54*), according to the reaction below:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Furthermore, H_2O_2 and organic hydroperoxides can be reduced to water or to the corresponding alcohols through a chemical reaction catalyzed by GPx, where reduced GSH acts as an electron donor (*55*):

$$H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2H_2O$$

or

$$ROOH + 2GSH \rightarrow ROH + GS\text{-}SG + H_2O$$

GPx is the general name for a family of isozymes that have a selenium-dependent glutathione peroxidase activity in mammals. The presence of selenocysteine in the catalytic centre has been suggested to guarantee a fast reaction with the hydroperoxide and a fast reducibility by GSH. There are four major GPx isozymes and they seem to have an antioxidant function at different locations in cellular compartments: GPx1 ubiquitously in the cytosol and mitochondria (considered as the prototype of glutathione peroxidase), GPx2 in the intestinal epithelium, GPx3 in plasma and GPx4 in cellular membranes (*55, 56*).

As mentioned above, the transcription factor Nrf2 downregulates genes related to cell defense against OxS including those that encode for the enzymes SODs, GPxs and thioredoxin reductase (TrxR) (*57*). TrxR belongs to a family of flavoenzymes that catalyze the transfer of two electrons from NADPH to the substrate. These oxido-reductases are required for the reduction of the active site disulfide in Trx, thereby maintaining the pool of reduced and reactive Trx (*58, 59*). It has been also described that peroxides, including lipid hydroperoxides and hydrogen peroxide, can directly be reduced by TrxR. By this mechanism, TrxR could function as an alternative enzymatic pathway for detoxification of lipid hydroperoxides, otherwise mainly managed by GPx (*60, 61*).

The effectiveness of this complex antioxidant response not only lies in its enzymatic machinery, it is worth noting the role of non-enzymatic compounds that largely contributes to maintaining the redox homeostasis. A clear example of that is the liposoluble vitamin E (α-tocopherol), whose important function is related to its capacity to retain the integrity of long-chain polyunsaturated fatty acids in the cellular membranes (fluidity, phase separation and lipid domains) and thus preserve their bioactivity. As these bioactive lipids are

important signaling molecules, changes in their amount or in their function due to oxidation affect crucial signaling pathways in cells (*62*). Another essential non-enzymatic compound is Vitamin C or ascorbic acid, which acts as an electron donor and a potent water-soluble antioxidant in humans. It is involved in the neutralization of free radicals and regeneration of vitamin E. In fact, both vitamins have been suggested to participate in the prevention of cardiovascular diseases (*63, 64*).

1.4. Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is a complex and well-organized system that catalyzes protein folding and detects the presence of misfolded or unfolded proteins (*65*). The continuous accumulation of misfolded proteins can affect the normal cellular functioning and trigger a condition called ER stress, which is known to contribute to the pathophysiology and development of numerous diseases such as neurodegenerative disorders, cardiovascular disease, diabetes mellitus and other aging-related diseases. Accumulated evidence has suggested a crosstalk between the generation of ROS and the cellular response to ER stress, although the exact mechanisms have not been fully clarified yet.

All proteins that undergo the secretory pathway in eukaryotic cells first enter the ER, where they have to pass a quality control after being folded and assembled into subunit complexes (*66*). In this way, misfolded proteins are either retained in the ER lumen by chaperones that assist them for a proper folding or they are directly decomposed by the ER-associated degradation (ERAD) machinery or by autophagy (*67*). Some of these chaperones are calnexin and calreticulin (CRT), which are lectin proteins responsible for glycoprotein quality control and also contribute to the maintenance of Ca^{2+} homeostasis (*68, 69*).

In response to the ER stress caused by the aggregation of misfolded proteins, cells activate the "unfolded protein response" (UPR) to nullify the stress deleterious effects in the ER lumen (*70*). By and large, the UPR system acts through four main mechanisms: a) attenuation of protein synthesis in order to avoid more extra protein aggregation, b) enhancement of the folding capacity by inducing the transcriptional expression of ER chaperone genes, c) increment of ERAD capacity by inducing the expression of ERAD genes, and eventually through d) induction of apoptosis to delete damaged stressed cells (*71*) (**Figure 5**).

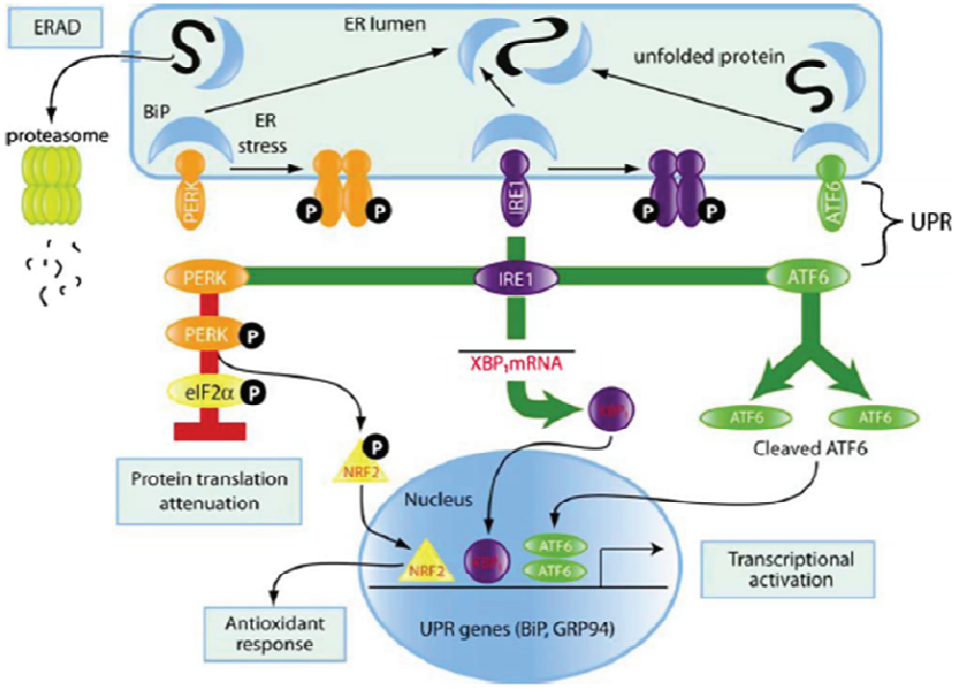

Figure 5. Schematic model of the unfolded protein response (UPR). *Misfolded or unfolded proteins induce the release of BiP from the 3 transducers of ER stress: PERK, IRE-1 and ATF6. Activated PERK phosphorylates eIF2a to attenuate protein translation and phosphorylates Nrf2 to up-regulate the anti-oxidant response. Cleaved activated ATF6 leads to induction of molecular chaperones BiP, PDI, etc. IRE-1 activation leads to XBP-1 splicing, transcriptional activation of chaperones and stimulation of protein degradation. ER*

chaperones, such as BiP, are protective and control protein folding and components of the UPR.. Adapted from N. Naidoo / Ageing Research Reviews 8 (2009) 150–159.

1.4.1. UPR pathway

The molecular components of the UPR signaling pathway have been successfully determined over the last decades. Three proximal ER-located transmembrane signal transducers act together in harmony with the purpose of regulating the UPR through their respective signal cascades. These transducers are two protein kinases IRE1 (inositol-requiring kinase 1) and PERK (double-stranded RNA-activated protein kinase-like ER kinase), and the transcription factor ATF6 (activating transcription factor 6) (*71, 72*). In this framework, the protein chaperone BiP (binding immunoglobulin protein) - also known as 78 kDa glucose-regulated protein (GPR78) - is the master regulator of the whole pathway. Under non-stressed conditions, BiP binds to the luminal domains of the three transmembrane sensors to prevent their activation. However, under stress conditions (accumulation of misfolded and unfolded proteins in the ER lumen) BiP is released from IRE1, PERK and ATF6 and preferentially binds to the damaged proteins (*73*). Despite the fact that the exact mechanism by which the transducers sense ER stress is still under research, several models have been propose for the UPR activation, suggesting that each branch of the system is separately regulated (*74*).

IRE1 is a type I transmembrane protein kinase that separates from BiP and oligomerizes during ER stress. Following oligomerization, its kinase activity becomes activated, initiating a signaling cascade that may activate the inflammatory protein c-Jun amino terminal kinase (JNK) (*75*). Its endoribonuclease domain becomes active as well, favouring a selective splicing through the cleavage of the mRNA that codes for the factor called X-

box binding protein-1 (XBP-1) (*76*). This process leads to the translation of the spliced form of XBP-1 (XBP-1s), which is a highly active transcription factor that upregulates the expression of genes encoding chaperones, the expression of components involved in ERAD and also plays a key role in ER expansion (*77, 78*).

ATF6 is categorized as a type II transmembrane protein that contains a DNA-binding domain and a transcriptional activation domain. In response to ER stress, the dissociation from BiP leads to its transport to the Golgi complex where it is sequentially cleaved by two proteases (*79*). The resulting fragment is a transcription factor that translocates to the nucleus and activates UPR target genes related to chaperone expression and mechanisms of apoptosis.

PERK is a type I transmembrane protein kinase whose activation during RE stress reduces protein synthesis globally, thereby preventing continued influx of newly synthesized polypeptides into the stressed lumen (*80*). This translational attenuation is achieved through activation of the main downstream effector of PERK, the eukaryotic initiation factor 2 (eIF2α). In addition to the global inhibition of protein synthesis, PERK-mediated phosphorylation of eIF2α activates the transcription factor 4 (ATF4), which contributes to ER function, growth arrest mechanisms and apoptosis (*81*).

PERK is also able to initiate the anti-OxS response due to the fact that upon ER stress, the transcription factor Nrf2 may be a substrate for its kinase activity. In such a way, PERK may phosphorylate Nrf2 resulting in dissociation of the Nrf2/Keap1 complex (*82*).

Finally, if the UPR-mediated efforts to correct the protein folding damage fail, the cell undergoes apoptosis. At that point, ER generate apoptotic signals through several mechanisms that include PERK/ eIF2α-dependent induction of the pro-apoptotic transcription factor (CHOP), IRE1-mediated activation of JNK and activation of pro-caspase 12 (*65*).

With age, the adaptive response of the UPR declines in terms of a progressive failure of chaperoning systems. In fact, in many age-related diseases, proteins or fragments of proteins convert from their soluble state to insoluble fibrils that accumulate in a variety of organs causing serious damage, as is the case of Alzheimer's, Parkinson's and type II diabetes (*83*).

1.5. Inflammatory response

The aging process has been tightly integrated with inflammation over the last years. In this connection, many research studies have shown that a low-grade chronic inflammatory status is characteristic of the aging process as a consequence of the global reduction in the ability of the elderly to cope with chemical, physical and nutritional stress factors and the concomitant progressive increase in pro-inflammatory markers. Different age-related pathologies, such as cardiovascular disease, type II diabetes, MetS, osteoporosis and cognitive decline, share a common inflammatory pathogenesis. Moreover, a large body of evidence suggests that environmental and lifestyle factors may also contribute to modulation of both inflammatory processes and age-related dysfunction (*84, 85*).

Inflammation is the primary immune system reaction by the organism to remove pathogens or other injurious stimuli that may threaten the cellular normal state. The interaction of the cellular immune system with endogenous or exogenous antigens results in the generation of ROS and RNS, leading to signaling cascades that produce pro-inflammatory cytokines and inducing OxS phenomena (*86*).

1.5.1. NF-κB activation

The master regulators of immune and inflammatory processes in response to both injury and infection are the NF-κB (nuclear factor kappa B) family transcription factors. This family can function as homo- or heterodimers according to the combination of the different subunits: RelA (p65), RelB, c-Rel, p50 (p105 precursor) and p52 (p100 precursor) (*87*), where the most common association is a heterodimer of the p50 and p65 proteins. All of them share a highly conserved DNA-binding and dimerization domain known as Rel homology region. On the contrary, the subunits p65, RelB and c-Rel are the only ones that contain a transactivation domain that allows them to activate target gene expression (*87, 88*). The activity of these dimers is directly controlled by a set of proteins termed as IκB (inhibitor of NF-κB) through its non-covalent association with NF-κB, shifting the steady-state subcellular location of the transcription factor to the cytoplasm (*89*). Members of the IκB family are the classical IκB proteins (IκB-α, IκB-β, IκB-ε), the precursor proteins p100 and p105 and the nuclear IκB (IκB-ζ and Bcl-3). The best characterized interaction is the one between IκB-α and the heterodimer p50/p65, due to the fact that IκB-α is related to the transient activation of NF-κB and interestingly, it is regulated at the same time by NF-κB (*87*).

NF-κB pathway can be activated by at least three routes named the classical (canonical), alternative (non-canonical) and atypical pathway (*90*), where the first two pathways require the activation of the IκB kinase (IKK) complex consisting of IKK-α, IKK-β and IKK-γ (also known as NEMO) subunits.

The classical pathway relies on the phosphorylation of IκB-α by IKK-β and IKK-γ subunits that leads to ubiquitination and consequent degradation of IκB-α through the proteosomal pathway (*91*). Free NF-κB then rapidly translocates to the nucleus, where it binds to concrete DNA sequences, known as κB DNA/sites, located in the promoter regions of numerous genes and

elevates or represses their expression (*89*). This pathway is activated by diverse stimuli that include pro-inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukins (IL-1β, IL-6), bacterial lipopolysaccharide (LPS), Toll-like receptor (TLR) and ROS (*91*) (**Figure 6**).

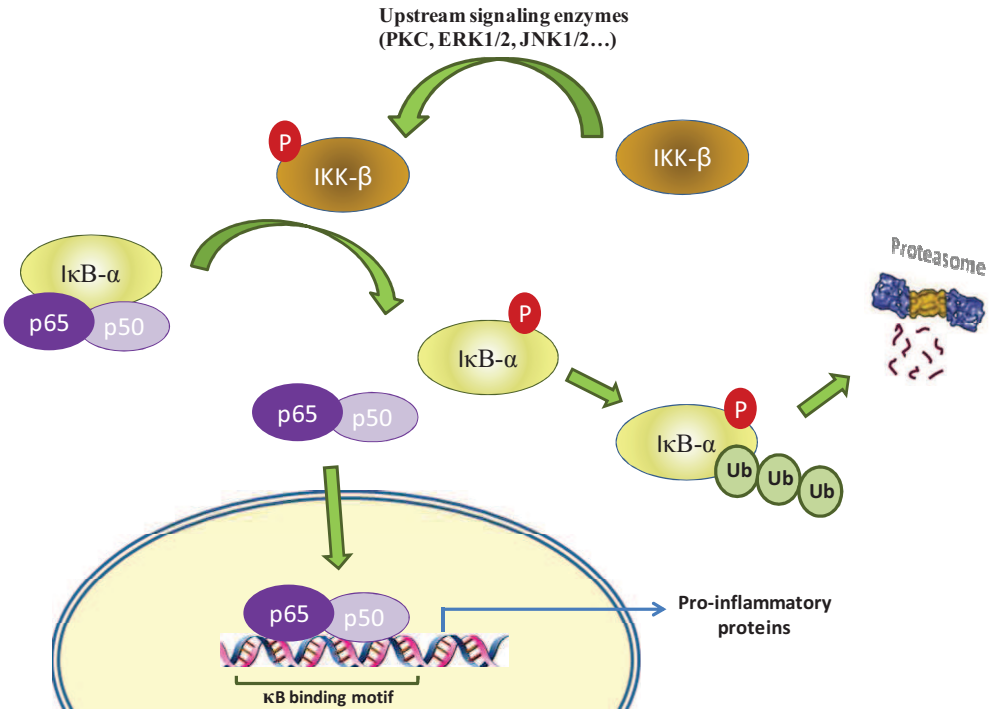

Figure 6. Schematic model of the classical NF-κB activation. *Upon stimulation of cells, activation of protein kinases induces phosphorylation of IKK-β. IκB-α is rapidly phosphorylated by IKK-β and degraded via the ubiquitin-proteasome pathway. The resulting free NF-κB dimer (p50-p65) translocates to the nucleus for the transcriptional regulation of multiple target genes.*

In the alternative pathway, the IKK-α is the main effector and this sort of activation is completely independent of IKK-β and IKK-γ. Its activation may be triggered by a subset of the TNF superfamily receptors such as lymphotoxin β receptor, B-cell activating factor receptor (BAFF) or CD40 and requires the NF-κB-inducing kinase (NIK) (*92*). This process results in the processing of

p100 and nuclear localization of the dimer RelB/p52. The alternative pathway is generally slower than the classical one but it has been demonstrated that is important for B cell homeostasis and adaptive immunity (*93*).
Finally, the atypical NF-κB activation is IKK-independent and can be caused by hypoxia/reoxygenation, UV irradiation and in some cell types, by H_2O_2 (*94*).

1.5.2. Cytokines: IL-β

Cytokines are a diverse group of soluble proteins, peptides and glycoproteins that act as hormonal regulators in response to inflammatory or antigenic stimuli. They can exert their activity through autocrine, paracrine or endocrine pathways and their excessive production may lead to diverse pathological situations such as inflammatory, immune and infectious diseases (*95*).
In this framework, interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine that is crucial for host-defense responses to infection and cell injury, and it also plays key roles in acute or chronic inflammatory and autoimmune disorders. The IL-1 family of cytokines comprises 11 proteins encoded by 11 distinct genes in humans members, being IL-1β the best characterized and most studied (*96*). A large body of evidences has shown that this important molecule is produced and secreted by a variety of cell types, especially cells of the innate immune system such as monocytes and macrophages. It is produced as an inactive 31 kDa precursor, known as pro-IL-1β, in response to the interaction of the pathogen associated molecular patterns (PAMPs) with their corresponding receptors in macrophages. Then, pro-IL-1β is processed and cleaved by the pro-inflammatory caspase-1, resulting in an active IL-1β molecule (*97*). Both isoforms, IL-1α and IL-1β, have potent pro-inflammatory activities restricted at three levels: 1) synthesis and release, 2) membrane receptors and 3) intracellular signal transduction. Moreover, their pathways

include diverse positive- and negative- feedback mechanisms that amplify or terminate the inflammatory response. In the end, as a consequence of its activation, diverse cascades of phosphorylation and ubiquitination are triggered, resulting in the initiation of the NF-κB and JNK pathways (*98*).

1.5.3. JNK signaling

JNK is a multifunctional kinase involved in numerous physiological and pathological processes. Its pathway plays a major role in apoptosis and can be triggered in response to growth factors and cytokines such as TNF and IL-1β (*99*).

In mammals, the JNKs are encoded by three genes (JNK1, JNK2 and JNK3) and each of them belongs to a different chromosome. Interestingly, each mammalian JNK gene has alternative splicing products, resulting in 10 different identified proteins. JNK1 and JNK2 are ubiquitously expressed, whilst JNK3 is only expressed in the central nervous system, cardiac smooth muscle and testis (*100*).

Depending on the context of its expression, JNK is essential for critical cellular processes such as cell death, survival, proliferation and differentiation. Moreover, it is necessary for the induction of a great diversity of genes that encode cytokines, vascular endothelial growth, transmembrane receptors, matrix metalloproteinase 9 (MMP9), cell cycle regulator cyclins and intercellular adhesion molecules, among others (*101-103*).

In addition to its key role in inflammatory pathways, JNK is also connected to OxS and ER stress mechanisms, where it is activated through the IRE1 route and it is involved in apoptotic processes (*104*). Regarding to OxS, there are many ways in which ROS may modify JNK activity, through both chemical (*105*) and enzymatic (*106*) reactions. Generally, increased levels of ROS very often correlate with increased JNK phosphorilation and subsequent activation.

One mechanism by which JNK is activated by ROS results from the fact that JNK can be held in an inactive form by GSTPi (glutathione-S-transferase enzyme class Pi). Under OxS situation, JNK dissociates from GSTPi and translocates to the nucleus or mitochondria in order to activate substrates (*106*). In addition, another mechanism that reflects this connection is conducted by hydroxynonenal, a molecule that is generated as an end product of lipid peroxidation and possesses the ability to form aggregates with JNK, causing its activation (*105*).

1.5.4. Matrix metalloproteinases: MMP-9

Matrix metalloproteinases (MMPs) are a family of Ca^{2+} and Zn^{2+} dependent endopeptidases that are able to degrade most of the extracellular matrix components (ECM). They regulate numerous physiological processes such as cell migration, proliferation and apoptosis, playing an important role in tissue remodeling in inflammatory diseases and cancer metastasis. MMPs alter cell function through the cleavage and release of microenvironment molecules from the ECM and through modulation of gene expression (*107, 108*).

MMP-9 (gelatinase B) belongs to the MMP family and is synthesized as a zymogen with a molecular mass of 92-kDa, which is converted to an active enzyme of 82-kDa. Some of its substrates include collagen IV or V, fibronectin, ICAM-1, plasminogen and interleukin-2 (*109*). It has been described the relationship of MMP-9 with inflammatory processes in various diseases, which has led to a profound investigation in the mechanisms responsible for its regulation. MMP-9 gene expression is controlled by transcriptional factors including JNK/AP-1 and NF-κB (*110*) and also can be induced by pro-inflammatory cytokines such as TNFα, IL-1β or IL-6 (*111*). In this way, MMP-9 participates in several stages of atherosclerosis, where foam

cells increase its expression in response to oxidized lipoproteins and cytokines, facilitating the migration of muscle cells (*112*).

1.6. Diet, postprandial state and oxidative stress

Taking into account the great influence of processes such as OxS, ER stress and inflammation over the aging process, as well as the capacity of the organism in terms of dealing with them, it has been suggested that factors that increase the resistance to stress could be highly beneficial for the organism and could improve the lifespan. According to this, several relations between the stress response and genetic factors have been established in different models such as Drosophila, Caenorhabditis elegans (C. elegans) and mice. In fact, a mutation in the gene clk-1 of C. elegans has been described to increase longevity and to improve the antioxidant capacity of the nematode. This gen encodes for a mitochondrial protein that is homologous to a protein involved in the synthesis of Coenzyme Q, an electron transporter required for the cellular respiratory chain, in yeast. Those clk-1 mutants could lengthen lifespan by decreasing the metabolic rate, which in consequence would decrease the damage caused by ROS (*113*).

Hence, the interest of the scientific community in the development of anti-aging therapies has been growing up in the last years. In this framework, caloric restriction has been suggested to be the most reliable intervention to prevent age-related disorders and extend lifespan. This technique consists of the reduction of calories by 10-30% compared to *ad libitum* diet and despite the fact that the underlying mechanisms by which its benefits occur have not been clearly defined yet, some indications point towards an alteration of the metabolic rate and the accumulation of ROS (*114*). Experiments conducted in non-human primates have shown that caloric restriction may extend the

median lifespan and reduce the onset of age-related diseases (*115*). Further research in mimetics of caloric restriction such as resveratrol (*116*) is needed in order to broaden studies to humans since, owing to ethical and experimental limitations the investigation of human caloric restriction have not been actively conducted.

1.6.1. Diet and oxidative stress

Dietary components with antioxidant activity have received great attention due to their demonstrated role in modulating the OxS associated with aging and age-related conditions. Several research studies have indicated potential beneficial effects produced by dietary antioxidants related to the decrease of degenerative disease such as vascular dysfunction, cardiovascular disease and cancer (*117*). The importance of some of these antioxidants lies in the fact that they cannot be synthesized by the organism, as is the case of vitamin C. Humans and primates depend on the diet as a source of vitamin C, which has the ability to protect against lipid peroxidation (LPO) by acting as a scavenger of ROS and by reducing lipid hydroperoxyl radicals in the "vitamin E recycling process" (*118*). LPO is a biomarker of OxS that is often increased in cardiovascular disease, neurodegenerative diseases and diabetes (*119, 120*). These observations have raised the question whether there is a direct relationship between diet and OxS, and because of this, numerous studies including antioxidant dietary interventions have been conducted. Indeed, vitamin C supplementation was found to decrease the urinary concentrations of LPO by 20-30% in a placebo-controlled study (*121*) and vitamin E supplementation was shown to decrease heart attack risk in subjects with inadequate antioxidant protection (*122*). However, a variety of trials have resulted in conflicting outcomes regarding to antioxidant therapies. A possible explanation for this could be that the chronic intake of a healthy food pattern

such as the Mediterranean diet (Med diet), which includes a combination of antioxidant compounds, is more effective that the supplementation with just one antioxidant element during an acute intake (*123*).

In fact, extensive prospective studies suggest that a high degree of adherence to Med diet is associated with reduced mortality and also prevents the onset and progression of coronary heart disease (*124*) and other aging-related diseases (*125*). Common components of this diet include monounsaturated fatty acids (MUFA), α-tocopherol, phenolic compounds, phytoesterols and other antioxidants, thus the leading hypothesis of this association is a decrease of OxS because of the antioxidant capacity of this diet (*126*).

Recent studies have shown that the fat quality modification of meals may affect significantly the OxS levels. According to Perez-Martinez *et al.*, the comparison of four diets with different fat quality and quantity in the postprandial state resulted in an improvement of the OxS biomarkers after consumption of a MUFA-rich diet. In this interventional study, the GSH/GSSG ratio was improved following the MUFA intake. In addition, this dietary pattern also induced lower plasma levels of LPO and protein carbonyl (PC), and also lower SOD activity compared with subjects adhering to the other three diets. Conversely, the consumption of a saturated fatty acid (SFA)-rich diet increased H_2O_2 plasma levels (*127*).

It is worth noting the main role that the oxidation of LDL plays in the scenario of cardiovascular disease. This mechanism generates oxidized LDL (oxLDL) particles which are further harmful than natives LDL in the vascular wall during the atherosclerosis process. In a recent review by Lapointe *et al.*, twelve of the fourteen studies selected for obtaining an overview of the dietary patterns that participate in the oxLDL generation, showed that MUFA diet

consumption induced lower LDL oxidizability than diets rich in polyunsaturated fatty acid (PUFA diet) (*123*).

Interestingly, marine-derived (n-3) PUFA has been reported to contribute to cardiovascular health through its anti-inflammatory properties and also through the decrease of very low density lipoproteins (VLDL) cholesterol and triglycerides (TG) plasma levels (*128*).

1.6.2. Oxidative stress and postprandial state

The postprandial state is a dynamic, non steady-state condition, with rapid remodeling of lipoproteins compared to the relatively stable fasting condition (*129*).

Much of our knowledge of the relationship between lipids, lipoprotein metabolism and the development of atherosclerosis and cardiovascular disease is based on measurements taken in the fasting state. Despite such measurements remain the foundation of clinical assessment and are an important basis for medical treatments, it is remarkable that we spend most of the time in a non-fasting, postprandial state with continuous lipemia fluctuations throughout the day. In Western society, the most typical eating pattern consists of three or more meals a day, with each containing 20-70 g of fat (*130*). Unlike circulating carbohydrates that normally show only transient elevations following a typical fat containing meal, circulating TG show a pronounced elevation (postprandial lipemia) within an hour after meal ingestion and can remain elevated for 5–8h (*129*). Thus apart from breakfast, each of these meals is most likely consumed before plasma TG have returned to the baseline condition resulting from the previous intake. Several studies support the concept that the levels of circulating TG-rich lipoproteins (TRL) after meals are significantly associated with the development of atherosclerosis (*131*). Determination of the postprandial response is complex,

and consequently, it is more challenging to assess the cardiovascular risk associated with postprandial lipemia than during fasting conditions.
After a fatty meal has been ingested, chylomicrons from absorption, VLDL and their remnants contribute to fast plasmatic elevation of TG, whilst LDL particles become smaller and denser and HDL are quickly catabolized (*132*). The capacity of individuals to regulate the circulating TG levels and clear TRLs is obviously an important reflection of their metabolic efficiency, which can be affected by age, sex, insulin levels, abdominal adiposity, exercise or genetic background (*133, 134*).

The concept voiced by Zilversmit marked postprandial lipemia as a putative atherogenic factor in 1979 (*135*) and since then, growing evidences have suggested that remnant lipoproteins contribute largely to the atherosclerotic process. Due to the strong relation between OxS and atherosclerosis, those phenomena have been widely studied during postprandio, demonstrating that postprandial state is associated with OxS (*136*). Consumption of a meal rich in lipids and carbohydrates involves oxidative damage, because of the fact that macronutrients have an effect on the redox homeostasis in the organism. They can be either targets of oxidative modifications after absorption or they can be presented in a pro-oxidant form in the diet (*137*). These oxidized and/or oxidizable lipids give rise to increased plasma concentration of lipid hydroperoxides, which are associated with elevated susceptibility of LDL to oxidation, apparently due to a structural perturbation at the particle surface (*138*). Furthermore, many studies have focused on the direct influences of OxS on the endothelial function, since the endothelium is the site for the etiology of atherosclerotic vascular disease (*139*). In this way, postprandial hyperglycemia and hypertriacylglycerolemia induced by high carbohydrate or SFA diet intake leads to increased postprandial OxS and impaired endothelial function in the majority of cases (*140*). By contrast, consumption of Med diet have been

shown to reduce the damage and dysfunction of the endothelium (*141*). These studies highlight the fact that dietary fat may be able to modulate the OxS produced at the postprandial state.

2. MEDITERRANEAN DIET

2.1. Mediterranean diet as a healthy dietary pattern

The interest in nutrition has been gaining in ground over the last several decades. This is evidenced by numerous guidelines that combine balanced diet education with others healthy practices such as quitting smoking, increasing physical activity and avoiding behavioural risk factors that may compromise the personal well-being (*142*).

Due to the fact that the genetic background of individuals is pre-determined and genetic manipulations nowadays cannot influence the aging rate of people, lifestyle and environmental factors are the ones in our hands to be modified. In this context, nutrition appears to be one of the strongest factors in order to ameliorate the rate of aging as well as the incidence of age-related diseases (*143*).

With such a background, Med diet have emerged not only as a mere collection of some selected foods but as an recognized Intangible Cultural Heritage by the UNESCO in 2010, which ascribes the eating habits typical of the Mediterranean basin a cultural promotion role (*144*). This dietary pattern originally represented the food habits of Italy and Greece around the 1970s, which were part of the Seven Countries Study. This study was the first to demonstrate the association between Med diet and a decrease in all-cause mortality (especially cardiovascular mortality), when comparing the Med dietary pattern with diets from United States and northern European countries (*145*).

There is no single Med diet, indeed more than 20 countries are included in what is termed Mediterranean region and therefore, there are many variations to the Med diet due to social, political and economic differences between these countries. By and large, Med diet typically emphasizes plant foods (cereals,

fruits, vegetables, legumes, tree nuts, seeds and olives) and olive oil as the main source of added fat. It also recommends reasonable consumption of fish, sea food, eggs, poultry and dairy products such as cheese and yoghurt, low intake of red meat and moderate ingestion of alcohol (especially red wine) (*146*).

Despite the Med diet is high in fat content, the adherence to this dietary pattern has been related to greater longevity, improved life quality and lower incidence of cardiovascular diseases, cancer or cognitive impairment (*147*).

In this way, intervention studies that assess the value of a Med dietary pattern have a great impact on the scientific community but also on society habits.

The PREDIMED (Prevención con Dieta Mediterránea) Spanish study is a large-scale, multicenter, randomized, primary prevention trial in a high risk population that have addressed the effects of the three healthy diets on cardiovascular outcomes: low-fat diet, Med diet rich in olive oil and Med diet rich in tree nuts. After 3 months of follow-up, both Med diets resulted in lower blood pressure, improved lipid profiles, decreased insulin resistance and reduced concentrations of inflammatory biomarkers compared with the control low-fat diet (*125*). Eventually, after 4.8 years of follow-up the researchers observed that a Med diet supplemented with extra- virgin olive oil or nuts, resulted in an important reduction in the risk of major cardiovascular events among high-risk people (*148*).

Similar outcomes resulted from the Medi-RIVAGE French intervention study, in which a Med diet group was compared to a low-fat diet group for risk factors of cardiovascular disease. After 3 months of intervention, LDL cholesterol, TG and insulin levels were lower in the Med diet group (*149*).

Regarding to cohort prospective studies, a recent meta-analysis by Sofi *et al.* showed a significant protection against major chronic degenerative diseases and overall mortality for individuals who reported a greater adherence to a

Med diet. Indeed, a 2-point increase of adherence to the Med diet significantly reduced death from any cause in a 8%, death or incidence from cardiovascular diseases in a 10% and neurodegenerative diseases in a 13% (*150*).

2.2. Olive oil

Olive oil is undoubtedly the core element of the Med diet. The healthy qualities derived from the consumption of olive oil have been known for thousands of years. It is remarkable that a great interest in this fat is referred in the myths and legends of the ancient Greece and in the writings of important Arabic doctors such as Maimónides or Averroes. However, all these beliefs only have had scientific basis in the twentieth century (*151*).

Unlike other fat-rich diets such as the Western diet, in which most of the fat comes from the consumption of red meat or dairy products, most of the fat content of the Med diet comes from a single food component, namely olive oil. It means that the diet is low in SFA and high in MUFA, particularly in oleic acid. In fact, numerous estimations suggest that olive oil provides some 85% of the fat content. The gastronomic characteristics of this dietary component are due to its richness in several micro-components that provide odour, colour and taste to the meals. That is why the addition of olive oil to certain dishes easily stimulates the consumption of healthy food such as fruits, vegetables, legumes and cereals. All of them contain high amounts of carbohydrates of low glycemic index and have important potential for promoting good health. Thus the benefits of the Med diet are not given exclusively by olive oil itself, but to the combination with its other health-promoting components (*152, 153*).

Olive oil is mainly composed of two fractions, saponifiable and non-

saponifiable. The first fraction represents the 98-99% of oil and is basically formed by TG esterifying oleic acid (55-83% of the total fatty acid composition) and moderate quantities of other fatty acids such as linoleic acid (3.5-21%). The non-saponifiable fraction, or micro-components, contains a great variety of non-fat compounds that represent the 1-2% and provide the biological interest that is missing in other oils extracted from certain seeds such as sunflower, soya and rapeseed oils. Although they have high contents of oleic acid, they also need to be refined for human ingestion, which means that during the refinery process they will lose the vast majority of their original micro-components (*153, 154*).

These non-fat components with great biological potential include a complex combination of vitamin E (tocopherols), hydrocarbons (squalene, carotenes and chlorophyll), triterpenes, phytosterols and a number of phenolic compounds. Phenolic compounds represent the polar fraction and are divided in four groups: the simple phenols (tyrosol, hydroxytyrosol, p-coumaric, vanillic, caffeic and gallic); flavonoids (luteolin and apigenin); secoiridoids (oleuropein); and lignans (pinoresinol and 1-acetoxypinoresinol) (*155, 156*).

With regard to olive oil, a number of commercial varieties are offered on the market, being *Virgin olive oil* (VOO) and *Extra virgin olive oil* the ones that are real natural juices obtain directly from olives through physical processes and without organic solvents. Another product is sold simply as *Olive oil* and contains a small percentage of VOO (5-10%), which is added to previously refined olive oil. In the end, the content of micro-components is lower than in VOO, although it is higher than those derived from seeds (*157*).

2.3. Olive oil and oxidative stress

As a result of the Seven Countries Study, a great deal of interest in elucidating

the effects of MUFA on cholesterol metabolism has been exponentially growing. Over the past decade a new paradigm has emerged, with the idea that the beneficial effects of a Med diet go much further than cholesterol and even traditional cardiovascular risk factors.

Notably, there are approximately 20 human intervention studies that indicate that extra VOO (rich in polyphenols) is superior to the rest of oils in modulating selected biomarkers of cardiovascular disease. It is of note that while some human studies have been conducted using excessive doses of olive oil that do not approximate the usual consumption, several others were performed using more realistic quantities (*158*).

One of the most complete human experiments on olive oil phenols and cardiovascular prevention is the Eurolive study. In this trial, 200 healthy subjects from 5 European countries were given three different oils in a crossover design, with different polyphenol content each. Oils were namely with low, medium and high polyphenol content. The results showed on one hand that all of the oils reduced serum TG and the oxidative damage to DNA; on the other hand, there was an increase in HDL-C levels and in the GSH/GSSG ratio. Interestingly, these improvements in HDL levels and oxidative damage to lipids were related to the phenolic content in a dose-dependent manner (*159, 160*).

These beneficial effects on the lipid profile showing a decrease in LDL-C and an increase in HDL/cholesterol ratio after Med diet versus SFA diet were also observed and demonstrated in studies such as PREDIMED (*125*), MediRIVAGE (*149*) and the study conducted by Ahuja *et al.*, where both diets were rich in lycopene (*161*).

An important consideration that links LDL particles and the antioxidant capacity of the VOO components, particularly polyphenols, is the susceptibility of these particles to suffer oxidative modifications. It has been proven that after VOO intake, LDL molecules increase its content in

antioxidants (phenols and vitamin E) and oleic acid as well as its resistance to oxidation, by decreasing the progression rate of the curve of LDL oxidation (*162, 163*).

Recent studies have suggested that chronic consumption of Med diet avoids the postprandial deterioration of endothelial function. In this way, the bioavailability of NO was higher and thus the endothelium-dependent vasodilatory response was greater following the ingestion of a MUFA-rich diet compared to a SFA or a low-fat diet (*164, 165*). In line with the improvement in the endothelial function, the influence of different dietary models on hemostasis has been widely studied due to the fact that this process is the result of a complex balance between coagulation and fibrinolysis, which both are crucial in the development of atherosclerosis. VOO consumption promotes a less pro-thrombotic environment compared with SFA diets, reducing platelet aggregation and modulating several different thrombogenic factors such as thromboxane B_2 production, von Willebrand factor, tissue factor, tissue factor pathway inhibitor, plasminogen activator inhibitor-1 (PAI-1), Factor VII and Factor XII (*166, 167*).

Moreover, the long-term consumption of the Med diet produces a lower NF-κB activation when compared with other types of fat either in fasting or postprandial state, and these results have been observed both in healthy people (*168*) and in MetS patients (*169*).

Regarding to elderly people, the more recent studies consistently support that the Med diet, based in VOO, promotes a healthier aging and increased longevity (*170*). In fact, a prospective study of 8.5-year follow-up on older non-demented subjects, a MUFA-rich diet intake was shown to be protective against age-related cognitive decline (*171*).

3. COENZYME Q_{10}

3.1. Introduction

Coenzyme Q_{10} or 2, 3-dimethoxy-5-methy-6-decaprenyl-1, 4-benzoquinone is a liposoluble molecule naturally present in most aerobic organisms from bacteria to mammals. It was first isolated and characterized by Festenstein *et al.* in 1955 (*172*) and two years later, in 1957, Crane *et al.* established that this compound functions as a member of the mitochondrial respiratory chain (*173*). In higher organisms, such as humans, the redox active benzoquinone ring is connected to a 10 isoprenoid units in its side chain, hence the name Coenzyme Q_{10} (CoQ).

At the time of its discovery, the CoQ distribution and synthesis was attributed exclusively to the inner mitochondrial membrane due to the fact that its only known function was transferring electrons from complexes I and II to complex III in the mitochondrial membranes (*174*). However, nowadays it is known that CoQ is a lipid-soluble molecule located in the hydrophobic domain of the phospholipid bilayer of virtually all cellular membranes and also in blood, both in HDL and LDL particles (*175*).

CoQ is the only endogenously synthesized lipid with a redox function in mammals that exhibits broad tissue and intracellular distribution. It is also the only liposoluble antioxidant to date that can be synthesized *de novo* by animal cells, which is why CoQ is not considered a vitamin although its chemical structure is very similar (*176*).

Cells generally rely on biosynthesis for their supply of CoQ: the precursor of the benzoquinone ring is 4-hydroxybenzoate (derived from the amino acid tyrosine) and the isoprenoid side chain comes from the mevalonate pathway. Endogenous CoQ levels are determined by both the rate of production and the rate of consumption in the body. The alteration of these levels can trigger

numerous disease states, especially those related to cardiovascular and neurodegenerative disease (*174*).

In order to maintain CoQ levels, animal cells have unique enzymatic mechanisms to regenerate CoQ from its oxidized product, which is formed during the course of its antioxidant activities (*177*).

CoQ can be also obtained through dietary sources such as heart, chicken leg, spinach and herring, with an estimated daily intake from food of 3-5 mg (*175*). Its absorption follows the same process as lipids in the gastrointestinal tract, but unlike them CoQ is taken up from the intestine into the circulation in a low rate, ranging between 2-3% of the total dose (*178*). CoQ is practically insoluble in aqueous solutions because of its long isoprenoid tail chain and due to this lipid characteristic, exogenous CoQ is incorporated into chylomicrons for transport to the peripheral blood (*179*). Following absorption, CoQ appears in plasma lipoproteins and in liver, but not usually in heart, kidney, muscle or brain.

Administration of an exogenous substance may have consequences for the endogenous synthesis, but in vivo labeling of endogenous CoQ with ^{3}H-mevalonate have demonstrated that there is no product inhibition upon exogenous administration of the lipid (*180*).

3.2. Function

The two main functions of CoQ that constitute the basis for the research that supports its clinical use, are its key role in mitochondria bioenergetics and its antioxidant role. At mitochondrial membrane level, CoQ has three well-characterized functions: transfer electrons among complexes of the mitochondrial respiratory chain, making it possible to produce energy in the form of ATP; generation of $\cdot O_2^-$ by autoxidation of ubisemiquinone; and

neutralize free radicals, thereby exercising its antioxidant role. This apparently paradox in its properties that allows CoQ to act both as a pro-oxidant and an antioxidant suggests that it may also modulate the cellular redox state under physiological or pathological conditions, and in particular, the aging process (*181*). The redox functions of CoQ are due to its ability to exchange two electrons in a redox cycle between the oxidized form (ubiquinon) and the reduced form (ubiquinol). This reaction can occur in a single step by the simultaneous transfer of two electrons or by two sequential steps of one electron each, through a semiquinone mediatory (*182*).

Furthermore, at the inner mitochondrial membrane, CoQ is recognized as a crucial co-factor for uncoupling proteins and a modulator of the transition pore (*183*).

Recent data have revealed that some of its effects may be related to gene induction mechanisms involved in human cell signaling, metabolism and cellular transport (*184*).

In its reduced form, CoQ inhibits protein and DNA oxidation (*185*), although its capacity in order to avoid lipid peroxidation is the antioxidant function most deeply studied. Ubiquinol protects cell membrane lipids from oxidation and also interferes in the oxidation process of lipoprotein lipids present in the circulation. In fact, studies performed on LDL peroxidability in relation to its CoQ content clearly show that ubiquinol is the most active antioxidant and this content can be boosted through supplementation with exogenous CoQ. In this way, the resistance of the LDL particles is increased against the initiation of lipid peroxidation (*186*).

3.3. Therapeutic use of CoQ

CoQ has been widely used as a dietary supplement for more than 30 years and its use has grown with a corresponding increase in daily dosage. For this

reason, many studies concerning CoQ safety levels have been reported demonstrating its low toxicity in humans. Indeed, several clinical trials have indicated that the safety level for CoQ is 1200 mg/day/person and pharmacokinetic studies in animals and humans suggest that exogenous supplementation does not influence its endogenous biosynthesis nor does it accumulate in the organism (*187*).

Cardiovascular disease is basically the main field of study of the CoQ therapeutic use and recent findings confirm a role of this antioxidant lipid in improving endothelial function. Patients affected by coronary artery disease have decreased levels of endothelium-bound extracellular SOD (ecSOD). In a randomized and controlled study of patients with ischemic heart disease, CoQ treatment at doses of 300 mg/day resulted in an improvement in ecSOD activity and this effect was accompanied by an increase of the flow-mediated dilation of the endothelium (*188*). In this regard, a recent meta-analysis conducted by Gao *et al.* provided important evidence that CoQ therapy improves endothelial function in patients both with and without cardiovascular disease (*189*). Furthermore, CoQ has been demonstrated to have an anti-atherogenic effect in apo-E deficient mice fed with high-fat diet. In this model, CoQ supplementation was capable of decreasing the concentration of lipid hydroperoxides in atherosclerotic lesions (*190*).
In reproductive medicine, several treatments with exogenous CoQ have also been employed. A study with 212 infertile men showed that the administration of 300 mg/day of CoQ or placebo for 26 weeks significantly improved sperm count and motility values (*191*).

A common shared characteristic among degenerative diseases is mitochondrial dysfunction with abnormal energy metabolism and increased levels of cellular OxS. Therefore, the use of antioxidants is being widely explored for its

potential treatment, in which CoQ may be a promising neuroprotectant at high dosages in order to slow progression of Alzheimer's disease, Parkinson's disease, Friedeich's ataxia, Huntington's disease and other neurodegenerative disorders (*175, 192*).

Regarding to the aging process, it seems that the CoQ content decreases during aging especially in mitochondria of some tissues and also in certain species (*181*). Moreover, it has been suggested that the high rate of ROS production by the mitochondrial respiratory chain induces a progressive peroxidation of mitochondrial phospholipids. This fact could lead to the dissociation of Complexes I-III and the subsequent loss of electron transferring. Thus, according to this statement, an increase in CoQ levels through supplementation may partially counteract this detrimental effect (*193*).

In our study population of elderly people, we have previously demonstrated that the supplementation of 200mg/day of CoQ in sinergy with Med diet (Med+CoQ diet) has an additive effect on the antioxidant benefits provided for this dietary pattern. Indeed, the consumption of Med+CoQ diet resulted in a greater postprandial decrease in PC plasma levels; in SOD, CAT and GPx plasma activities and a greater increase in capillary flow and NO plasma levels with respect to the intake of a Med diet or a SFA diet (*194*). Furthermore, we have also proved that Med+CoQ diet consumption reduces the activation and stabilization of p53 in response to the DNA damage produced by OxS during the postprandial period in an elderly population (*195*).

Taken together, all of these intervention studies with supplementation of CoQ make evident its beneficial effects on numerous health disorders, particularly those that are related to OxS process such as aging and age-related diseases.

III. HYPOTHESYS

III. HYPOTHESIS

Aging population is a global and progressive phenomenon that is expanding over the last decades. Accelerated aging population is occurring mainly because of declining fertility rates and increasing life expectancy, especially in the developing world (*5*). Nowadays, people over 60 years old constitute 11 per cent of the global population and, by 2050 it is expected to account for 22 per cent of the global population (*6*). Accordingly, the demand for long-term healthcare is exponentially growing and compromising the sustainability of health and social services. Nutrition and other lifestyle habits have a major impact on the risk of developing chronic diseases that appear more frequently in the elderly. In order to prevent these chronic disorders, a balanced diet and healthy lifestyles may be the main strategy to follow.

Previous evidences support the fact that the aging process is accompanied by an increase in OxS and ER stress, phenomena that have been associated with the activation of inflammatory pathways and the production of pro-inflammatory cytokines, which contribute to the low-grade chronic inflammatory state that underlies aging and age-related diseases (*196*). Another parameter highly related to aging and to some health disorders such as MetS, is the telomere length, which seems to be affected by OxS processes. In fact, progressive telomere attrition leads to a critical length that triggers the cell arrest phenomenon known as senescence (*12*). Due to the universal fact that telomere shorten with age, its study is crucial for understanding mechanisms of age-related diseases (*9*).

Diet and particularly fat quality may affect the ROS homeostasis and, in consequence, both OxS and ER stress levels. Despite the majority of our knowledge about this fact comes from fasting state studies, fasting is not the typical physiological state of the occidental society, where successive food intake makes humans spend most of the time in the postprandial state (*197,*

198). Thus, it is fundamental to study the effect induced by the long-term consumption of different dietary patterns as well as the metabolic changes that occur during the postprandial state. In this connection, the long-term consumption of a Mediterranean diet has been shown to decrease the postprandial OxS by reducing processes of cellular oxidation in elderly people (*199*).

CoQ is part of the mitochondrial electron transport chain and a potent antioxidant. Its beneficial effect has been reported in large preclinical and clinical studies regarding to cardiovascular disease, endothelial dysfunction and other conditions linked to mitochondrial dysfunction. In this regard, antioxidant supplementation interventions have been carried out suggesting that these therapies may ameliorate the oxidant and inflammatory status (*189, 200*).

Our hypothesis focuses on demonstrating that the consumption of diets with different fat quality modulates the processes related to oxidative stress, ER stress and inflammatory response in terms of gene expression in an elderly population, during fasting and postprandial state. Moreover, the supplementation with a natural antioxidant such as CoQ further improves this modulation.

IV. OBJECTIVES

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Main objective

To evaluate whether the long-term consumption of three dietary patterns with different fat quality [1. Mediterranean diet supplemented with coenzyme Q_{10} (Med+CoQ diet); 2. Mediterranean diet without supplementation (Med diet); 3. Saturated fatty acid-rich diet (SFA diet)] modulates oxidative stress in fasting and postprandial state, by modifying the gene expression of Nrf2, $p22^{phox}$ and $p47^{phox}$ NADPH oxidase subunits, SOD1, SOD2, Gpx1, TrxR and the protein concentrations of cytoplasmic and nuclear Nrf2 and Keap-1 in PBMC from elderly people.

Secondary objectives

1. To investigate the effect of the long-term consumption in fasting and postprandial state of three dietary patterns with different fat quality and supplementation with CoQ, on the expression of genes related to endoplasmic reticulum stress (sXBP-1, CRT, BiP/Grp78) in PBMC from elderly people.

2. To study the effect of the long-term consumption in fasting and postprandial state of three dietary patterns with different fat quality and supplementation with CoQ, on the expression of genes related to the inflammatory response (p65 subunit of NF-κB, IκB-α, IKK-β, MMP-9, IL1-β and JNK-1) in PBMC from elderly people.

3. To investigate whether diets with different fat quality and supplementation with CoQ affect the metabolomic profile in urine analyzed by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) from elderly people.

4. To determine whether there is a direct relationship between endothelial function, through oxidative stress mechanisms, and relative telomere length (RTL) in a cross-sectional study involving an elderly population and a subgroup of metabolic syndrome patients from a LIPGENE subcohort.

V. MATERIALS AND METHODS

V. MATERIALS AND METHODS

A. STUDY POPULATION I

1. Participants and recruitment

Volunteers were recruited using various methods including the use of general practitioner databases, poster and newspaper advertisements. Recruitment of participants and dietary intervention took place between January 1st, 2006 and November 15st, 2007. A total of 63 persons were contacted among those willing to enter the study. All participants underwent a comprehensive medical history, physical examination and clinical chemistry analysis before enrolment and gave their informed consent before joining the study. Inclusion and exclusion criteria were fulfilled by 20 participants (age ≥ 65 years; 10 men and 10 women). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by The Human Investigation Review Committee at Reina Sofía University Hospital (Córdoba, Spain).

1.1. Sample size calculation

The calculation was made based on the following premises:

- Primary endpoint of the study: Gene expression of Nrf2 (mRNA levels)
- Standard deviation of the primary endpoint: 30%
- Difference in minimum expected: 20%
- Alpha error = 0.05 (Confidence level: 95%)
- Beta error = 0.10 (Power: 90%)
- Hypothesis Contrast: Bilateral.

Based on these premises are required 18 participants per group.

1.2. Inclusion criteria

- Age ≥ 65 years.
- Body mass index: 20–40 kg/m^2.
- Apo E E_3/E_3 genotype.
- Total cholesterol concentration equal to or < 8.0 mmol/L.
- Non-smokers.
- No severe chronic pathology associated. The concomitance of diseases such as hypertension without micro- or macroangiopathy, dyslipidemia, degenerative joint disease, obesity, benign prostatic hyperplasia or chronic venous insufficiency.

1.3. Exclusion criteria

- Age < 65 years.
- Diabetes or other endocrine disorders.
- Chronic inflammatory conditions.
- Kidney or liver dysfunction.
- Iron deficiency anemia (hemoglobin < 12 g/dL men, < 11 g/dL women).
- Prescribed hypolipidemic medication.
- Prescribed anti-inflammatory medication.
- Fatty acid supplements including fish oil.
- Consumers of high doses of antioxidant vitamins (A, C, E, β-carotene).
- Highly trained or endurance athletes or those who participate in more than three periods of intense exercise per week.

- Weight change equal or > 3 kg within the last 3 months.
- Alcohol (> 20g/day) or drug abuse (based on clinical judgment).
- Smokers.

2. Study design

2.1. Randomization and diet intervention

Participants were randomly assigned to receive, in a crossover design, three isocaloric diets with different fat quality for 4-week period each (**Figure 7**).

The three diets were as follows:

- Mediterranean diet supplemented with CoQ (Med+CoQ diet): containing 15% of energy as protein, 47% of energy as carbohydrates and 38% of total energy as fat [24% MUFA (provided by virgin olive oil), 10% SFA, 4% PUFA]. CoQ supplementation was supplied with daily capsules of 200 mg.

- Mediterranean diet supplemented with placebo (Med diet): with the same composition of the Med+CoQ diet but supplemented with placebo capsules.

- Western diet rich in saturated fat (SFA diet): containing 15% of energy as protein, 47% of energy as carbohydrate and 38% of total energy as fat (12% MUFA, 22% SFA, 4% PUFA).

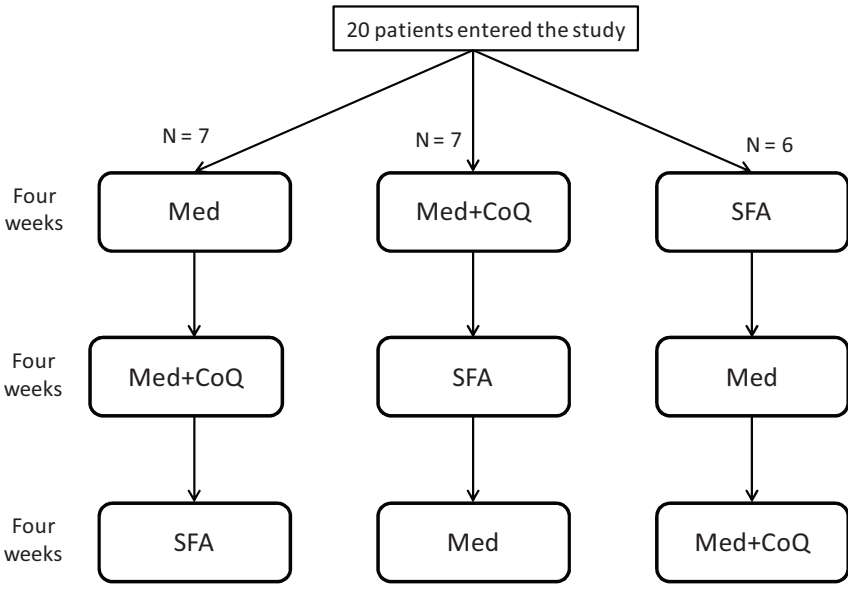


Figure 7. Flow-chart of subjects who participated in each 4-week feeding trial.

The cholesterol intake was kept constant (< 300 mg/day) during the three periods. Eighty percent of the MUFA diet was provided by VOO, which was used for cooking, dressing salads and as a replacement for butter. Butter was used as the main source of fat during the SFA dietary period.

Both the CoQ and the placebo capsules were specially produced by the same company (Kaneka Corporation, Osaka, Japan) and were identical in weight and external aspect. Participants taking capsules were unaware whether they were in the Med+CoQ or Med dietary period.

The composition of the experimental diets was calculated by using the US Department of Agriculture food tables (*201*) and Spanish food composition tables for local foodstuff (*202*). The nutrient composition for each dietary pattern is detailed in **Table 1**.

Table 1: Composition of diet at the end of intervention period, alongside dietary targets[1]

	Med+CoQ diet	Med diet	SFA diet
Energy (MJ/d) (E)	8.16 ± 0.32	8.22 ± 0.35	8.21 ± 0.31
%E from fat	39.73 ± 2.65	39.11 ± 2.02	40.38 ± 2.03
%E from SFA	9.04 ± 0.71[a]	8.78 ± 0.61[a]	20.75 ± 0.87[b]
%E from MUFA	24.36 ± 1.47[a]	24.24 ± 0.92[a]	13.46 ± 0.71[b]
%E from PUFA	4.28 ± 0.29[a]	4.15 ± 0.23[a]	3.92 ± 0.25[b]
%E from protein	16.07 ± 0.64	16.33 ± 0.52	16.16 ± 0.53
%E from CHO	44.31 ± 2.29	44.64 ± 1.86	43.64 ± 1.73
Cholesterol (mg/d)	345.86 ± 36.92	345.71 ± 35.93	378.18 ± 59.29
EPA (g/d)	0.15 ± 0.23	0.15 ± 0.23	0.15 ± 0.23
DHA (g/d)	0.26 ± 0.48	0.26 ± 0.48	0.26 ± 0.48
Total fibre (g/d)	27.17 ± 2.38	27.93 ± 2.22	27.42 ± 2.34
Ascorbic acid (mg/d)	181.17 ± 6.12	187.54 ± 5.83	195.62 ± 6.86
α-tocoferol (mg/d)	18.95 ± 0.84[a]	18.91 ± 0.73[a]	6.97 ± 0.76[b]
β-carotene (mg/d)	3.13 ± 0.66	3.11 ± 0.92	3.21 ± 0.23
Nicotinic acid (mg/d)	21.54 ± 3.72	21.90 ± 3.95	21.87 ± 3.90
C14 (g/d)	0.48 ± 0.26[a]	0.47 ± 0.27[a]	7.00 ± 2.41[b]
C16:0 (g/d)	14.10 ± 4.79[a]	14.10 ± 3.96[a]	25.83 ± 6.85[b]
C16:1 (g/d)	1.81 ± 0.45[a]	1.81 ± 0.40[a]	3.11 ± 0.75[b]
C18:0 (g/d)	4.81 ± 2.45[a]	4.82 ± 2.23[a]	10.23 ± 3.41[b]
C18:1 (g/d)	61.10 ± 12.39[a]	60.40 ± 6.60[a]	37.15 ± 9.02[b]
C18:2 n6 (g/d)	9.28 ± 2.03	9.27 ± 1.64	5.82 ± 2.43
C18:3 n3 (g/d)	0.65 ± 0.16	0.66 ± 0.11	0.86 ± 0.26

[1] *Values are means ± SE (n = 20). Means in a row with superscripts without a common letter differ, p < 0.05. Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet*

supplemented with CoQ; SFA diet, SFA-rich diet; CHO, carbohydrate; SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid; g, grams; mg, milligrams; EPA, eicosapentaenoic acid; DHA, docosahexaenoic; C14:0, myristic saturated fatty acid; C16:0, palmitic saturated fatty acid; C16:1, palmitoleic monounsaturated fatty acid; C18:0, stearic saturated fatty acid; C18:1, oleic monounsaturated fatty acid; C18:2, linoleic polyunsaturated fatty acid; C18:3, linolenic polyunsaturated fatty acid.

Before the start of the intervention period, volunteers completed a 3-day weighed food diary and extensive Food Frequency Questionnaires, which allowed the identification of foods to be modified. At the start of the intervention period, each participant was provided with a handbook for the diet to which they had been randomized. Advice was given on foods to choose and those to avoid if eating outside home. They were also instructed to write down in the diary about any menu eaten out of home and to call the monitoring study nurse reporting such event. At baseline, volunteers were provided with a supply of study foods to last for 2 weeks (extra VOO for Med and Med+CoQ diet and butter for SFA diet). They collected additional study foods every fortnight or when required.

At these times, a 24-h recall of the previous day food intake and a short food use questionnaire based on the study foods were completed to monitor and motivate volunteers to adhere to the dietary advice. A point system was used to assess the number of food exchanges achieved in the 24-h recall and additional advice was given if either the 24-h recall or food use questionnaire showed inadequate intake of food exchange options. Volunteers were asked to complete 3-day weighed food diaries at baseline, weeks 2 and 4. Weighed food intake over two weekdays and one weekend day was obtained using scales provided by the investigators. Fat foods were administered by dietitians in the intervention study. The dietary analysis software Dietsource version 2.0 was used (Novartis S.A., Barcelona, Spain).

2.2. Postprandial study

At the end of the dietary intervention period, subjects were given a fatty breakfast with the same fat composition as consumed in each of the dietary intervention period. Participants presented at the clinical centre at 8-hours following a 12 hours fast (time 0), abstained from alcohol intake during the preceding 7 days. After canulation of a blood vessel, a fasting blood sample was taken before the test meal, which was then ingested within 20 min under supervision.

The test meal reflected the fatty acid composition of each subject after the chronic dietary intervention. Subsequent blood samples were drawn at 2 and 4 hours after the fat challenge. Test meals provided an equal amount of fat (0.7 g/kg body weight), cholesterol (5 mg/kg of body weight) and vitamin A (60,000 IU/m^2 body surface area). It provided 65% of energy as fat, 10% as protein and 25% as carbohydrates. The composition of the breakfasts was as follow:

- Med+CoQ diet: with 12% of the total fat as SFA, 43% as MUFA and 10% as PUFA, supplemented with 400 mg of CoQ in capsules.

- Med diet: with 12% of the total fat as SFA, 43% as MUFA and 10% as PUFA, supplemented with 400 mg of placebo in capsules.

- SFA diet: with 38% of the total fat as SFA, 21% as MUFA and 6% as PUFA.

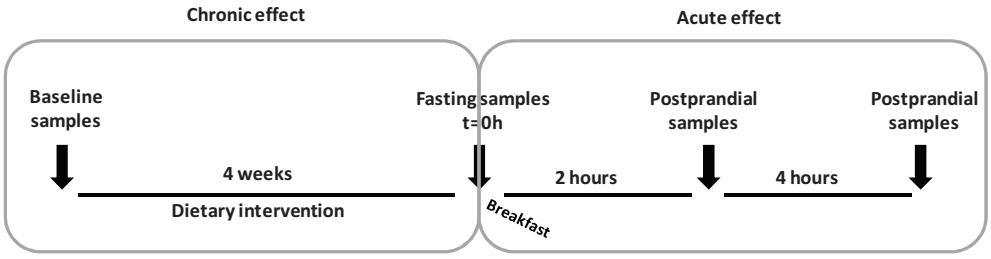


Figure 8. Diagram of the study design.

3. Biochemicals determinations

3.1. Plasma samples

Venous blood samples were obtained at the end of each dietary intervention period at fasting state (time 0h), following a 12-fast, and at 2 and 4 hours after the intake of the breakfast. Samples from the fasting and postprandial state were collected in tubes containing 1g EDTA/L, stored in containers with ice and kept in the dark. Particular care was taken to avoid exposure to air, light, and ambient temperature. Plasma was separated from whole blood by low-speed centrifugation at 1500 x *g* for 15 min at 4°C within 1 h of extraction.

3.2. Lipid analysis

Lipid variables were analyzed with a modular autoanalyzer (DDPPII Hitachi; Roche, Basel, Switzerland) with the use of Boehringer-Mannheim reagents®. Total cholesterol (TC), TG and lipoprotein fractions were assayed in plasma by means of enzymatic procedures. Apolipoprotein A1 (Apo A1) and apolipoprotein B (Apo B) levels were determined by inmunoturbidimetry. HDL-C was measured by analyzing the supernatant obtained following precipitation of a plasma aliquot with dextran sulfate-Mg^{2+}. LDL-C levels

were estimated using the Friedewald formula based on TC, TG, and HDL-C concentrations. Plasma glucose concentrations were measured with an Architect-CG16000 analyzer (Abbott Diagnostics, Tokyo, Japan) by the hexoquinase method. Plasma insulin concentrations were measured by chemoluminesence with an Architect-I2000 analyzer (Abbott Diagnostics, Tokyo, Japan). High-sensitivity C-reactive protein concentrations were measured according to Rifai et al (*203*).

3.3. Coenzyme Q_{10}

CoQ levels were carried out in plasma samples according to the method described by Santos-Gonzalez et al (*204*). Quantification was perfomed by reversed-phase HPLC. Separation was performed at 1 mL/min in a C18 column (5 μm particles, 5×0.45 cm) and with a mobile phase that consisted of a mixture of methanol and n-propanol (1:1) containing lithium perchlorate (2.12 g/L). Monitoring was carried out with a Coulochem II electrochemical detector (ESA, Chelmsford, MA) fitted with a Model 5010 analytical cell with the electrodes set at potentials of −500 and +300 mV. CoQ was detected from the signal obtained at the second electrode. Eluted compounds were quantified by integration of peak areas and comparison with an external CoQ standard (Sigma Aldrich, Madrid, Spain).

3.4. Isolation of PBMC

Peripheral blood mononuclear cells (PBMC) are a sub-set of white blood cells, including lymphocytes and monocytes, which play a critical role in the immune system. Various studies have referred to PBMC as a model to study the response of energy homeostasis-related genes to acute changes in feeding conditions and also have shown disease-characteristic gene expression patterns

(*205, 206*). These cells can be easily and repeatedly collected in sufficient quantities in contrast to the more invasive sampling of adipose, muscle and liver tissues, amongst others (*207*).

PBMC were isolated from 20 mL of venous blood in tubes containing 1 mg/mL of EDTA. Blood samples were diluted 1:1 in PBS, and cells were separated in Ficoll gradient by centrifugation at 800×g for 25 min at 20°C. Cells were collected and washed with cold PBS twice and finally resuspended in buffer A. This buffer contained 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 mM KCl, 2 mM MgCl2 and 1 mM EDTA. At the time of use 1 mM PMSF and 1 mM DTT were added. Finally, cells obtained were stored at −80°C for further analysis.

4. RNA isolation and real time-polymerase chain reaction (RT-PCR)

4.1. RNA isolation

Total RNA from PBMC was isolated using the trizol method according to the recommendations of the manufacturer (Tri Reagent®, Sigma, St Louis, MO, USA) and quantified in a NanoDrop 1000A Spectrophotometer. RNA integrity was verified on agarose gel electrophoresis and stored at −80°C. Next, since qRT-PCR can detect even a single molecule of DNA, RNA samples were digested with DNAse I (AMPD-1 KT, Sigma) before RT-PCR.

4.2. Gene expression analysis by RT-PCR

4.2.1. Reverse transcription polymerase chain reaction

The reverse transcription polymerase chain reaction was performed using the commercial kit iScript cDNA Synthesis Kit (Bio Rad) according to the

manufacturer's instruction. Briefly, cDNA was synthesized from 1µg of total RNA in a final volume of 20µL. Each mixture was prepared in sterile tubes of 0.2µL, RNase, DNase and exogenous DNA free. The reverse transcription reaction was carried out in a thermo-cycler MJ Thermal Cycler Personal mini™ closure (*BioRad Inc., Hercules, CA, USA)* consisted of 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. The cDNA ready for qRT-PCR, was stored at -20 °C until its performing.

4.2.2. RT-PCR

The reaction of real-time PCR was carried out using the platform OpenArray™ NT Cycler (Applied Biosystems). This system of gene expression analysis is used as a method for quantification TaqMan probes. OpenArray™ subarrays were preloaded by Biotrove with the selected primer pairs. The individual primer pairs (synthesized by Sigma-Aldrich, St. Louis, MO, USA) were preloaded into Bio Trove OpenArray™ plates. Each primer pair was spotted in duplicate. Primers that amplify the genes of interest were selected from the database TaqMan Gene Expression Assays (Applied Biosystems;https://products.appliedbiosystems.com/ab/en/US/adirect/ab?Cmd =catNavigate2&catID=601267) in Assays search tab taking as search criteria: selection and Homo sapiens Gene Expression Assays for each of the genes of interest.

Samples were loaded into OpenArray plates with the OpenArray NT Autoloader according to the manufacturer's protocols. Each subarray was loaded with 5.0 µl of master mix consisted of 1× LighCycler FastStart DNA Master SYBR Green Kit (Roche Applied Sciences, Indianapolis, IN, USA), 1× SYBR Green I 80×, 0.5% glycerol, 0.2% Pluronic F-68, 1 mg/mL bovine serum albumin (New England Biolaboratories, Beverly, MA, USA), 1 mM MgCl2, 400 nM FP, 400 nM RP, 8% Formamide, 0.25× Rox, 1× TfR

amplicon, and cDNA samples. The PCR OpenArray thermal cycling protocol consisted of 95°C for 10 min, followed by cycles of 10 s at 95°C, 10 s at 53°C, and 10 s at 72°C. All samples were tested in duplicate. The Biotrove OpenArrayTM NT Cycler System Software (version 1.0.2) uses a proprietary calling algorithm that estimates the quality of each individual CT value by calculating a CT confidence value for each amplification reaction.

In our assay, CT values with CT confidence values below 700 were regarded as background signals. The remaining positive amplification reactions were analyzed for amplicon specificity by studying the individual melting curves.

The same program allowed the selection of the most stable housekeeping gene in the samples processed for the relativization of the expression of genes of interest.

Following this methodology, we analyzed the relative gene expression of the following genes: Nrf2, $p47^{phox}$ and $p22^{phox}$ (NADPH oxidase subunits), SOD1, SOD2, GPx1, TrxR, p65 (RelA), IκB-α, IKK-β, MMP-9, IL-1β, JNK-1, sXBP-1, CRT and BiP/Grp78.

5. Western Blot analysis

5.1. Protein extraction

PMBC were thawed on ice and buffer A was supplemented with 5µg Aprotinin, 10µg Leupeptin and 0.8% Nonidet NP-40. Cells were incubated on ice for 5 minutes, subjected to gentle agitation for 20 seconds in the vortex, and then centrifuged at 13000 rpm for 5 minutes at 4ºC. The supernatant containing cytoplasmic proteins was distributed in aliquots that were stored at -80ºC. The pellet was treated with 100µL of lysis buffer (20mM HEPES, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM PMSF, 1mM DTT, 20µg Aprotinin and 40µg Leupeptin). The sample was incubated on ice for 20

minutes with periodic mixing with a vortex by stirring for 30 seconds every 5 minutes of incubation. Cells were then centrifuged at 13000 rpm for 5 minutes at 4ºC. The obtained supernatant, containing the nuclear proteins, was distributed in aliquots and stored at -80ºC. The extracted proteins were quantified using the method of Bradford (1976) (Bio-Rad Protein Assay).

5.2. Western Blot

Electrophoretic separation was carried out with 50µg of protein for both cytoplasmic and nuclear fractions. After separation in SDS-PAGE gels (11% polyacrylamide) proteins were transferred to nitrocellulose membranes (BioTrace NT Membrane; PALL Gelman Laboratory). The following proteins were detected using their corresponding antibodies: Nrf2 (C-20, sc-722: mouse monoclonal, Santa Cruz Biotechnology, inc.); Keap-1 (Kelch-like ECH associating protein 1) (H-190, sc-33569: rabbit polyclonal, Santa Cruz Biotechnology, inc.); Actin (C-2, sc-8432: mouse monoclonal, Santa Cruz Biotechnology, inc.); TFIIB (C-18, sc-225: rabbit polyclonal Santa Cruz Biotechnology, inc.). After incubation with these primary antibodies, samples were incubated with respective secondary antibodies (goat anti-mouse or anti-rabbit HRP-conjugate, Santa Cruz Biotechonology, inc.). The development process was carried out with ECL-Plus Western Blotting Detection System (Amersham^{TM}) and used for autoradiography Hyperfilm MP High performance autoradiography film (Amersham^{TM}).

5.3. Quantification of protein bands

Proteins were identified in the autoradiography by its relative position to molecular weight markers: 57 kDa for Nrf2, 69 kDa for Keap-1, 40 kDa for actin and 30 kDa for TFIIB. The relative amount of each on was quantified by

densitometry using the software Quantity One.

6. Metabolomic analysis

6.1. Biofluid collection

First void urine samples were collected from ten elderly participants at baseline (pre-intervention) and after a 12 h fast (time 0, post-intervention) and centrifuged at 1800g for 10 min at 4ºC. Aliquots (500µL) of the urine were stored at -80ºC until analysis by Proton Nuclear Magnetic Resonance spectroscopy (^{1}H-NMR).

6.2. ^{1}H-NMR spectroscopy

Urine samples were prepared by the addition of 250µL phosphate buffer (0.2M KH_2PO_4, 0.8M K_2HPO_4) to 500µL urine. Following centrifugation at 7155 x g for 5 minutes, 50µL D_2O and 10µL TSP were added to 540µL of the supernatant. Spectra were acquired on a 600 MHz NMR spectrometer (Varian, Palo Alto, CA 94304-1039, United States) using a Noesypresat pulse sequence at 25ºC. Spectra were acquired with 16 K data points and 128 scans over a spectra width of 8 kHz. Water suppression was achieved during the relaxation delay (2.5s) and the mixing time (100ms). All ^{1}H-NMR urine spectra were referenced to TSP at 0.0 ppm, line broadened (0.3 Hz) and phase and baseline corrected using the processor on Chenomx NMR suite 5.1 (Chenomx Inc, Edmonton, Alberta, T5K 2J1). Spectra were integrated into spectral regions of 0.01 ppm. The water region (4–6 ppm) was excluded and the data was normalized to the sum of the spectral integral.

7. Statistical analysis

The Statistical Package for the Social Sciences (SPSS 17.0 for Windows Inc., Chicago, Illinois) was used for the statistical comparisons. The Kolmogorov-Smirnov test did not show a significant departure from normality in the distribution of variance values. In order to evaluate data variation, Student's t test and an analysis of variance for repeated measures (ANOVA) was performed, followed by Bonferroni's correction for multiple comparisons. We studied the statistical effects of the type of fat meal ingested, independent of time (represented by $p1$), the effect of time (represented by $p2$), and the interaction of both factors, indicative of the degree of the postprandial response in each group of subjects with each fat meal (represented by $p3$). Differences were considered to be significant when $p < 0.05$. All data presented in text and tables are expressed as means ± standard error (± SE).

7.1. Statistical analysis for metabolomics

The ^{1}H-NMR spectral data were processed using SIMCA-P+ (version 12.0.1; Umetrics, Umea; Sweden) mean centered and pareto scaled ($1/\sqrt{SD}$). Principal component analysis (PCA) was applied to the urine dataset and the score plots were visually inspected for patterns and outliers. Differences between baseline and diet intervention were further analyzed by using partial least squares discriminant analysis (PLS-DA) which is a supervised technique that uses previous knowledge about the samples. The quality of the models formed by PCA and PLS-DA was assessed by interrogation of the R^2 and Q^2 parameters. The R^2 parameter is a representation of how much of the variation within the data set is explained by the components of the model, and the Q^2 parameter gives an indication of the prediction power of the model. Additionally, permutation testing was performed on each PLS-DA model (with 20

permutations) to assess the validity of the model. Permutation testing was performed in SIMCA-P+.

Where robust PLS-DA models were built, the variable importance in the projection (VIP) and loading plots were used to identify the regions of the spectra that had the largest discriminatory effect in the model. Variables with a VIP value > 2 were considered important in discriminating between groups. Metabolites responsible for the peaks in these regions were assigned using in-house databases and the Chenomx Database. Assignments were confirmed using 2D TOCSY experiments.

Hippurate, phenylacetylglycine, glycerol results and gender comparison were analyzed using Student's t test in order to evaluate data variation after dietary intervention. A study of the relationship among parameters was also carried out using Pearson's linear correlation coefficient. Differences were considered to be significant when $p<0.05$.

B. STUDY POPULATION II

1. Participants and study design

This cross-sectional study was conducted within the framework of the LIPGENE study ('Diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis'), a Framework VI Integrated Project funded by the European Union (*208*).

Subject eligibility was determined using a modified version of the NCEP criteria for MetS, according to published criteria (*209*).

Subjects were required to meet at least two of the following five criteria: waist circumference > 102 cm (men) or > 88 cm (women); fasting glucose 5.5–7.0 mmol/l; TG ≥ 1.5mmol/l; HDL-C < 1.0mmol/l (men) or < 1.3mmol/l (women); blood pressure (BP) ≥ 130/85mmHg or treatment of previously diagnosed hypertension. We used a subgroup of pre-intervention data for 68 subjects (26 men and 42 women), which conformed to the LIPGENE inclusion and exclusion criteria (*210*).

All participants provided written informed consent and underwent a comprehensive medical history, physical examination and clinical chemistry analysis before enrollment. Participants displayed no signs of cardiac dysfunction or hepatic, renal and thyroid diseases and were requested to maintain their regular physical activity and lifestyle. Participants were also asked to record in a diary any event that could affect the outcome of the study. All of them were free from cardiovascular complications at the time of the enrollment. The study was carried out in the Lipid and Atherosclerosis Unit at the Reina Sofia University Hospital, from February 2005 to April 2006. The experimental protocol was approved by the local ethics committee according to the Helsinki Declaration. The study was registered with the US National Library of Medicine Clinical Trials registry (NCT00429195).

1.1. Sample size calculation

The calculation was made based on the following premises:

- Primary endpoint of the study: IRH measurement according to RTL
- Standard deviation of the primary endpoint: 60%
- Difference in minimum expected: 60%
- Alpha error = 0.05 (Confidence level: 95%)
- Beta error = 0.10 (Power: 90%)
- Hypothesis Contrast: Bilateral.

Based on these premises are required 12 patients per group.

1.2. Inclusion criteria

- Age: 35-70 years.
- Gender: males and females (not pregnant or lactating).
- Body mass index: 20–40 kg/m^2.
- Total cholesterol concentration equal to or < 8.0 mmol/L.
- Medications/nutritional supplements allowed, on conditions that the subjects adhere to the same regimen during the study: anti-hypertensive medication (including beta-blockers), oral contraceptives, hormone replacement therapy, multi-vitamin supplements and other non-fatty acid based nutritional supplements.
- Smokers and non-smokers.
- No severe chronic pathology associated. The concomitance of diseases such as hypertension without micro- or macroangiopathy, dyslipidemia, degenerative joint disease, obesity, benign prostatic hyperplasia or chronic venous insuffiency.
- Regular consumer of alcohol, which is not excessive as defined by elevated liver enzymes (AST and ALT).

- Ethnicity: intention to include white Europeans.

1.3. Exclusion criteria

- Age < 35 or > 70 years.
- Diabetes or other endocrine disorders.
- Chronic inflammatory conditions.
- Kidney or liver dysfunction.
- Iron deficiency anemia (hemoglobin < 12 g/dL men, < 11 g/dL women).
- Prescribed hypolipidaemic medication.
- Prescribed anti-inflammatory medication.
- Fatty acid supplements including fish oils, evening primrose oil, etc.
- Consumers of high doses of antioxidant vitamins (A, C, E, β-carotene).
- Red rice yeast (Monascus purpureus) supplement usage.
- High consumers of oily fish (>2 serving of oily fish per week).
- Highly trained or endurance athletes or those who participate in more than three periods of intense exercise per week.
- Volunteers planning to start a special diet or lose weight (e.g. the Slimfast Plan, Atkins Diet, etc.).
- Weight change equal or > 3 kg within the last 3 months.
- Alcohol (> 20g/day) or drug abuse (based on clinical judgment).

2. Anthropometric measurements

After recording clinical histories and conducting physical examinations, we obtained the following anthropometric measurements for each individual:

weight, height, body mass index and waist circumference. Weight was measured while the subject was wearing light indoor clothing, without shoes and after voiding. Height was obtained with a stadiometer graduated in millimeters. The subject was barefoot with the back and head in contact with the stadiometer in the Frankfurt horizontal plane. Body mass index was calculated by dividing weight (kg) by height squared (m^2). Waist circumference (cm) was measured to the nearest 0.5cm with a tape measure at the umbilical scar level. A non-stretchable tape measure was used to measure waist circumference. The measurement was taken directly on the skin with the subject in a standing position with the abdomen relaxed, the arms at the sides and the feet together. We used the homeostatic model assessment index for insulin resistance ($HOMA_{IR}$: fasting insulin (mUl^{-1})/fasting glucose (mmol l^{-1})/22.5) and HOMAβ-cell function as the index of insulin secretory function derived from fasting plasma glucose and insulin concentrations, calculated as 20 x fasting insulin (mUl^{-1})/fasting glucose (mmol l^{-1})-3.5 (*211*). Insulin sensitivity was estimated by a quantitative insulin sensitivity check index (QUICKI) (1/[log insulin (mU l^{-1}) + log baseline glucose (mg dl^{-1})]) (*212*). BP was measured using an automatic BP device. In accordance with the European Society of Hypertension Guidelines (*213*), BP measurement was obtained with an appropriately sized cuff positioned at the heart level and after the patient had been relaxed for at least 5 min. The same arm was used for each measurement, and the average of two measurements was used for data processing.

3. Biochemical determinations

3.1. Plasma samples

Blood was collected in tubes containing ethylene diaminetetraacetic acid

(EDTA) to yield a final concentration of 0.1% EDTA. Plasma was separated from red cells by centrifugation at 1500 x *g* for 15 min at 4°C within 1 h of extraction. Plasma was immediately aliquoted and stored at -80 ºC until analysis.

3.2. Lipid analysis

The lipid variables were analyzed with a modular autoanalyzer (DDPPII Hitachi; Roche, Basel, Switzerland) with the use of Boehringer-Mannheim reagents. Plasma TG were assayed by means of enzymatic procedures (*214*). HDL-C was measured by analyzing the supernatant obtained following precipitation of a plasmatic aliquot in dextran sulfate-Mg^{2+}, as described by Warnick et al. (*215*). Plasma glucose concentrations were measured with an Architect-CG16000 analyzer (Abbott Diagnostics, Tokyo, Japan) by the hexokinase method. Plasma insulin concentrations were measured by chemoluminescence with an Architect-I2000 analyzer (Abbott Diagnostics, Tokyo, Japan). High-sensitivity C-reactive protein concentrations were measured according to Rifai et al. (*203*).

4. Relative telomere length measurement

4.1. DNA isolation

DNA was isolated from buffy coats using the Gentra Puregene Blood kit (Gentra Systems Inc., Minneapolis, MN, USA), and low yielding samples (< 10 ng) were subjected to whole genome amplification using the REPLI-g kit (Qiagen Ltd. West Sussex, UK).

4.2. qRT-PCR for telomere length

RTL was determined using the Cawthon method where the measurements are performed by qRT-PCR (*216*). We estimated the relative ratio of telomere repeat copy number (T) normalized against a single copy gene copy number (Homo sapiens ribosomal protein L13a gene RPL13a; S) for all the samples. Results for each qRT-PCR were relativized to a standard curve built using a reference DNA sample. The standard curves for telomere and genomic qRT-PCRs consisted of eight standards of reference DNA (1-25ng). The correlation between relative T/S ratios measured by qRT-PCR and relative telomere DNA restriction fragment length by Southern blotting has been confirmed previously to be highly consistent (*216, 217*).

All qRT-PCRs were performed in duplicate with the use of iQ5-BIORAD thermal cycler and SensiFAST^{TM} SYBR Lo-ROX kit (Bioline). The thermal cycler profile for both amplicons began with a 95ºC incubation for 3 min to activate the polymerase, followed by 40 cycles of 95ºC for 5 s, 54ºC 15 s.

Specificity of qRT-PCR amplifications was verified by a melting curve program (60-95ºC with a heating rate of 0.5 ºC/s and a continuous fluorescence measurement).

The reaction mix composition was identical except for the oligonucleotide primers:

20 ng template DNA

1X SensiFAST^{TM} SYBR Lo-ROX

200 nM reverse primer

200 nM forward primer

Primers used are shown in **Table 2**. (*218*)

Table 2: Primers sequences used for the qRT-PCR

Gene	Sequence
Telomere	5'–CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT–3'-forward
	5'–GGCTTGCCTTACCCTTACCCTTACCC TTACCCTTACCCT–3'-reverse
RPL13a	5'–CCTGGAGGAGAAGAGGAAAGAGA–3'-forward
	5'–TTGAGGACCTCTGTGTATTTGTCAA–3'-reverse

5. Study of endothelial function using laser Doppler

A laser Doppler linear Periflux 5000 (Perimed S.A., Stockholm, Sweden) was used to measure IRH. The methodology has been published previously elsewhere (*164, 165, 219*), where we found an inter-study variability of 8.85% and an intra-study variability of 8.7%.

Briefly, capillary flow of the second finger of the dominant arm of the participant was assessed for 1 min before (t0) and after (td) applying 4 min of ischemia to the arm by means of a sphygmomanometer. The ischemic reactive hyperemia was obtained IRH = (AUC_{td} 2 AUC_{t0}) × 100AUC_{t0}.

6. Determination of oxidative stress biomarkers

Total nitrite (nitrite and nitrate) was used as an indicator of NO production and was assayed using the Griess method (*220*), with an intra-assay coefficient of variation of 6.1%, and an inter-assay coefficient of variation of 7.7%. The reaction was monitored at 540 nm (UV-1603 spectrophotometer, Shimadzu).

PC content was carried out in plasma samples using the method of Levine (*221*). We found and intra-assay coefficient variation of 4.7%, and an inter-assay coefficient variation of 8.0%. Carbonyls were evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan) at 360 nm.

7. Antioxidant enzyme activities

Total SOD (E.C: 1.15.1.1) activity was determined by colorimetric assay in plasma at wavelength of 525 nm according to the method described by McCord and Fridovich et al. (*222*), with an intra-assay coefficient variation of 7.3% and an inter-assay coefficient variation of 8.7%. GPx (E.C.: 1.11.1.9) activity was evaluated in plasma by the Flohé and Gunzler method (*223, 224*), with an intra-assay coefficient of variation of 6.5% and an inter-assay coefficient of variation of 7.2%. The GPx assay is based on the oxidation of NADPH to NAD+, catalyzed by a limiting concentration of glutathione reductase, with maximum absorbance at 340 nm. The absorbance was evaluated in a Shimadzu UV-1603 spectrophotometer (Kyoto, Japan).

8. Statistical analysis

The Statistical Package for the Social Sciences (SPSS 17.0 for Windows Inc., Chicago, IL, USA) was used for the statistical comparisons. RTL was analyzed as quartiles after removal of experimental outliers. Telomere length was categorized into quartiles (shortest to longest) and analyzed as a categorical variable. This resulted in the following distribution of RTL, quartile 1: RTL ≤ 1.10, quartile 2: 1.10 < RTL ≤ 1.26, quartile 3: 1.26 < RTL ≤ 1.56, quartile 4: RTL > 1.56.

Univariate analysis of variance adjusted for age was performed to compare variables across quartiles of RTL with Bonferroni post hoc test used for multiple comparisons. Differences were considered to be significant when $p < 0.05$. All data presented in text and tables are expressed as the means ± standard error (SE).

VI. RESULTS

VI. RESULTS

Expression of antioxidant, pro-inflammatory and endoplasmic reticulum stress-related genes and protein levels in an elderly population following consumption of diets with different fat quality

In order to carry out the study, 20 participants (10 men and 10 women) from the *Study Population I* completed the long-term dietary intervention and the postprandial post-intervention study.

1. Baseline characteristics

The epidemiological, anthropometric and lipid data from the participants of the study at baseline are shown in **Table 3**. As expected, males had higher height (166.65 ± 1.43cm vs 154.8 ± 1.41cm, $p < 0.001$) and waist circumference (109.62 ± 11.61cm vs 92.35 ± 7.07cm, $p = 0.001$) than females.

At the start of the study, males had higher TG (1.25 ± 0.11mmol/L vs 0.87 ± 0.13mmol/L, $p = 0.013$) and Apo B plasma levels (0.90 ± 0.04 mmol/L vs 0.76 ± 0.02mmol/L, $p = 0.036$) than females. We did not find any other differences by gender.

Table 3: Epidemiological, anthropometric data and levels of lipids, apolipoproteins, glucose, insulin and CRP from participants of the study at baseline[1].

	Male (*n*=10)	Female (n=10)	p
Age (years)	65.82 ± 0.91	68.44 ± 1.36	0.208
Weight (kg)	87.73 ± 4.72	78.44 ± 3.19	0.232
Height (cm)	166.65 ± 1.43[a]	154.8 ± 1.41	<0.001
BMI (kg/m^2)	31.25 ± 1.57	32.52 ± 1.33	0.614
Waist circumference (cm)	109.68 ± 2.92[a]	92.41 ± 1.84	0.001
TC (mmol/L)	4.95 ± 0.21	4.52 ± 0.16	0.141
TG (mmol/L)	1.25 ± 0.11[a]	0.87 ± 0.13	0.013
HDL-C (mmol/L)	1.26 ± 0.05	1.42 ± 0.07	0.137
LDL-C (mmol/L)	3.06 ± 0.12	2.70 ± 0.11	0.107
Apo B (g/L)	0.90 ± 0.04[a]	0.76 ± 0.02	0.036
Apo A (g/L)	1.43 ± 0.03	1.52 ± 0.05	0.301
Glucose (mmol/L)	5.77 ± 0.23	5.07 ± 0.14	0.064
Insulin (pmol/L)	80.72 ± 8.68	56.75 ± 4.45	0.078
CRP (mg/L)	4.36 ± 1.35	4.37 ± 1.01	0.996

[1]*Values are means ± SE (n = 20). Means in a row with superscripts without a common letter differ, p < 0.05. Kg, kilograms; cm, centimetres; BMI, body mass index; m^2, square metres; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein-cholesterol; LDL-C low density lipoprotein-cholesterol; ApoB, apolipoprotein B; Apo A, apolipoprotein A-1; CRP, C-reactive protein.*

2. Lipid analysis after the long-term intervention of different dietary patterns

Plasma levels of lipids, glucose, insulin and CRP at fasting state at the end of each period of dietary intervention are shown in **Table 4**. Plasma concentrations of TC ($p < 0.001$), LDL-C ($p = 0.013$), ApoB ($p = 0.017$), and ApoA-I ($p = 0.002$) were higher after participants consumed the SFA diet than when they consumed the other diets.

Plasma levels of TG, HDL-C, glucose and insulin did not differ after the three periods of intervention.

Table 4: Fasting plasma concentrations of lipids, apolipoproteins, glucose, insulin and CRP at the end of each dietary intervention period*.

	Med diet	Med+CoQ diet	SFA diet	p
TC (mmol/L)	4.65 ± 0.13^a	4.73 ± 0.14^a	5.21 ± 0.17^b	<0.001
TG (mmol/L)	1.10 ± 0.01	1.15 ± 0.12	1.16 ± 0.09	0.675
HDL-C (mmol/L)	1.31 ± 0.06	1.34 ± 0.06	1.40 ± 0.07	0.069
LDL-C (mmol/L)	2.80 ± 0.12^a	2.82 ± 0.06^a	3.19 ± 0.17^b	0.013
Apo B (g/L)	0.83 ± 0.04^a	0.85 ± 0.04^a	0.91 ± 0.05^b	0.017
Apo A (g/L)	1.44 ± 0.05^a	1.47 ± 0.05^a	1.56 ± 0.06^b	0.002
Glucose (mmol/L)	4.97 ± 0.12	5.06 ± 0.17	5.15 ± 0.22	0.440
Insulin (pmol/L)	10.33 ± 2.39	14.40 ± 5.02	8.63 ± 1.39	0.470
CRP (mg/L)	3.01 ± 0.83	3.60 ± 1.39	3.60 ± 1.06	0.240

** Values are means ± SE (n = 20). Means in a row with superscripts without a common letter differ, p < 0.05. Main effect of diet by repeated measures ANOVA.*

TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein-cholesterol; LDL-C low density lipoprotein-cholesterol; ApoB, apolipoprotein B; Apo A, apolipoprotein A-1; CRP, C-reactive protein.

3. CoQ determination

A higher fasting plasma CoQ concentration ($p < 0.001$) was found after the intake of the Med+CoQ diet compared with the Med and SFA diets (**Figure 9**). At 2 and 4h after consumption of the Med+Q diet, we observed a greater postprandial increase in plasma CoQ levels compared with the Med and SFA diets ($p = 0.018$ and $p = 0.032$, respectively). No significant differences were detected in postprandial CoQ levels at 2 and 4h between the Med and SFA diets.

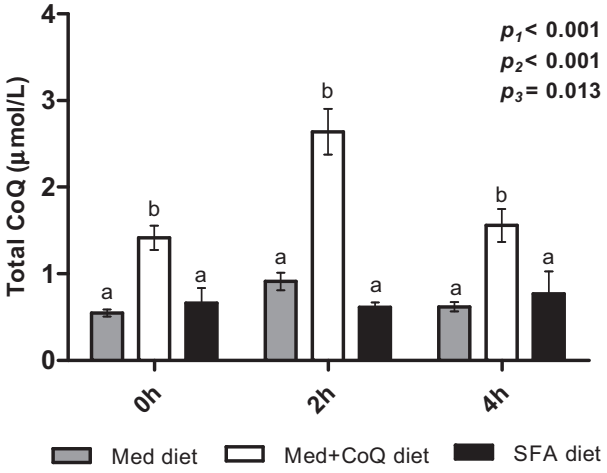


Figure 9: Fasting and postprandial plasma levels in total CoQ according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, p < 0.05. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

4. Expression of genes related to oxidative stress in PBMC

In the current study, we have analyzed the effect produced by the consumption of different dietary patterns on the expression of the following genes related to

OxS processes: Nrf2, NADPH oxidase ($p22^{phox}$ and $p47^{phox}$ subunits), SOD (isoforms 1 and 2), Gpx1 and TrxR.

4.1. Nrf2

Nrf2 is a central transcription factor, essential for the coordinated induction of those genes encoding many stress-responsive or cytoprotective enzymes and related protein, such as SOD, GPx, glutathione S-transferase and thioredoxin (*225, 226*). At 4h after intake of the Med+CoQ diet we observed a greater postprandial decrease in Nrf2 mRNA levels compared with the other diets (p = 0.029) (**Figure 10**). Furthermore, Nrf2 mRNA levels were lower after consumption of the Med diet compared with the SFA diet (p = 0.039). No significant differences were detected in Nrf2 mRNA levels in fasting and at 2h after intake of the three diets.

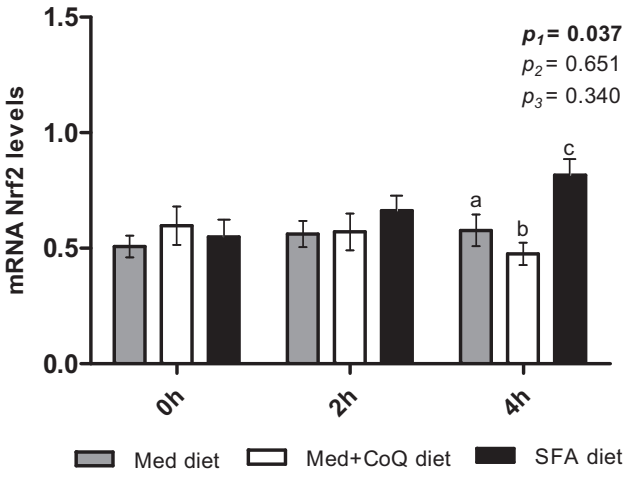


Figure 10: Fasting and postprandial levels of Nrf2 mRNA in PBMCs according to the type of fat consumed.

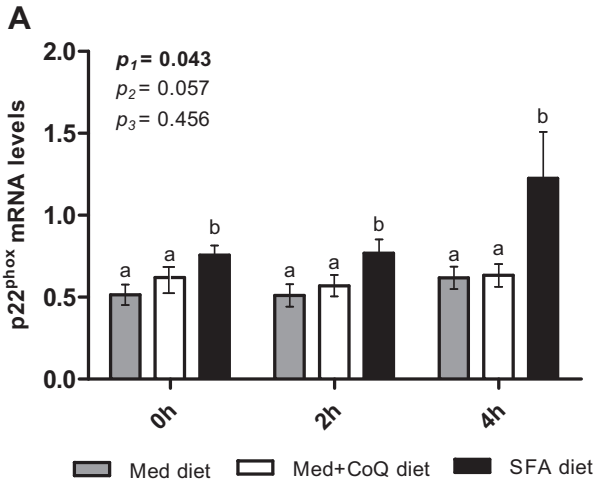
Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different

superscript letters depict statistically significant differences, $p < 0.05$. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

4.2. NADPH oxidase

NADPH oxidases are likely to be the predominant source of ROS in the vasculature (*29, 227, 228*). Activation of the NADPH oxidase enzyme complex requires the assembly of the cytosolic regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac) with the membrane-bound cytochrome b558 (subunits $gp91^{phox}$ and $p22^{phox}$) (*29*).

We observed a decrease in fasting $p22^{phox}$ mRNA levels after consumption of the Med and Med+CoQ diets compared to the SFA diet ($p = 0.032$) (**Figure 11A**). At 2 and 4h after the SFA diet, we found higher postprandial $p22^{phox}$ and $p47^{phox}$ mRNA levels compared with the Med and Med+CoQ diets ($p = 0.038$ and $p = 0.003$, respectively) (**Figure 11A,B**). No significant differences were detected in $p47^{phox}$ mRNA levels in fasting state after the intake of any of the three diets (**Figure 11B**).



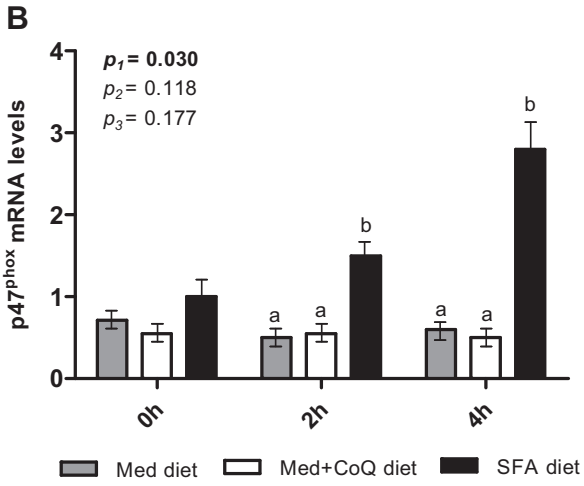


Figure 11: Fasting and postprandial levels of $p22^{phox}$ (A) and $p47^{phox}$ (B) mRNA in PBMC according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, $p < 0.05$. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

4.3. SOD

SOD is a key antioxidant enzyme that participates in the scavenging of the superoxide radical ($\cdot O_2^-$). It is one of the main enzymes of the first line of defense against OxS (*51*). We studied the expression of the two human isoforms of SOD: cytoplasmic SOD gene (SOD1) and mitochondrial SOD gene (SOD2). Fasting SOD2 mRNA levels were lower after participants consumed the Med and Med+CoQ diets than when they consumed the SFA diet ($p = 0.007$) (**Figure 12A**). At 2 and 4h after the SFA diet, we found higher postprandial SOD2 mRNA levels compared with the Med and Med+CoQ diets ($p = 0.008$ and $p = 0.003$, respectively) (**Figure 12A**). At 4h after intake of the Med and Med+CoQ diets we observed a greater postprandial decrease in SOD1

mRNA levels with respect to the SFA diet ($p = 0.011$) (**Figure 12B**). However, no significant differences were detected in SOD1 mRNA levels in fasting and at 2h after intake of the three diets (**Figure 12B**).

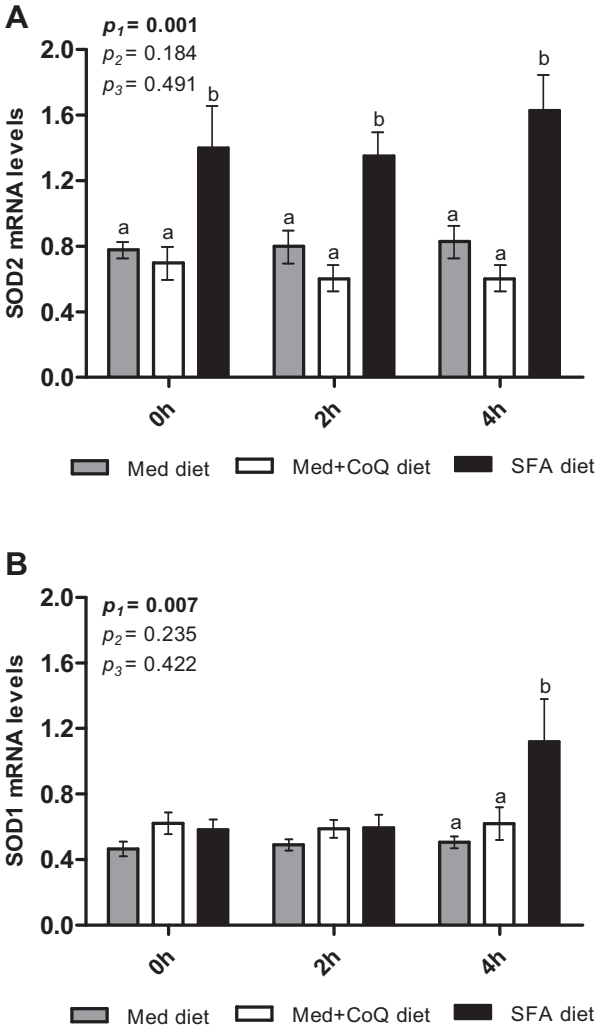


Figure 12: Fasting and postprandial levels of SOD2 (A) and SOD1 (B) mRNA in PBMC according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, p < 0.05. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

4.4. Gpx1

Gpx1 is a ubiquitous antioxidant enzyme that catalyzes the reduction of H_2O_2 and scavenges organic hydroperoxides in both the cytosol and mitochondria (*56, 229*).

Fasting Gpx1 mRNA levels were greater after SFA diet consumption (p = 0.013) than after the Med diet intake, with or without CoQ supplementation (**Figure 13**). During postprandial state, we found lower postprandial Gpx1 mRNA levels at 2 and 4h after Med+CoQ diet intake, compared with the SFA diet (p = 0.013).

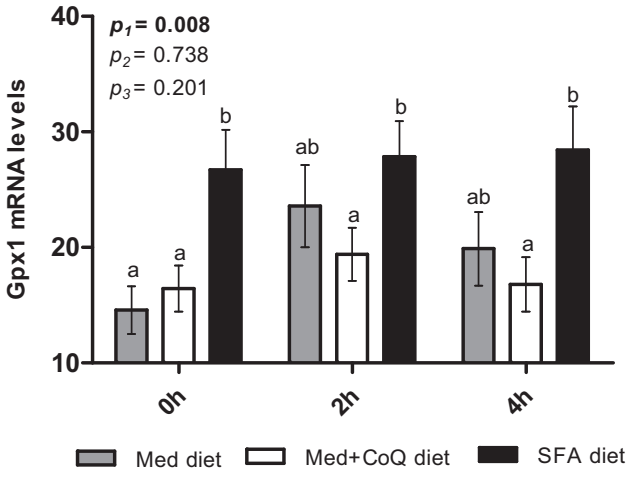


Figure 13: Fasting and postprandial levels of Gpx1 mRNA in PBMC according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, p < 0.05. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

4.5. TrxR

TrxR is a ubiquitous oxidoreductase enzyme with antioxidant and redox regulatory roles. It is also involved in prevention, intervention and restoring the damage caused by H_2O_2-based OxS (*58, 230*).

At 4h after the intake of the SFA diet we found a greater postprandial increase in TrxR mRNA levels compared to Med and Med+CoQ diets ($p = 0.023$). No significant differences were detected in TrxR mRNA levels in fasting state or 2h after consumption of any of the three diets (**Figure 14**).

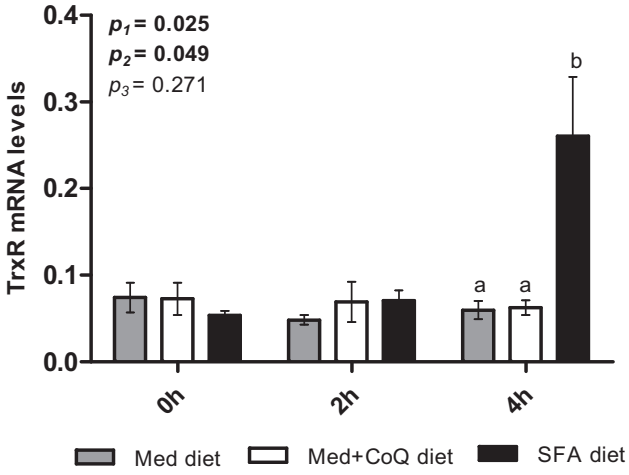


Figure 14: Fasting and postprandial levels of TrxR mRNA in PBMC according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, p < 0.05. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

5. Cellular protein levels related to oxidative stress in PBMC

In the current study, we have studied the effect produced by the consumption of different dietary patterns on the levels of the following proteins related to OxS processes: Nrf2 (cytoplasmic and nuclear) and Keap-1.

5.1. Cytoplasmic and nuclear Nrf2

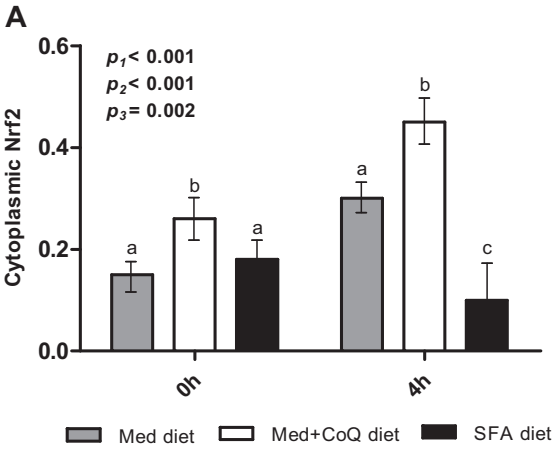
Nrf2 normally resides in the cytoplasm of unstressed cells interacting with Keap-1, who promotes its degradation (*45*). In response to an oxidative insult, Nrf2 evades Keap-1 mediated repression and accumulates within the nucleus, thus facilitating the expression of antioxidant genes.

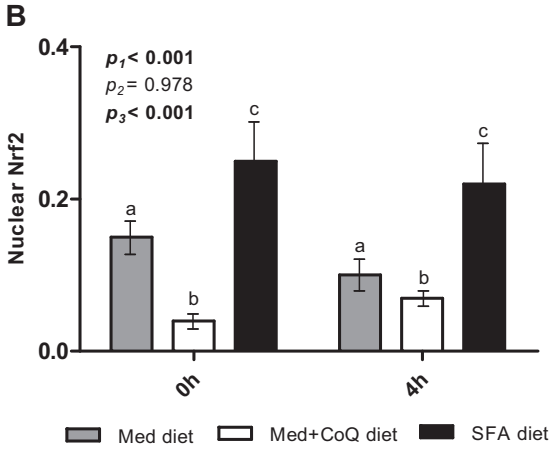
In fasting and at 4h after intake of the Med+CoQ diet we observed higher cytoplasmic Nrf2 protein levels compared to the other diets ($p = 0.033$ and $p = 0.011$, respectively) (**Figure 15A**). In addition, at 4h Med diet induced a significant increase in the protein levels of cytoplasmic Nrf2 compared to the SFA diet ($p = 0.026$).

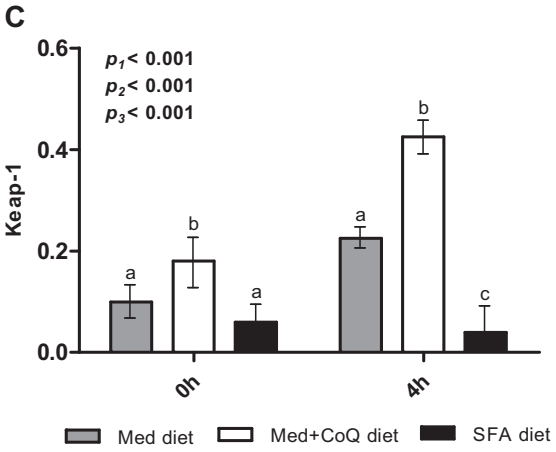
Consequently, both in fasting and at 4h after Med+CoQ diet consumption we found lower nuclear Nrf2 protein levels compared to the SFA diet ($p = 0.001$ and $p = 0.019$, respectively), with an intermediate effect for the Med diet (**Figure 15B**).

5.2. Cytoplasmic Keap-1

In fasting state and at 4h after intake of the Med+CoQ diet we observed higher Keap-1 protein levels compared to the other diets ($p = 0.035$ and $p = 0.003$, respectively) (**Figure 15C**). Additionally, at 4h Med diet induced a significant increase of postprandial Keap-1 protein levels compared to the SFA diet ($p = 0.017$).


A
Cytoplasmic Nrf2
p_1< 0.001
p_2< 0.001
p_3= 0.002
0.6
0.4
0.2
0.0
a
b
a
a
b
c
0h
4h
Med diet
Med+CoQ diet
SFA diet


B
Nuclear Nrf2
p_1< 0.001
p_2= 0.978
p_3< 0.001
0.4
0.2
0.0
a
b
c
a
b
c
0h
4h
Med diet
Med+CoQ diet
SFA diet


C
Keap-1
p_1< 0.001
p_2< 0.001
p_3< 0.001
0.6
0.4
0.2
0.0
a
b
a
a
b
c
0h
4h
Med diet
Med+CoQ diet
SFA diet

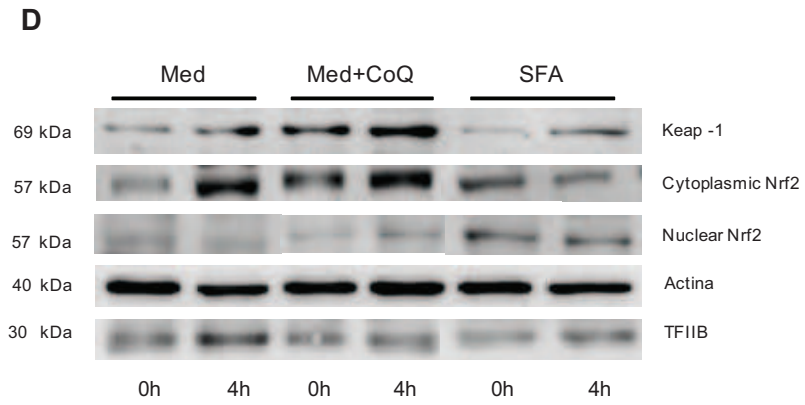


Figure 15: Fasting and postprandial levels of cytoplasmic Nrf2 (A), nuclear Nrf2 (B) and Keap-1 in PBMC according to the type of fat consumed. (D) Representative immunoblot of nuclear and cytoplasmatic Nrf2, Keap-1, actin and TFIIB. Depicted bands are different parts of the same blot stained for each antigen.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, $p < 0.05$. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

6. Expression of genes related to the inflammatory response in PBMC

In the current study, we have studied the effect produced by the consumption of different dietary patterns on the expression of the following genes related to the inflammatory response: p65 (RelA), IκB-α, IKK-β, MMP-9, IL-1β and JNK-1.

6.1. Genes related to the activation of NF-κB

The NF-κB transcription factor functions as homo- or heterodimers of the Rel family of proteins, which includes p50, p65 (RelA), c-Rel, p52 and RelB. The most common combination of subunits is a heterodimer of the p50 and p65 (RelA) proteins. Activation of NF-kB dimers is the result of the

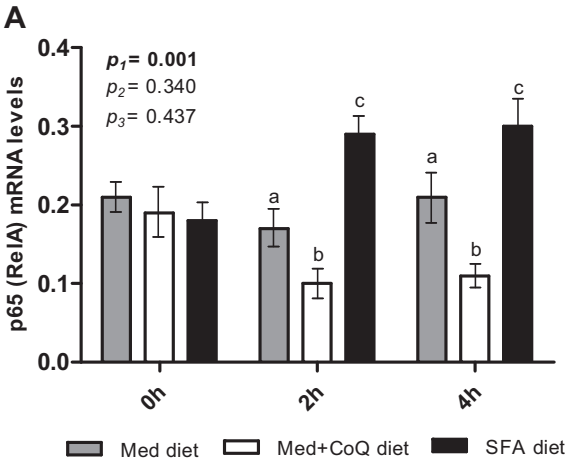
phosphorylation of IκBα by IKK, which enables NF- α B dimers to enter the nucleus and activate specific target gene expression (22).

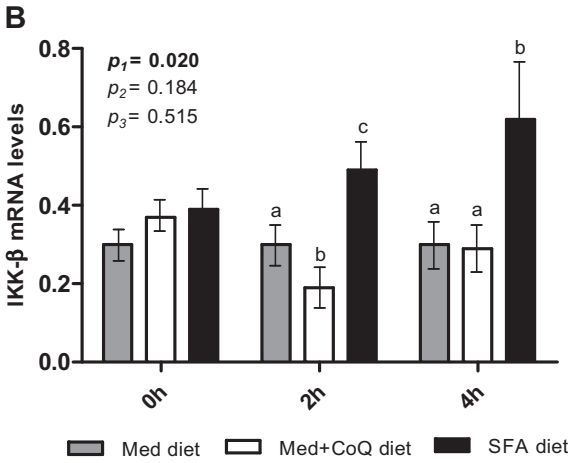
At 2 and 4h after intake of the Med+CoQ diet, we found lower postprandial p65 (RelA) mRNA levels compared with the other diets (p = 0.008 and p = 0.012, respectively) (**Figure 16A**). Furthermore, p65 (RelA) mRNA levels were lower at 2 and 4h after consumption of the Med diet compared with the SFA diet (p = 0.033).

At 2h after intake of the Med+CoQ diet we observed a greater postprandial decrease in IKK-β mRNA levels compared with the other diets (p = 0.010) (**Figure 16B**). Moreover, IKK-β mRNA levels were lower after consumption of the Med diet compared with the SFA diet (p = 0.034). At 4h after intake of the Med and Med+CoQ diets we observed a greater postprandial decrease in IKK-β mRNA levels with respect to the SFA diet (p = 0.011).

At 2h after ingestion of the Med and Med+CoQ diets, we observed a greater postprandial increase in IκB-α mRNA levels compared with the SFA diet (p = 0.028). At 4h after the Med diet, we found higher postprandial IκB-α mRNA levels compared with the SFA diet (p = 0.018) (**Figure 16C**).

No significant differences were detected in p65 (RelA), IKK-β or IκB-α mRNA levels in fasting after intake of any of the three diets (**Figure 16A, B** and **C**, respectively).


A
p65 (RelA) mRNA levels
p_1= 0.001
p_2= 0.340
p_3= 0.437
0.4
0.3
0.2
0.1
0.0
a
b
c
a
b
c
0h
2h
4h
Med diet
Med+CoQ diet
SFA diet


B
IKK-β mRNA levels
p_1= 0.020
p_2= 0.184
p_3= 0.515
0.8
0.6
0.4
0.2
0.0
a
b
c
a
a
b
0h
2h
4h
Med diet
Med+CoQ diet
SFA diet

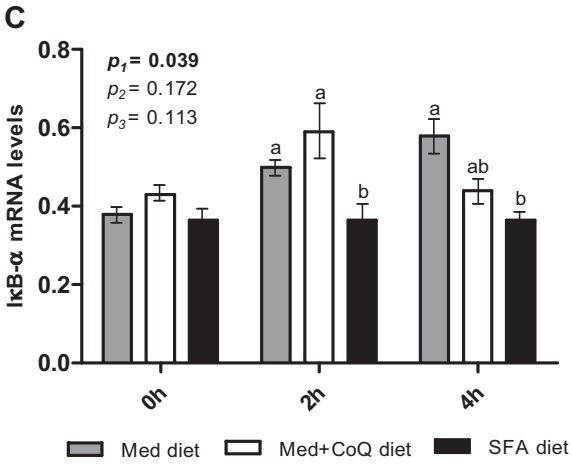

C
IκB-α mRNA levels
p_1= 0.039
p_2= 0.172
p_3= 0.113
0.8
0.6
0.4
0.2
0.0
a
a
b
a
ab
b
0h
2h
4h
Med diet
Med+CoQ diet
SFA diet

Figure 16: Fasting and postprandial levels of p65 (RelA) (A), IKK-β (B) and IκB-α (C) mRNA in PBMC according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, $p < 0.05$. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

6.2. MMP-9

MMP-9 is a metalloproteinase involved in several stages of atherosclerosis through remodelling of the extracellular matrix (23). We observed a decrease in fasting MMP-9 mRNA levels after intake of the Med diet compared to the SFA diet (p = 0.034) (**Figure 17**). At 2 and 4h after the SFA diet, we found higher postprandial MMP-9 mRNA levels compared with the Med and Med+CoQ diets (p = 0.008 and p = 0.032, respectively) (**Figure 17**).

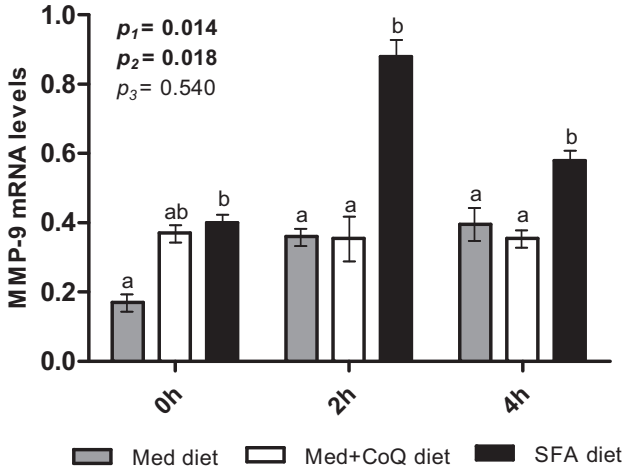


Figure 17: Fasting and postprandial levels of MMP-9 mRNA in PBMC according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different

superscript letters depict statistically significant differences, $p < 0.05$. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

6.3. IL-1β

IL-1 is an inflammatory cytokine that consists of two distinct ligands (IL-1α and IL-1β) and both subunits appear to play an essential role in many inflammatory processes (24).

Fasting IL-1β mRNA levels were lower after participants consumed the Med and Med+CoQ diets than when they consumed the SFA diet (p = 0.017) (**Figure 18**). At 2h after intake of the Med+CoQ diet we observed a greater postprandial decrease in IL-1β mRNA levels compared with the other diets (p = 0.011). Furthermore, IL-1β mRNA levels were lower at 2h after consumption of the Med diet compared with the SFA diet (p = 0.029). At 4h after consumption of the Med and Med+CoQ diets we observed a greater postprandial decrease in IL-1β mRNA levels with respect to the SFA diet (p = 0.015) (**Figure 18**).

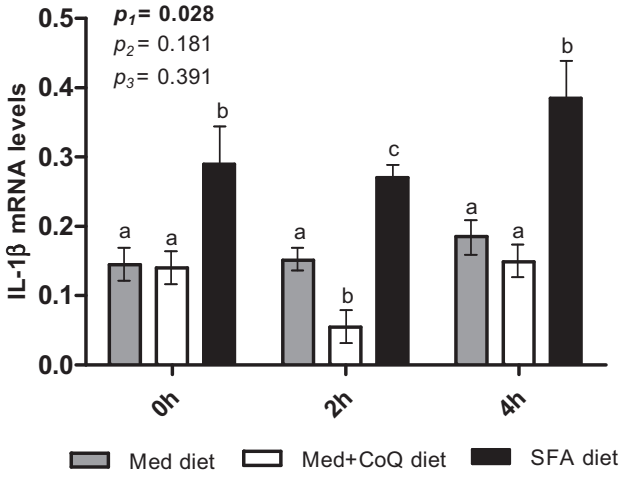


Figure 18: Fasting and postprandial levels of IL-1β mRNA in PBMC according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, p < 0.05. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

6.4. JNK-1

JNK is involved in inflammatory pathways, changes in ROS levels and a variety of stress stimuli. Ten isofroms, derived from three genes, are known: JNK-1 (four isoforms), JNK-2 (four isoforms) and JNK-3 (two isoforms) (25). Fasting JNK-1 mRNA levels were lower after participants consumed the Med and Med+CoQ diets than when they consumed the SFA diet ($p = 0.037$). At 2 and 4h after the SFA diet, we found higher postprandial JNK-1 mRNA levels compared with the other diets ($p = 0.009$ and $p = 0.011$, respectively) (**Figure 19**).

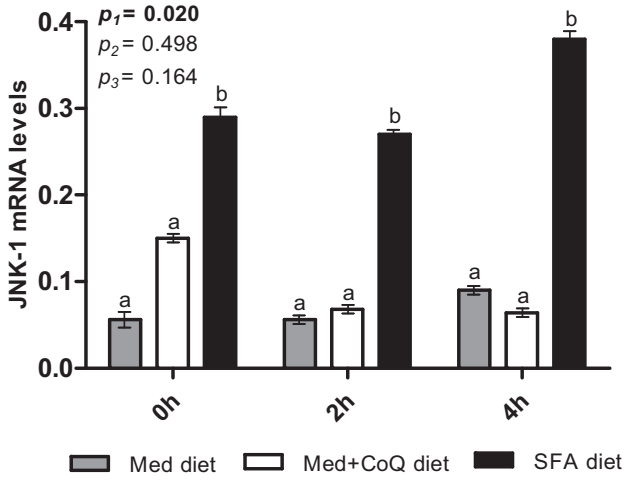


Figure 19: Fasting and postprandial levels of JNK-1 mRNA in PBMCs according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, $p < 0.05$. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

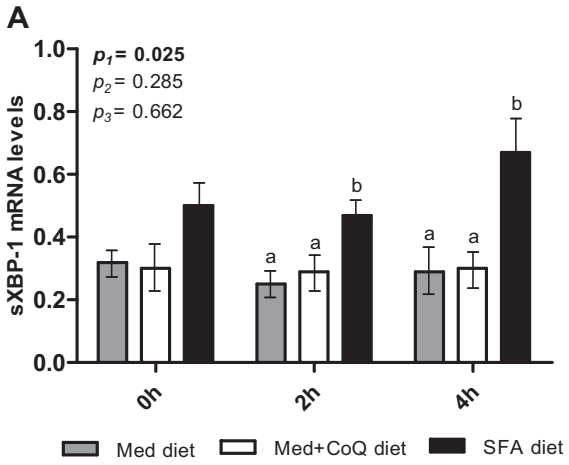
7. Expression of genes related to ER stress in PBMC

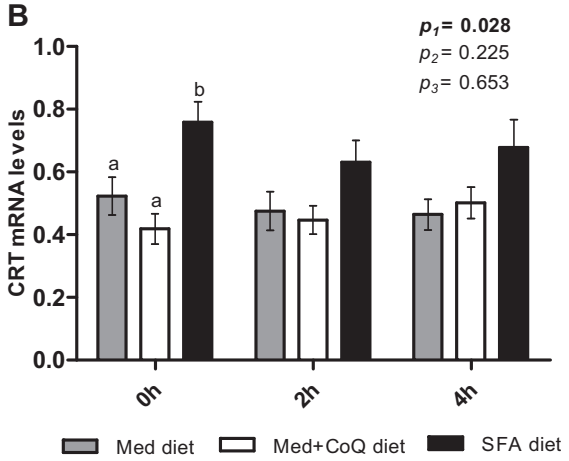
The transcription factor sXBP-1 has been identified as a key regulator of the mammalian UPR or ER stress response, which is activated by environmental stressors such as protein overload that require increased ER capacity (26). CRT, BiP/Grp78, calnexin and other ER Ca^{2+} binding chaperons are important components of the protein folding process and quality control (27).

At 2 and 4 h after the Med and Med+CoQ diets, we found lower postprandial sXBP-1 mRNA levels compared with the SFA diet ($p = 0.033$ and $p = 0.008$) (**Figure 20A**). No significant differences were detected in sXBP-1 mRNA levels in fasting state after intake of any of the three diets.

In addition, fasting CRT mRNA levels were lower when participants had consumed the Med and Med+CoQ diets than when they had consumed the SFA diet ($p = 0.031$) (**Figure 20B**).

At 2 h after intake of the SFA diet we found a greater postprandial increase in BiP/Grp78 mRNA levels compared to the other diets ($p = 0.021$). No significant differences were detected in BiP/Grp78 mRNA levels in fasting and at 4 h after intake of the three diets (**Figure 20C**).


A
p_1= 0.025
p_2= 0.285
p_3= 0.662
sXBP-1 mRNA levels
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
a
a
b
0h
2h
4h
Med diet
Med+CoQ diet
SFA diet


B
p_1= 0.028
p_2= 0.225
p_3= 0.653
CRT mRNA levels
1.0
0.8
0.6
0.4
0.2
0.0
a
a
b
0h
2h
4h
Med diet
Med+CoQ diet
SFA diet

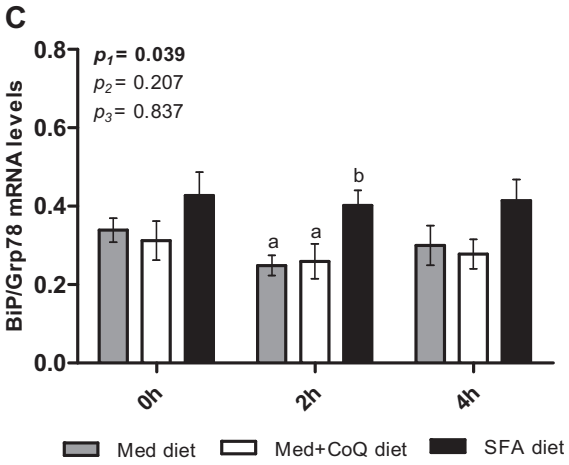

C
p_1= 0.039
p_2= 0.207
p_3= 0.837
BiP/Grp78 mRNA levels
0.8
0.6
0.4
0.2
0.0
a
a
b
0h
2h
4h
Med diet
Med+CoQ diet
SFA diet

Figure 20: Fasting and postprandial levels of sXBP-1 (A), CRT (B) and BiP/Grp78 mRNA in PBMC according to the type of fat consumed.

Values are means ± SE (n = 20). Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet. Bars with different superscript letters depict statistically significant differences, $p < 0.05$. Main effect of diet by repeated measures ANOVA. p_1, diet effect; p_2, time effect; p_3, diet×time interaction.

8. Metabolomic analysis of urine samples

Urine samples, corresponding to baseline and to 12h fast after each dietary intervention (time 0h), were collected from 10 participants (5 women and 5 men) from the study population I. PCA of ^{1}H-NMR urine spectra resulted in a two component model with a R^2 of 0.31. **Figure 21A** shows the PCA score plot for the dataset. Visual inspection of the model showed grouping of samples according to gender. Pair wise comparison of samples using PCA according to diet did not reveal any pattern with all the participants and PLS models were not built. Following this, PCA analysis for women and men were conducted separately. Analysis for women displayed differences between dietary interventions when comparing fasting state after Med+CoQ diet against SFA diet. PLS-DA analysis of these groups revealed one component model with a R^2X of 0.21, a R^2Y of 0.66 and a Q^2 of 0.17 (**Figure 21B**). Validation of the model was performed using permutation testing and resulted in a Q^2 intercept of (0.0, -0.1). No significant differences were found comparing the other diets.

Analysis of the VIP plots revealed greater levels of hippurate excretion after consumption of Med+CoQ diet compared to SFA diet. Lower excretion in phenylacetylglycine levels was observed after Med+CoQ diet compared to SFA diet intervention (**Table 5**). However, visual inspection of PCA score plots for men revealed no patterns based on diets.

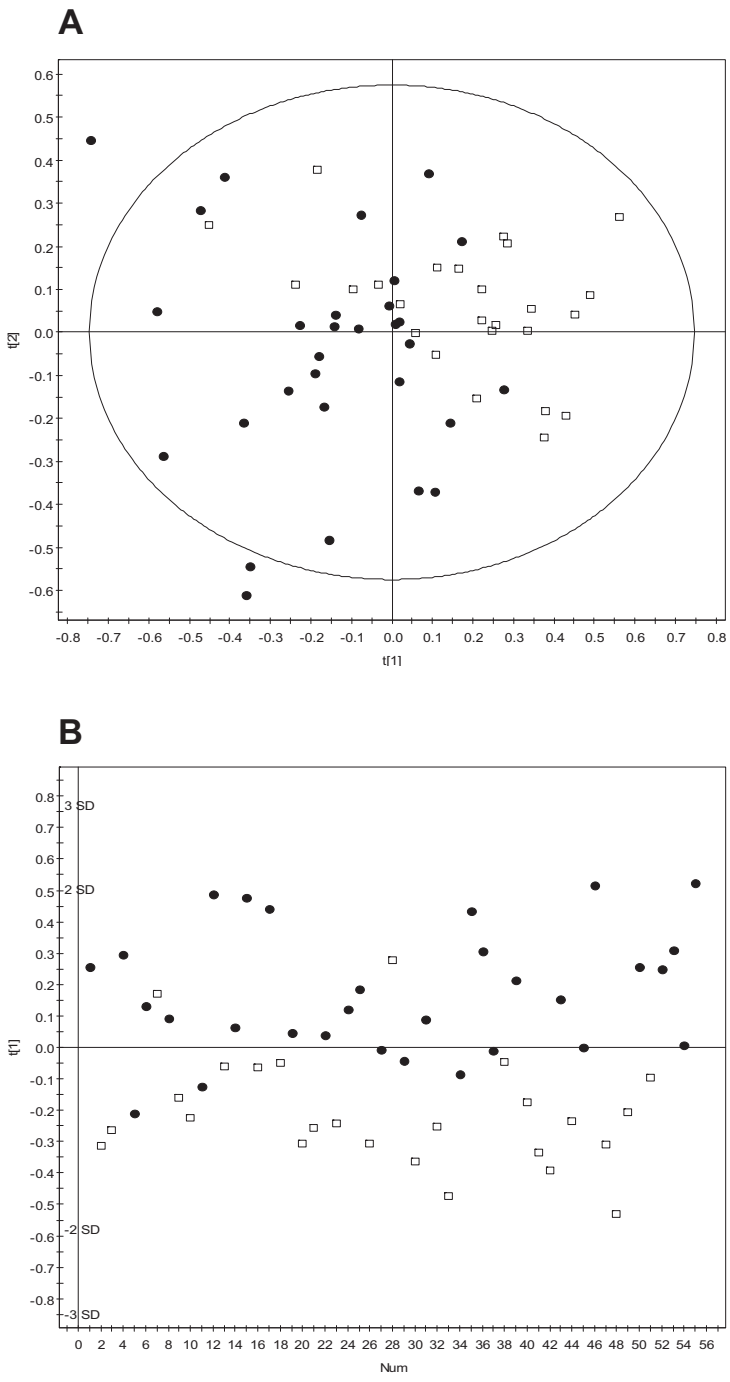


Figure 21: (A) PCA score plot (R^2 = 0.31, Q^2 = 0.17) derived from ^{1}H-NMR urine spectra collected pre- and post- dietary intervention. Open squares represent male samples and closed circles represent female samples. (B) PLS-DA score plot derived from ^{1}H-NMR urine spectra

collected from women after Med+CoQ diet (open circles) and after SFA diet intervention (close squares). $R^2 = 0.21$, $Q^2 = 0.17$

Table 5: Metabolites identified as being discriminant between Med+CoQ and SFA diet.

Metabolite	Chemical shift (ppm)	VIP values	Med+CoQ	SFA	p-value
Hippurate	3.975	6.380	0.022 ± 0.006	0.014 ± 0.004	0.037
Hippurate	3.965	6.176	0.021 ± 0.006	0.013 ± 0.004	0.038
Hippurate	7.835	5.241	0.016 ± 0.005	0.009 ± 0.003	0.041
Hippurate	7.545	3.542	0.006 ± 0.002	0.004 ± 0.001	0.049
Phenylacetylglycine	7.435	2.963	0.003 ± 0.002	0.005 ± 0.002	0.045
Phenylacetylglycine	7.375	2.380	0.003 ± 0.001	0.005 ± 0.001	0.045
Un-assigned peak	3.725	2.413	0.007 ± 0.001	0.008 ± 0.001	0.084
Un-assigned peak	2.175	2.204	0.002 ± 0.001	0.003 ± 0.002	0.167
Un-assigned peak	3.635	2.134	0.006 ± 0.001	0.007 ± 0.002	0.160

Average intensities of metabolite bin regions fot the Med+CoQ diet and SFA diet are shown. Values are means ± SD and are presented as arbitrary units. p-values are reported for paired t-test.

8.1. Correlation analysis between urinary metabolites and other parameters

After chronic consumption of Med+CoQ diet, we observed positive strong correlations between hippurate and CoQ and β-carotene plasma levels (**Table 6**). Aditionally, we found a high inverse correlation between urinary levels of hippurate and Nrf2 gene expression, antioxidant enzymes gene expression as Trx, SOD1 and $gp91^{phox}$ subunit of NADPH oxidase. Similarly, after consumption of SFA diet, we observed a strong negative correlation between urinary levels of phenylacetylglycine and CoQ plasma levels and a great

positive correlation between urinary levels of phenylacetylglycine and urinary isoprostanes (**Table 6**).

Table 6: Correlations between urinary levels of hippurate, phenylacetylglycine and other factors[a] after Med+CoQ diet intervention.

		CoQ	β-carotene	Nrf2	Trx	SOD1	gp91^{phox}
Hippurate	Pearson correlation	0.899*	0.928*	-0.978**	-0.976**	-0.913*	-0.898*
	p-value	0.038	0.023	0.004	0.004	0.030	0.039
		CoQ	**Isoprostanes**				
Phenylacetylglycine	Pearson correlation	-0.897*	0.952*				
	p-value	0.039	0.013				

[a]*Pearson´s correlation: *p < 0.05; **p < 0.01. CoQ, coenzyme Q_{10} plasma levels; β-carotene, plasma levels; Nrf2, transcription factor Nrf2 gene expression; Trx, Thioredoxin gene expression; SOD1, superoxide dismutase 1 gene expression; gp91^{phox}, subunit of NADPH oxidase; Isoprostanes, urinary levels.*

8.2. Gender comparison of OxS biomarkers

Gender-related differences after Med+CoQ diet intervention are shown in **Figure 22**. We observed that levels of the antioxidants compounds CoQ **(A)** and β-carotene **(B)** are significantly greater in women compared to men. By contrast, we found that OxS biomarkers such as LPO **(A)** or oxLDL **(B)** after Med+CoQ are higher in men than in women (**Figure 23**). Data from these parameters used for the correlations were previously analyzed and published (199, 231).

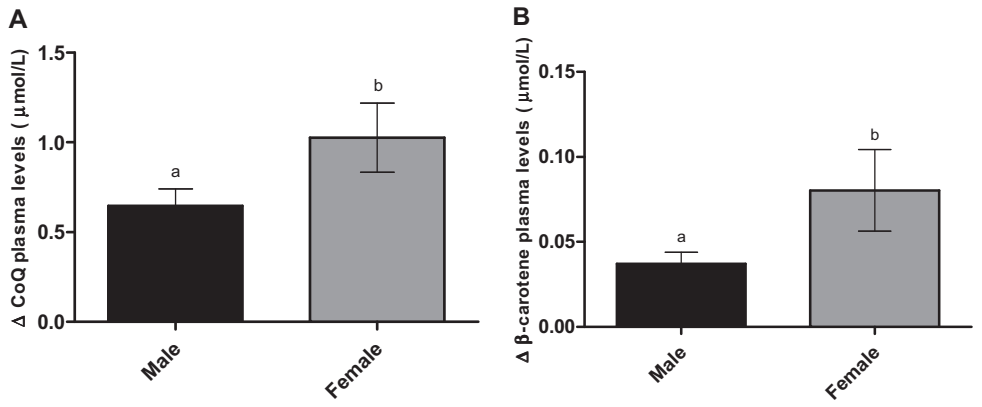


Figure 22: Gender-related differences after Med+CoQ diet intervention of CoQ (A) and β-carotene (B).

*Differences in the parameter levels between pre- and post-intervention are shown as Δ (value of post-intervention minus value of pre-intervention). Data were analyzed using unpaired t-test and differences were considered to be significant when *p < 0.05.*

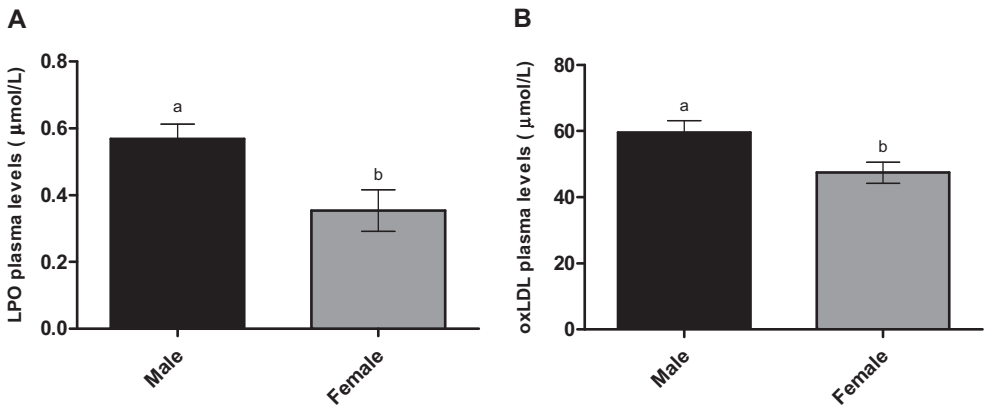


Figure 23: Gender-related differences in fasting plasma levels after Med+CoQ diet intervention of LPO (A) and oxLDL (B).

Data were analyzed using unpaired t-test and differences were considered to be significant when p < 0.05. Bars with different superscript letters depict statistically significant differences.

Influence of endothelial dysfunction on telomere length in subjects with metabolic syndrome

In order to carry out the study, 20 participants from the *Study Population I* and a subgroup of 68 participants from the LIPGENE *(Study Population II)* were divided into four groups by quartiles of relative telomere length.

1. Baseline characteristics

The anthropometric, biochemical, BP and metabolic parameters of the participants are shown in **Table 7**. Plasma TG was higher in the subjects with shortest RTL (quartile 1) compared to those in quartile 4 with largest RTL (p = 0.031).

Baseline HOMAβ was greater in the subjects grouped in the quartile 4 with largest RTL compared with the subjects in the quartiles 1, 2 and 3 (p = 0.003). We did not find significant differences with respect to the remaining parameters between the four groups of MetS subjects according to the RTL (**Table 7**).

Table 7: Baseline characteristics of the study population according to quartiles of RTL[1,2,3]

	Quartile 1 (n = 22)	Quartile 2 (n = 24)	Quartile 3 (n = 20)	Quartile 4 (n = 22)	*p*
Age (years)	59.73 ± 1.87	59.71 ± 1.55	58.60 ± 1.60	56.59 ± 1.94	0.540
Sex (female/male)	14/8	14/10	12/8	12/10	
$HOMA_{IR}$	3.37 ± 0.07	3.97 ± 0.10	3.42 ± 0.11	4.32 ± 0.12	0.700
HOMAβ	89.94 ± 9.31[a]	91.77 ± 8.93[a]	94.21 ± 9.69[a]	139.14 ± 9.49[b]	0.003
Weight (kg)	87.78 ± 2.74	86.50 ± 2.63	89.66 ± 2.85	96.06 ± 2.79	0.940
Height (m)	1.57 ± 0.03	1.62 ± 0.02	1.54 ± 0.05	1.56 ± 0.04	0.129
BMI	34.28 ± 0.88	33.68 ± 0.84	34.28 ± 0.92	35.28 ± 0.90	0.800
Waist circumference (cm)	106.34 ± 2.18	103.54 ± 2.09	108.40 ± 2.27	109.71 ± 2.22	0.937
Insulin (mU/L)	12.22 ± 2.10	14.71 ± 2.05	12.69 ± 2.19	15.04 ± 2.14	0.657
Glucose (nmol/L)	6.29 ± 0.22	6.20 ± 0.22	5.93 ± 0.23	5.03 ± 0.23	0.143
TG (nmol/L)	1.89 ± 0.14[a]	1.51 ± 0.13[a. b]	1.55 ± 0.15[a. b]	1.27 ± 0.14[b]	0.031
LDL-C (nmol/L)	17.38 ± 8.63	34.02 ± 8.27	21.30 ± 8.78	26.16 ± 8.97	0.647
HDL-C (nmol/L)	1.11 ± 0.05	1.22 ± 0.05	1.08 ± 0.06	1.09 ± 0.06	0.325
hsCRP (mg/L)	6.63 ± 1.17	5.24 ± 1.12	7.09 ± 1.19	6.52 ± 1.16	0.573
SBP (mm Hg)	142 ± 3	144 ± 3	138 ± 3	142 ± 3	0.306
DBP (mm Hg)	87 ± 3	87 ± 2	88 ± 3	89 ± 3	0.900

[1]Data are means ± SE. $HOMA_{IR}$, homeostatic model assessment index for insulin resistance; HOMAβ, homeostatic model assessment index of insulin secretory function; BMI, body mass index; TG, triglycerides; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; hsCRP, high sensitivity C-reactive protein.

[2] Means in a column with different superscript letters are significantly different, $p < 0.05$ (Univariate analysis of variance adjusted for age).

[3] Quartile 1: RTL ≤ 1.10, quartile 2: 1.10 < RTL ≤ 1.26, quartile 3: 1.26 < RTL ≤ 1.56, quartile 4: RTL > 1.56.

2. Study of endothelial function

NO plasma levels were higher in the quartile 3 (p = 0.012) and 4 (p = 0.016) than the quartile 1 (shortest RTL) (**Figure 24A**). Additionally, IRH shows the same trend as NO levels with greater percentage of change from baseline in the quartile 3 (longest RTL) (p = 0.015) and 4 (p = 0.011) than quartile 1 (**Figure 24B**).

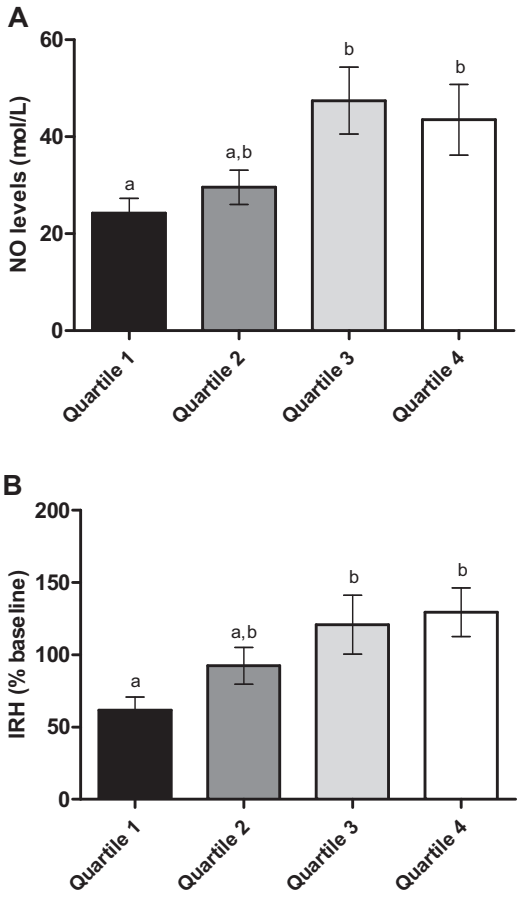


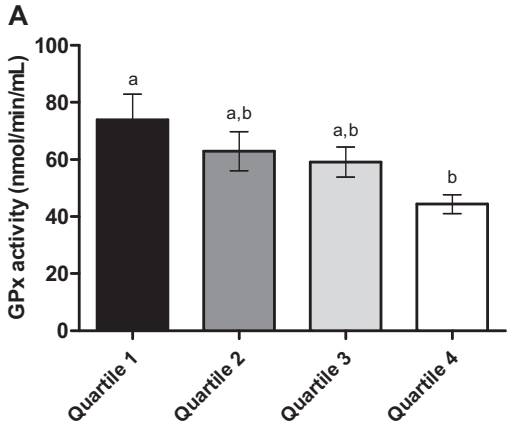
Figure 24: Total nitrite (NO) levels in plasma (A) and ischemic reactive hyperemia (IRH) (B) across quartiles of RTL.

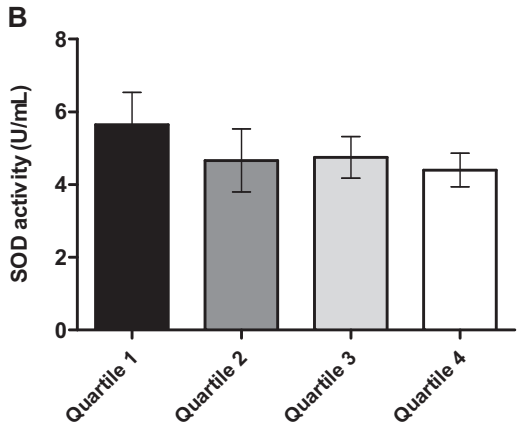
Data were analyzed using univariate analysis of variance adjusted for age. All values represent the means ± SE. Differences were considered to be significant when $p < 0.05$. Bars with different superscript letters depict statistically significant differences.

3. Antioxidant enzymes activities and levels of OxS biomarker

We found that GPx plasma activity of subjects grouped in the quartile 4 with longer RTL was lower compared to quartile 1 with shorter RTL (p = 0.003), with intermediate activity levels for quartile 2 and 3 (**Figure 25A**). Although differences of SOD plasma activity between quartiles were not statistically significant, there was a marked trend that showed an increase in the activity of the enzyme in the quartile 1 with respect to the other RTL groups (**Figure 25B**).

Regarding to PC plasma levels, which are an important biomarker of OxS, they were higher in the subjects grouped in the quartile 1 with the shortest RTL compared with the subjects in quartile 4 (p =0.036; **Figure 26**).





gFigure 25: Gluthatione peroxidase **(**GPx) plasma activity (A) and superoxide dismutase (SOD) plasma activity (B) across quartiles of RTL.

Data were analyzed using univariate analysis of variance adjusted for age. All values represent the means ± SE. Differences were considered to be significant when p < 0.05. Bars with different superscript letters depict statistically significant differences.

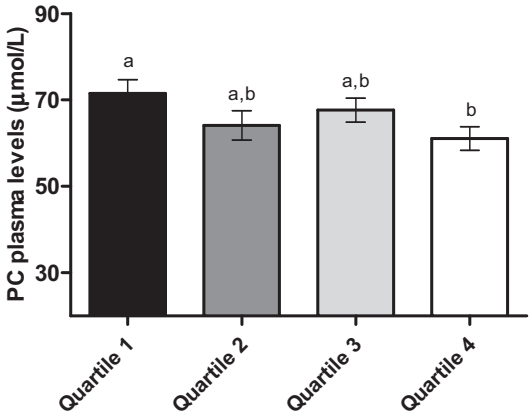


Figure 26: Protein carbonyls (PC) plasma levels across quartiles of RTL.

Data were analyzed using univariate analysis of variance adjusted for age. All values represent the means ± SE. Differences were considered to be significant when p < 0.05. Bars with different superscript letters depict statistically significant differences.

VII. DISCUSSION

VII. DISCUSSION

Expression of antioxidant, pro-inflammatory and endoplasmic reticulum stress-related genes and protein levels in an elderly population following consumption of diets with different fat quality

Aging is characterized for being an unavoidable and irreversible process in which multiple factors are involved, both genetic and environmental (*2*). Regarding to its ethiopathogeny, OxS is likely to be one of the main mechanisms of functional and structural impairment as the organism ages. On this premise, the "free-radical theory" was postulated in order to give an explanation of why this complex process occurs (*3*). Organisms have a potent antioxidant system to cope with the overall oxidation inherent to life with the purpose of maintaining the redox homeostasis. These antioxidant systems are formed by a number of enzymes as well as several endogenous and exogenous antioxidant compounds that react against ROS, by neutralizing them (*44, 118*). OxS has also been widely linked to the development and progression of multiple pathophysiological conditions, such as endothelial dysfunction, hypertension, cardiovascular disease and MetS. Although it is generally accepted that the main mechanism underlying metabolic changes in MetS patients relies on insulin resistance, there is a growing body of evidence that demonstrates a close link between MetS and OxS (*38, 41*).
Once accepted that OxS have a significant role in the aging process and age-related diseases, and also that the capacity to prevent OxS is determinant in longevity, it is reasonable to lead anti-aging strategies towards the improvement of the antioxidant defense or the decrease of OxS processes.

The present study demonstrates that the long-term consumption of a Med diet, with or without CoQ supplementation, reduces the fasting gene expression of

$p22^{phox}$ (NADPH-oxidase subunit), SOD2, Gpx1, MMP-9, JNK-1, IL-1β and CRT compared to a SFA diet. The influence of the long-term intervention is also reflected in the fasting protein levels of Nrf2 and Keap-1, whose cytoplasmic fraction are greater after Med+CoQ diet than after SFA diet, with an intermediate effect of Med diet. With regard to the postprandial state, Med diet consumption reduces the expression of genes related to OxS (Nrf2, SOD, TrxR and NADPH-oxidase), inflammatory response (NF-κB, IKK-β, MMP-9, IL-1β and JNK-1) and ER stress (sXBP-1, BiP/Grp78) that is produced in response to a fat challenge in PBMC from an elderly population, with respect to a SFA diet. Furthermore, the addition of CoQ has an additive effect on the Med diet since the participants that consumed the Med+CoQ diet showed a greater postprandial decrease in the gene expression of p65 (RelA), IKK- β, IL-1β, Nrf2 and Gpx1; as well as a greater postprandial increase in cytoplasmic Nrf2 and Keap-1 protein levels in PBMC with respect to the other diets.

In this study, the long-term intake of the Med+CoQ diet is associated with increased excreted levels of the metabolite hippurate, which is strongly correlated with antioxidant biomarkers; and decreased excreted levels of the metabolite phenylacetylglycine, which is highly related to oxidant biomarkers, when comparing to SFA diet in elderly women.

Postprandial lipemia has been considered crucial during atherogenesis since Zilversmit introduced the concept in 1979 (*135*) and subsequently, a large body of evidence has demonstrated that remnant lipoproteins greatly contribute to the atherosclerotic process (232). Due to the strong relationship between OxS and atherosclerosis, those phenomena have been widely studied during postprandio, demonstrating that postprandial state is associated with OxS (*136*). In fact, the dietary intake of one particular type of fatty acids

directly affects the fatty acid profile of an organism and indirectly influences its susceptibility to oxidative processes (233, 234).

As the human being spend most of the time in a postprandial state with a continual fluctuation in the degree of lipemia throughout the day, it is highly relevant to study the metabolic disorders related to OxS during postprandio instead of focusing in fasting state (*129*). However, the effect caused by food should not be viewed in isolation, since the study of an acute intake of fat cannot be divorced from the potential effects of chronic consumption.

Our study highlights the importance of mainstreaming the postprandial period analysis into the long-term intake of different dietary patterns during the four weeks of intervention. Few studies have explored into the molecular effects caused by diet on antioxidant gene expression and levels of proteins related to OxS during postprandial state in the elderly. While it is now commonly accepted that oxidative damage plays an important role in the aging process (235), the influence of age on the antioxidant enzymes gene expression has not been widely studied. Previous research has shown that long-term supplementation of PUFA along with dietary CoQ increases lifespan in rats (236). Moreover, CoQ has been described to modulate protein levels, control inflammation and also decrease OxS and cardiovascular risk during aging in rats (*204*). With regard to healthy humans, plasma oxidative damage may be partially prevented by CoQ supplementation (237) and these results have been replicated in subjects with psoriasis (238) or coronary heart disease patients (*188*).

In this connection, we have previously demonstrated in the same elderly population of this study, that the intake of Med diet improves postprandial OxS with a higher increase in IRH and NO plasma levels, lower LPO levels and lower GPx activity compared to the consumption of a SFA diet. Besides, we found that Med+CoQ diet consumption yielded a greater postprandial decrease in PC plasma levels; SOD, CAT and GPx activities and a higher

increase in IRH and NO with respect to a Med and SFA diet (*199*). Regarding to DNA damage, we have also proved that the intake of a Med+CoQ diet reduces the activation and stabilization of p53 in response to the OxS produced during postprandial state (*195*).

A central role in the defense against OxS has been attributed to the transcription factor Nrf2. Under unstressed conditions, Nrf2 is located in the cytoplasm interacting with Keap-1, which limits its activity and rapidly promotes its degradation by the ubiquitin-proteasome pathway (*45*). Conversely, Nrf2 dissociates from Keap-1 when the cell is exposed to OxS. In this situation, stabilized Nrf2 then translocates to the nucleus where stimulates the expression of genes encoding a set of antioxidant enzymes such as CAT, SOD and GPx through binding to the AREs placed within the gene promoter regions (*48, 50*).

According to these statements, our results show a greater increase in the cytoplasmic Nrf2 protein levels and a higher decrease in its nuclear fraction after the long-term consumption and during postprandial phase of the Med+CoQ diet with respect to the SFA diet, with an intermediate effect of the Med diet. These findings may be explained by the fact that the increase of Keap-1 protein levels, obtained after Med+CoQ diet consumption, promotes its interaction with Nrf2 in the cytoplasm, avoiding its translocation to the nucleus. On the other hand, the lower cytoplasmic levels of Keap-1 and the higher Nrf2 nuclear levels observed after SFA diet consumption may have favoured the binding of Nrf2 to the AREs and the subsequent transcriptional gene activation. This hypothesis seems to corroborate the increase in Nrf2, Gpx1, SOD1, SOD2 and TrxR mRNA levels obtained after SFA diet in our study.

The first line of enzymatic antioxidant defense is formed by SOD, GPx and CAT, which protect cells against the ROS produced during normal metabolism

and after an oxidative insult. SOD decomposes $\cdot O_2^-$ to O_2 and H_2O_2, preserving cells from $\cdot O_2^-$-mediated lipid peroxidation (*51*), while CAT and GPx neutralize the toxicity caused by H_2O_2 and/or ROOH (*54, 56*). Moreover, peroxides can be reduced directly by TrxR, providing an alternative mechanism for the detoxification of lipid hydroperoxides, otherwise mainly managed by GPx (*58, 60*).

The decrease in SOD mRNA levels observed after the long-term consumption and during postprandial phase of the Med and Med+CoQ diets compared to SFA diet, may well be caused by lower generation of $\cdot O_2^-$, due to the fact that the SOD gene expression has been proved to be up-regulated by ROS (239). In addition, Gpx1 mRNA showed the lowest postprandial levels after Med+CoQ diet with respect to the other diets. Presumably, it is likely that the supplementation with the antioxidant CoQ contributed to reduce H_2O_2 production and also to diminish Gpx1 expression, for which H_2O_2 serves as a substrate (240). These results, together with the increase in TrxR mRNA levels observed following SFA diet consumption, suggest that the OxS phenomena originated after this diet are higher than after the Med and Med+CoQ diets.

The metabolic response to dietary interventions with different fat composition is not only related to the cellular redox state, but may also affect other metabolic pathways. The complexity of their effects can be explored by metabolomics, so that there has been a growing interest recently in the application of this technique in human nutritional research. Nutritional metabolomics focuses on the interaction between products of metabolism in a biological sample and dietary intake of nutrients and non-nutrients, helping us to understand the regulatory roles of nutrition (241, 242).

After consumption, food components are absorbed and transformed in the gastrointestinal tract or liver, appearing in plasma or urine with or without further transformations. They can be detected and identified by using targeted

or untargeted metabolomic approaches (243). Indeed, numerous epidemiological studies have shown that the urinary profile is subject to dietary influences (244, 245), age, gender and hormonal status (246, 247).

In our metabolomic analysis, the results show that the long-term consumption of the Med+CoQ diet after four weeks of intervention is associated with increased levels of excreted hippurate and decreased levels of phenylacetylglycine compared to the SFA diet in postmenopausal women.

Hippuric acid (N-benzoylglycine) has been described to be catabolically synthesized by conjugation of benzoic acid with glycine from its precursor quinic acid, via the shikimate pathway in the gastrointestinal tract (248). This metabolite is considered as one of the final products of gut microbial metabolism in urine after intake of dietary polyphenols. In fact, several intervention studies have indicated that consumption of polyphenol-rich extracts (249) or tea (250) enhances its excretion. Regarding to its health aspects, this metabolite does not seem to be directly beneficial by itself, but it can be considered as an indicator of antioxidant molecules synthesis by gut microflora (248, 251), DNA repair enhancement and NF-κB inhibition (252). In accordance with these studies, our our strong correlation results suggest that hippurate is positively related to antioxidant compounds such as CoQ and β-carotene, and negatively associated with the gene expression of Nrf2 and several molecules related to OxS processes (Trx, SOD1, $gp91^{phox}$).

Little is known about the excretion of phenylacetylglycine in humans following dietary intervention. This metabolite is generated from phenylacetate via phase II detoxification mechanisms of from microbiota metabolism (253) and is often overexpressed in disease. It is one of the major metabolites excreted in spontaneous hypertensive rats (253), in which acts as a putative biomarker of phospholipidosis (254). Interestingly, urinary acylglycines are often used as diagnostic tool for mitochondrial fatty-acid oxidation disorders in humans (255) and, in particular, phenylacetylglycine

levels have been found to be significantly higher in heart failure patients than in healthy controls (256, 257). Due to the important role of OxS in cardiovascular disease (258) and considering these studies, greater phenylacetylglycine excretion might be linked to an increase in OxS-related mechanisms. Herein, our results show that phenylacetylglycine levels are inversely associated with CoQ levels and conversely, highly positive correlated to urinary levels of isoprostanes, which are considered as biomarkers for monitoring oxidative status (259).

It is worth noting the fact that remarkable differences in metabolite excretion were just found when comparing Med+CoQ diet versus SFA diet, suggesting that the antioxidant compounds presents in the Med diet, together with CoQ may produce a synergic antioxidant effect (the most antioxidant diet) when is compared to SFA diet (the most oxidant diet).

Interestingly, the benefit caused by Med+CoQ diet consumption in our study is greater in elderly women than in elderly men. In this regard, the antioxidant compounds CoQ and β-carotene are greater in women than in men, whilst LPO and oxLDL plasma levels in men are higher than in women. This interesting gender difference should be retested in further studies since these women are postmenopausal and therefore, they do not have the additional antioxidant benefits attributed to estrogens (260). Nevertheless, hippurate and phenylacetylglycine are microbial metabolites, thus differences in its levels could be due to gut microbial diversity between men and women.

Not only is aging related to oxidative processes, but also to a low-grade chronic inflammatory status. In this connection, the term “inflamm-aging” refers to the common inflammatory pathogenesis that underlies different age-related pathologies, such as atherosclerosis, cardiovascular diseases, osteoporosis, type 2 diabetes, MetS or cognitive decline (261, 262).

The master regulator of the inflammatory response is the transcription factor NF-κB (p50/p65), which is normally present in an inactive form bound to its inhibitor IκB in the cytoplasm. Diverse stimuli such as OxS or cytokines release (IL-1β, TNF-α) result in the phosphorylation of IκB proteins by IKK, followed by its ubiquitination and degradation through the 26S proteasome pathway. Free NF-κB is then able to translocate to the nucleus, where it binds to its consensus sequences and activates transcription (*87, 90*), thus regulating IL-1β, TNF-α, IL-6 and COX-2 gene expression (263).

Among the numerous factors that can modulate inflamm-aging, nutrition is probably the most powerful tool that we have to enhance the health status of the elderly population (264). Accordingly, the consumption of a hypercaloric breakfast increases the nuclear NF-κB activity at the same time that leads to a reduction in the cytoplasmic IκB-α expression in healthy subjects (265). However, the long-term consumption of a Med diet produces a lower NF-κB activation when compared with other types of fat either in fasting or postprandial state, and these results have been observed both in healthy people (*168*) and in MetS patients (*169*). Consistently, our results show a greater postprandial decrease in p65 (RelA) and IKK-β mRNA levels, as well as a higher postprandial increase in IκB-α after Med+CoQ diet intake compared to SFA diet, with an intermediate effect for the Med diet. We also observed boosted IL-1β mRNA levels following SFA diet consumption compared with the other diets.

One pivotal factor down-regulated by NF-κB is MMP-9, which is a metalloproteinase involved in several stages of atherosclerosis through remodeling of the extracellular matrix (*110*). Interestingly, its expression in atherosclerotic plaques is proportional to the free radical production and has been described as an important mediator of the phagocytic NADPH oxidase-dependent ROS production in atherosclerosis (266). Taking into account the significant rise in NADPH oxidase activity after a fat challenge (265), the

observed increase in MMP-9 mRNA levels after SFA diet in our study could be explained by an increment in ROS production and a major activation of the inflammatory response when this type of fat is consumed.

Activation of stress-kinase signaling has recently been recognized as an important pathophysiological mechanism in the development of diet-induced obesity, type 2 diabetes mellitus and other aging-related pathologies (*100*). In this regard, JNK is not only involved in inflammatory pathways, but also associated with OxS and ER stress mechanisms, in which its activation is related to the UPR initiation (*104*). In our study, Med and Med+CoQ diets are associated with a lower expression of JNK-1 and ER stress-related genes such as sXBP-1, CRT and BiP/Grp78, suggesting that the consumption of these dietary patterns trigger inflammation processes and ER stress to a lesser extent than SFA diet intake.

The present study has the advantage of a randomized crossover design in which all the participants have experienced all the diet periods, each individual acting as his/her own control and strengthening the fact that the effects observed are due to the influence of the type of diet. Multiple studies have shown that a three week dietary period is enough for assessing its effects, and in longer periods there is not influence of previous diets (267, 268). In our study, and to avoid any doubt, we extended the dietary periods to four weeks each, thus securing that the outcomes found were not provided by previous diets. On the other hand, we are aware that our study has certain limitations, since ensuring adherence to dietary instructions is difficult in a feeding trial and also the small sample population. However, adherence to the recommended dietary patterns was satisfactory according to the Food Frequency Questionnaires.

In conclusion, our results suggest that consumption of a Med diet rich in olive oil contributes to the redox homeostasis and enhances the protection of the

organism against oxidative damage. Supplementation of Med diet with CoQ has an additive effect in lowering OxS levels, modifying antioxidant protein levels and also in reducing the expression of antioxidant genes in PBMC from elderly people at fasting and postprandial state. Moreover, these dietary patterns exert a modulatory effect on the inflammatory response and on the ER stress in this population, supporting the fact that the consumption of a Med diet supplemented in CoQ is beneficial for healthy aging.

Influence of endothelial dysfunction on telomere length in subjects with metabolic syndrome from LIPGENE study

MetS comprises a cluster of metabolic abnormalities characterized by hypertriglyceridemia, low HDL-C, hypertension, fasting glucose and abdominal obesity that lead to cardiovascular disease or increased risk of type 2 diabetes (269). Despite insulin resistance is considered as the core of the MetS in terms of molecular mechanisms, OxS has been emerging as a crucial process involved in the pathogenesis and the etiology of the syndrome (*38*).
Previous findings from the same LIPGENE subcohort as this study have shown that the number of MetS components is an important risk factor for the increase of the OxS degree (determined by SOD and GPx plasma activities, IRH and NO plasma levels, among other parameters) (*41*). An increment in OxS levels coupled with an impairment in NO availability has been described to disturb the endothelium, causing endothelial dysfunction (*24*). Indeed, endothelial damage and atherosclerosis have been related to telomere attrition (*10*) and this association is greater in coronary artery disease patients with MetS than in those without the syndrome (*13*). Hence, further knowledge of the redox state in early MetS patients may provide a starting point for

understanding the pathways that contribute to both the development of the syndrome as well as its subsequent complications.

The present cross-sectional study highlights a gradual and direct relationship between high OxS levels and endothelial dysfunction with regard to RTL in MetS participants. Those subjects with shorter telomeres have increased biomarkers of OxS and higher plasma activity of antioxidant enzymes. Interestingly, their total NO plasma levels and IRH are lower than subjects with longer telomeres.

Previous studies have suggested that DNA damage manifested as telomere attrition plays a pivotal role in the development of endothelial dysfunction in the pathogenesis of vascular disease, due to the strong relationship found between telomere shortening and cell senescence in HUVECS (270). In this regard, our results show that subjects with the shortest RTL have the lowest NO plasma levels and as one would expect, they present the lowest IRH compared to subjects with longer RTL.
Interestingly, those participants with the shortest RTL are the ones with the highest PC plasma levels, which are biomarkers of severe OxS. These proteins are characterized by irreversible damage in its structure that affect to its function and their presence has been frequently associated with obesity, insulin resistance, diabetes mellitus and MetS (271). Accordingly, the activity of antioxidant enzymes such as GPx and SOD from participants with shorter RTL is greater than subjects with longer RTL. A plausible explanation to these findings could be the fact that cells attempt to strengthen their antioxidant arsenal in response to OxS and also to prevent oxidative damage. For this purpose, enzymes from the first line of antioxidant defense such as SOD, GPx and catalase increase their activity to balance the excess of OxS (*41*). Our present observations are consistent with a study in coronary artery disease

patients with MetS in which telomere attrition and OxS damage in endothelial progenitor cells were higher than in those without MetS. On the basis of the results obtained, the authors suggest that telomere shortening in response to OxS processes may induce an increment in endothelial damage, thus contributing to increase the risk of subsequent cardiovascular events (*13*).

In the present study, we measured telomere length in circulating leucocytes instead of endothelial cells due to the fact that this method is less invasive than obtaining human vascular tissue from participants. Indeed, it has been demonstrated that circulating blood leucocyte DNA content is predictive of vascular telomere content as well as an accurate surrogate for vascular aging in several population studies (10, 272).

There is evidence for the role of obesity, particularly intra-abdominal visceral fat accumulation, in promoting the development of metabolic diseases including MetS, glucose intolerance, dyslipidemia and atherosclerosis. In this regard, systemic OxS has been strongly associated with visceral fat accumulation in MetS patients (273, 274). Our results display higher TG plasma levels and lower HOMAβ in participants with increased OxS levels and shorter RTL. The same findings have been shown in a study with type 2 diabetes patients, where telomere attrition was inversely correlated to TG levels and considerably explained by insulin resistance, which in turn lead to an increment in ROS levels (275).

We are aware that this cross-sectional study has some limitations since these findings do not prove a cause-and-effect relationship and the use of a small sample population. In this regard, larger and prospective studies are needed to elucidate which of the parameters studied herein has the strongest effect above the others.

In summary, our results show that there is a clear link among endothelial function, OxS and RTL in MetS subjects. High levels of OxS may induce DNA damage in terms of telomere attrition and may also contribute to the dysregulation of vascular homeostasis.

Thus, further support of the molecular and cellular mechanisms involved in endothelial dysfunction is needed to develop strategies in order to decelerate vascular aging or prevent cardiovascular disease.

VIII. CONCLUSIONS

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Main Conclusion

The long-term consumption of Mediterranean diet contributes in part to the redox homeostasis of elderly people at fasting and postprandial state through the modulation of the Nrf2-mediated response to oxidative stress. Moreover, the supplementation of coenzyme Q_{10} enhances this contribution by modifying the antioxidant protein levels and reducing the expression of genes related to oxidative stress processes in peripheral blood mononuclear cells from elderly people.

Secondary Conclusions

1. The long-term consumption of a Mediterranean diet, with or without coenzyme Q_{10} supplementation, modulates the endoplasmic reticulum stress response by decreasing the fasting and postprandial expression of genes associated with the unfolded protein response in peripheral blood mononuclear cells from elderly people.

2. The long-term intake of a Med diet, with an additive effect provided by coenzyme Q_{10} supplementation, modifies the inflammatory response by decreasing the fasting and postprandial expression of pro-inflammatory genes in peripheral blood mononuclear cells from an elderly population.

3. The long-term consumption of a Mediterranean diet supplemented with coenzyme Q_{10} modifies the metabolomic urinary profile of elderly women through greater excretion of metabolites associated with antioxidant properties. By contrast, metabolites excreted after the intake of a saturated

fatty acid-rich diet are related to increased biomarker of oxidative damage.

4. There is a direct relationship between endothelial function, oxidative stress and relative telomere length in elderly people and metabolic syndrome subjects, where high levels of oxidative damage may induce endothelial dysfunction and may increase telomere attrition.

IX. REFERENCES

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X. ANNEXES

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Publications derived from the thesis:

Mediterranean diet supplemented with coenzyme Q10 modifies the expression of proinflammatory and endoplasmic reticulum stress-related genes in elderly men and women.

Yubero-Serrano EM, **Gonzalez-Guardia** L, Rangel-Zuñiga O, Delgado-Lista J, Gutierrez-Mariscal FM, Perez-Martinez P, Delgado-Casado N, Cruz-Teno C, Tinahones FJ, Villalba JM, Perez-Jimenez F, Lopez-Miranda J.

J Gerontol A Biol Sci Med Sci. 2012 Jan; 67(1):3-10. doi: 10.1093/gerona/glr167. Epub 2011 Oct 20.

Journal category name: Geriatrics & Gerontology

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Journal rank in category/Total journals in category: 6/47

Postprandial antioxidant gene expression is modified by Mediterranean diet supplemented with coenzyme Q(10) in elderly men and women.

Yubero-Serrano EM, **Gonzalez-Guardia** L, Rangel-Zuñiga O, Delgado-Casado N, Delgado-Lista J, Perez-Martinez P, Garcia-Rios A, Caballero J, Marin C, Gutierrez-Mariscal FM, Tinahones FJ, Villalba JM, Tunez I, Perez-Jimenez F, Lopez-Miranda J.

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Impact factor: 4.084 (Q1)

Journal rank in category/Total journals in category: 8/47

Effects of the Mediterranean diet supplemented with coenzyme Q_{10} on metabolomic profiles in elderly men and women.

Lorena González-Guardia, Elena María Yubero-Serrano, Javier Delgado-Lista, Pablo Perez-Martinez, Antonio Garcia-Rios, Carmen Marin, Antonio Camargo, Nieves Delgado-Casado, Helen M Roche, Francisco Perez-Jimenez, Lorraine Brennan, José López-Miranda.

Under review in **Journal Gerontology: Medical Sciences.**

Influence of endothelial dysfunction on telomere length in subjects with metabolic syndrome: LIPGENE study.

Lorena González-Guardia, Elena María Yubero-Serrano, Oriol Rangel-Zuñiga, Carmen Marin, Antonio Camargo, Pablo Pérez-Jiménez, Javier Delgado-Lista, Francisco Gómez-Delgado, Antonio Garcia-Rios, Francisco José Tinahones, Helen M Roche, José López-Miranda.

Under review in **Age (Dordr).**

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Postprandial antioxidant gene expression is modified by Mediterranean diet supplemented with coenzyme Q_{10} in elderly men and women

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Abstract Postprandial oxidative stress is characterized by an increased susceptibility of the organism towards oxidative damage after consumption of a meal rich in lipids and/or carbohydrates. We have investigated whether the quality of dietary fat alters postprandial gene expression and protein levels involved in oxidative stress and whether the supplementation with coenzyme Q_{10} (CoQ) improves this situation in an elderly population. Twenty participants were randomized to receive three isocaloric diets each for 4 weeks: Mediterranean diet supplemented with CoQ (Med+ CoQ diet), Mediterranean diet (Med diet), saturated

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fatty acid-rich diet (SFA diet). After 12-h fast, volunteers consumed a breakfast with a fat composition similar to that consumed in each of the diets. Nrf2, $p22^{phox}$ and $p47^{phox}$, superoxide dismutase 1 and 2 (SOD1 and SOD2), glutathione peroxidase 1 (GPx1), thiorredoxin reductase (TrxR) gene expression and Kelch-like ECH associating protein 1 (Keap-1) and citoplasmic and nuclear Nrf2 protein levels were determined. Med and Med+CoQ diets induced lower Nrf2, $p22^{phox}$, $p47^{phox}$, SOD1, SOD2 and TrxR gene expression and higher cytoplasmic Nrf2 and Keap-1 protein levels compared to the SFA diet. Moreover, Med+CoQ diet produced lower postprandial Nrf2 gene expression and lower nuclear Nrf2 protein levels compared to the other diets and lower GPx1 gene expression than the SFA diet. Our results support the antioxidant effect of a Med diet and that exogenous CoQ supplementation has a protective effects against free radical overgeneration through the lowering of postprandial oxidative stress modifying the postprandial antioxidant protein levels and reducing the postprandial expression of antioxidant genes in peripheral blood mononuclear cells.

Keywords CoQ10 · Mediterranean diet · Oxidative stress · Gene expression

Abbreviations

Apo	Apolipoprotein
CAT	Catalase
CoQ	Coenzyme Q10
GPx	Gluthatione peroxidase
H_2O_2	Hydrogen peroxide
Keap-1	Kelch-like ECH associating protein 1
Med diet	Mediterranean diet
Med+CoQ diet	Mediterranean supplemented with CoQ
MUFA	Monounsaturated fatty acid
PBMCs	Peripheral blood mononuclear cells
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SFA diet	Saturated fatty acid-rich diet
SOD	Superoxide dismutase
TG	Triacylglycerol
TrxR	Thiorredoxin reductase

Introduction

Aging may be defined as an inherently complex process that is manifested within an organism at genetic, molecular, cellular, organ, and system levels (Harman 2009). The aging process induces age-related changes and leads to increased occurrence of many diseases. Although the fundamental mechanisms are still poorly understood, a growing body of evidence points toward the oxidative damage caused by reactive oxygen species (ROS) as one of the primary determinant of aging (Droge 2002). A certain amount of oxidative damage takes place even under normal conditions; however, the rate of this damage increases during the pathological conditions like diabetes, cardiovascular diseases, cancer, and aging-related diseases (Elahi et al. 2009). Although aging is an inevitable event, linked to the pass of time, nutritional intervention may influence the intrinsic rate of aging as well as the incidence of these age-associated diseases.

Extensive prospective studies suggest that a high degree of adherence to a Mediterranean diet (Med diet) is associated with reduced mortality, prevents the onset and progression of coronary heart disease (Covas 2007) and other aging-related diseases (Trichopoulou et al. 2003; Estruch et al. 2006). Common components of this diet include monounsaturated fatty acids (MUFA), α-tocopherol, phenolic compounds, phytoesterols, and other antioxidants so the leading hypothesis on the mechanism of this association is a decrease of oxidative stress due to the antioxidant capacity of this diet (Visioli and Galli 2001).

Fasting is not the typical physiological state of the modern human being, which spends most the time in the postprandial state. In line with this notion, oxidative stress has received considerable attention over the past several years in the fasting state; however, there is a paucity of data on postprandial oxidative stress. With regard to the postprandial state, we have recently demonstrated the antioxidant effect of Med diet rich in olive oil and that exogenous coenzyme Q_{10} (CoQ: 2,3-dimethoxy-5-methy-6-decaprenyl-1,4-benzoquinone) supplementation in synergy with a Med diet (Med+CoQ diet) improves the postprandial oxidative stress in elderly men and women, with a higher increase in capillary flow, a lower plasma biomarker of oxidative stress levels and a greater postprandial decrease in plasma antioxidant enzymatic activities with respect to a Western diet

rich in saturated fatty acid (SFA diet; Yubero-Serrano et al. 2010).

Interest in CoQ comes from the fact that it is an important mitochondrial redox component (Battino et al. 2001) and endogenously produced lipid-soluble antioxidant of the human organism. CoQ plays a crucial role in the generation of cellular energy, enhances the immune system, and acts as a free radical scavenger with antioxidant properties (Quiles et al. 2005). Aging, poor eating habits, and stress affects the organism's ability to provide adequate amounts of CoQ.

Many research suggest that using CoQ supplements may help maintain health of elderly people or treat some of the health problems or diseases such as adjunctive therapy in the treatment of congestive heart failure (Kaikkonen et al. 2002), as an anticancerogenic and immune-stimulating agent (Folkers et al. 1993) and for slowing down the progression of Parkinson's disease in the early stage (Muller et al. 2003).

As discussed above, one of the causes of aging is the change at the molecular level, which may cause alterations in the expression levels of genes and protein involved in oxidative stress. Peripheral blood mononuclear cells (PBMCs) are a subset of white blood cells, which include lymphocytes and monocytes, and play a critical role in the immune system. Various studies showed disease-characteristic gene expression patterns in PBMCs (Mass et al. 2002; Burczynski and Dorner 2006) and they can be easily and repeatedly collected in sufficient quantities in contrast to the more invasive sampling of adipose, muscle and liver tissues, among others (de Mello et al. 2008). However, little is known of nutritional effects on PBMCs gene expression patterns.

According to these premises, the aim of this study was to determine whether diets with different fat quality influence on the postprandial gene expression and protein levels involved in oxidative stress and that this hypothetical improvement could be boosted by supplementation with a natural antioxidant, like CoQ, a natural antioxidant agent in PBMCs of healthy elderly people.

Experimental procedures

Participants and recruitment

Volunteers were recruited using various methods including the use of general practitioner databases, and poster and newspaper advertisements. A total of 63 persons were contacted among those willing to enter the study. All participants underwent a comprehensive medical history, physical examination, and clinical chemistry analysis before enrolment and gave their informed consent before joining the study. Inclusion and exclusion criteria were fulfilled by 20 patients (age ≥65 years; 10 men and 10 women). Power analysis was set a 90% to detect variations equal or larger than 20% in the mRNAs gene expression (Jimenez-Gomez et al. 2009). The sample size calculation indicated that we needed at least 18 participants. Clinical inclusion criteria were: age ≥65 years, body mass index 20–40 kg/m^2, total cholesterol concentration equal to or <8.0 mmol/L and nonsmokers. Clinical exclusion criteria were: age <65 years, diabetes or other endocrine disorders, chronic inflammatory conditions, kidney or liver dysfunction, iron deficiency anemia (hemoglobin <12 g/dL men, <11 g/dL women), prescribed hypolipidaemic and anti-inflammatory medication, fatty acid supplements including fish oil, consumers of high doses of antioxidant vitamins (A, C, E, β-carotene), highly trained or endurance athletes or those who participate in more than three periods of intense exercise per week, weight change equal or >3 kg within the last 3 months, smokers, alcohol, or drug abuse (based on clinical judgment). The study protocol was approved by the Human Investigation Review Committee of the Reina Sofia University Hospital according to institutional and Good Clinical Practice guidelines.

Study design

Participants were randomly assigned to receive, in a crossover design, three isocaloric diets for 4-week periods each (Supporting Fig. S1). The three diets were as follows: (1) Mediterranean diet supplemented with CoQ (Med+CoQ diet; 200 mg/day in capsules), containing 15% of energy as protein, 47% of energy as carbohydrate and 38% of total energy as fat [24% MUFA (provided by virgin olive oil), 10% SFA, 4% polyunsaturated fatty acid (PUFA)]. (2) Mediterranean diet not supplemented with CoQ (Med diet), with the same composition of the first diet but supplemented by placebo capsules and (3) Western diet rich in saturated fat (SFA diet) with 15% of energy as protein, 47% of energy as carbohydrate, and 38% of total energy as fat (12% MUFA, 22% SFA, 4% PUFA).

The cholesterol intake was kept constant (<300 mg/day) during the three periods. Both the CoQ and the

placebo capsules were specially produced by the same company (Kaneka Corporation, Osaka, Japan) and were identical in weight and external aspect. Patients taking capsules were unaware whether they were in the Med+CoQ or Med dietary period (Supporting Table S1). The composition of the experimental diets was calculated by using the US Department of Agriculture (1987) food tables and Spanish food composition tables for local foodstuffs (Varela 1980).

Before the start of the intervention period, volunteers completed a 3-day weighed food diary and an extensive Food Frequency Questionnaires, which allowed identification of foods to be modified. At the start of the intervention period, each patient was provided with a handbook for the diet to which they had been randomized. Advice was given on foods to choose and those to avoid if eating outside home. They were also instructed to write down in the diary about any menu eaten out of home and to call the monitoring study nurse reporting such event. At baseline, volunteers were provided with a supply of study foods to last for 2 weeks. They collected additional study foods every fortnight or when required. At these times, a 24-h recall of the previous day's food intake and a short food use questionnaire based on the study foods were completed to monitor and motivate volunteers to adhere to the dietary advice. A points system was used to assess the number of food exchanges achieved in the 24-h recall and additional advice was given if either the 24-h recall or food use questionnaire showed inadequate intake of food exchange options. Volunteers were asked to complete 3-day weighed food diaries at baseline, weeks 2 and 4. Weighed food intake over two weekdays and one weekend day was obtained using scales provided by the investigators. Fat foods were administered by dietitians in the intervention study. The dietary analysis software Dietsource version 2.0 was used. (Novartis S.A., Barcelona, Spain).

At the end of the dietary intervention period, the subjects were given a fatty breakfast with the same fat composition as consumed in each of the diets. Patients presented at the clinical centers at 8 h following a 12-h fast (time 0), abstained from alcohol intake during the preceding 7 days. After canulation of a blood vessel, a fasting blood sample was taken before the test meal, which was then ingested within 20 min under supervision. The test meal reflected fatty acid composition of each subject after the chronic dietary intervention. Subsequent blood samples were drawn at 2 and 4 h. Test meals provided an equal amount of fat (0.7 g/kg body weight), cholesterol (5 mg/kg of body weight), and vitamin A (60,000 IU/m^2 body surface area). The test meal provided 65% of energy as fat, 10% as protein, and 25% as carbohydrates. The composition of the breakfasts was as follows: Med with CoQ (400 mg in capsules) breakfast (12% SFA, 43% MUFA, and 10% PUFA), Med with placebo capsules breakfast (12% SFA, 43% MUFA, and 10% PUFA), and SFA-rich breakfast (38% SFA, 21% MUFA, and 6% PUFA).

Biochemical determinations

Plasma samples

Venous blood samples were obtained at the end of the each dietary intervention period on fasting state after a 12-h fast, before breakfast ingestion and at 2 and 4 h after ingestion of breakfast. Samples from the fasting and postprandial states were collected in tubes containing 1 g ethylenediaminetetraacetic acid (EDTA)/L and were stored in containers with ice and kept in the dark. Particular care was taken to avoid exposure to air, light, and ambient temperature. Plasma was separated from whole blood by low-speed centrifugation at 1,500×*g* for 15 min at 4°C within 1 h of extraction.

Isolation of PBMCs

PBMCs were isolated from 20 mL of venous blood in tubes containing 1 mg/mL of EDTA. The blood samples were diluted 1:1 in PBS, and cells were separated in Ficoll gradient by centrifugation at 800×*g* for 25 min at 20°C. The cells were collected and washed with cold PBS two times and finally resuspended in buffer A. This buffer contained 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 mM KCl, 2 mM MgCl2 and 1 mM EDTA and, at the time of use, 1 mM PMSF and 1 mM DTT were added. The cells thus obtained were stored at −80°C for further analysis.

RNA extraction and qRT-PCR analysis

RNA extraction

Total RNA from PBMCs was extracted using the trizol method according to the recommendations of the manufacturer (Tri Reagent®, Sigma, St Louis, MO, USA) and quantified in a NanoDrop 1000A

Spectrophotometer. RNA integrity was verified on agarose gel electrophoresis and stored at −80°C. Next, since polymerase chain reaction (PCR) can detect even a single molecule of DNA, RNA samples were digested with DNAse I (AMPD-1 KT, Sigma) before RT-PCR.

Biotrove open array™ real-time PCR

Each reaction was performed with 1 μl of a 1:5 (*v*/*v*) dilution of the first cDNA strand, synthesized from 1 μg of total RNA using the commercial kit iScript cDNA Synthesis Kit (Bio Rad) according to the manufacturer's instruction.

The reaction of real-time PCR was carried out using the platform OpenArray™ NT Cycler (Applied Biosystems). This system of analysis of gene expression used as a method for quantification TaqMan probes. OpenArray™ subarrays were preloaded by Biotrove with the selected primer pairs. The individual primer pairs (synthesized by Sigma-Aldrich, St. Louis, MO, USA) were preloaded into Bio Trove OpenArray™ plates. Each primer pair was spotted in duplicate. The primers that amplify genes of interest were selected from the database TaqMan Gene Expression Assays (Applied Biosystems; https://products.appliedbiosystems.com/ab/en/US/adirect/ab?Cmd=catNavigate2&catID=601267) in Assays search tab taking as search criteria: selection and *Homo sapiens* Gene Expression Assays for each of the genes of interest.

Samples were loaded into OpenArray plates with the OpenArray NT Autoloader according to the manufacturer's protocols. Each subarray was loaded with 5.0 μl of master mix consisted of 1× LighCycler FastStart DNA Master SYBR Green Kit (Roche Applied Sciences, Indianapolis, IN, USA), 1× SYBR Green I 80×, 0.5% glycerol, 0.2% Pluronic F-68, 1 mg/mL bovine serum albumin (New England Biolaboratories, Beverly, MA, USA), 1 mM $MgCl_2$, 400 nM FP, 400 nM RP, 8% Formamide, 0.25× Rox, 1× TfR amplicon, and cDNA samples. The PCR OpenArray thermal cycling protocol consisted of 95°C for 10 min, followed by cycles of 10 s at 95°C, 10 s at 53°C, and 10 s at 72°C. All samples were tested in duplicate. The Biotrove OpenArrayTM NT Cycler System Software (version 1.0.2) uses a proprietary calling algorithm that estimates the quality of each individual C_T value by calculating a C_T confidence value for de amplification reaction.

In our assay, C_T values with C_T confidence values below 700 were regarded as background signals. The remaining positive amplification reactions were analyzed for amplicon specificity by studying the individual melting curves.

The same program allowed the selection of the most stable housekeeping gene in the samples processed for the relativization of the expression of genes of interest. Following this methodology, we analyzed the relative gene expression of these genes: Nrf2, $p47^{phox}$, and $p22^{phox}$ (nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits), superoxide dismutase 1 and 2 (SOD1 and SOD2), glutathione peroxidase 1 (GPx1), and thioredoxin reductase (TrxR).

Western blot analysis

Protein extraction

PMBCs were thawed on ice and buffer A was supplemented with 5 μg Aprotinin, 10 μg Leupeptin and 0.8% Nonidet NP-40. Cells were incubated on ice for 5 min, subjected to gentle agitation for 20 s in the vortex and then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant containing cytoplasmic proteins was distributed in aliquots that were stored at −80°C. The pellet was treated with 100 μL of lysis buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 20 μg Aprotinin, and 40 μg Leupeptin). The sample was incubated on ice for 20 min with periodic mixing with a vortex by stirring for 30 s every 5 min of incubation. Cells were then centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant thus obtained, containing the nuclear proteins, was distributed in aliquots, and stored at −80°C. The extracted proteins were quantified using the method of Bradford (1976).

Western blot

Electrophoretic separation was carried out with 50 μg of protein for both cytoplasmic and nuclear fractions. After separation in sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (11% polyacrylamide), proteins were transferred to nitrocellulose membranes (BioTrace NT Membrane; PALL Gelman Laboratory). The following proteins were detected using their corresponding antibodies: Nrf2 (C-20, sc-722: mouse monoclonal; Santa Cruz Biotechnology, Inc.), Kelch-like ECH

associating protein 1 (Keap-1; H-190, sc-33569: rabbit polyclonal; Santa Cruz Biotechnology, Inc.); Actin (C-2, sc-8432: mouse monoclonal; Santa Cruz Biotechnology, Inc.). After incubation with these primary antibodies, samples were incubated with respective secondary antibodies (goat anti-mouse or anti-rabbit HRP-conjugate; Santa Cruz Biotechnology, Inc.). The development process was carried out with ECL-Plus Western Blotting Detection System (Amersham™) and used for autoradiography Hyperfilm MP high-performance autoradiography film (Amersham™).

Quantification of protein bands

The proteins were identified in the autoradiography by its position relative to molecular weight markers: 57 kDa for Nrf2, 69 kDa for Keap-1, and 40 kDa for actin. The relative amount of each was quantified by densitometry using the software WIN1D.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS 17.0 for Windows Inc., Chicago, IL, USA) was used for the statistical comparisons. The Kolmogorov–Smirnov test did not show a significant departure from normality in the distribution of variance values. In order to evaluate data variation, Student's *t* test and an analysis of variance for repeated measures was performed, followed by Bonferroni's correction for multiple comparisons. We studied the statistical effects of the type of fat meal ingested, independent of time (represented by *p*1), the effect of time (represented by *p*2), and the interaction of both factors, indicative of the degree of the postprandial response in each group of subjects with each fat meal (represented by *p*3). Differences were considered to be significant when $p<0.05$. All data presented in text and tables are expressed as means±standard error (±SE).

Results

Metabolic parameters levels

The baseline characteristics of the 20 participants who completed the three dietary intervention periods showed that males had higher height, waist circumference, TG and Apo B than females. We did not find any other differences by gender (Supporting Table S2).

Moreover, we previously observed higher fasting plasma CoQ concentration ($p<0.001$) after the intake of the Med+CoQ diet compared with the Med and SFA diets. At 2 and 4 h after consumption of the Med+CoQ diet we observed a greater postprandial increase in plasma CoQ levels compared with the Med and SFA diets ($p=0.018$ and $p=0.032$, respectively; Supporting Fig. S2; Yubero-Serrano et al. 2010).

Diet intake- and expression genes-related oxidative stress in PBMCs

Nrf2

Nrf2 is the central transcription factor, essential for the coordinated induction of those genes encoding many stress-responsive or cytoprotective enzymes and related protein, such as SOD, GPx, glutathione S-transferase, and Thioredoxin (Dinkova-Kostova and Talalay 2008; Chen and Kong 2004). At 4 h after intake of the Med+CoQ diet, we observed a greater postprandial decrease in Nrf2 mRNA levels compared with the other diets ($p=0.029$; Fig. 1a). Furthermore, Nrf2 mRNA levels were lower after consumption of the Med diet compared with the SFA diet ($p=0.039$). No significant differences were detected in Nrf2 mRNA levels in fasting and at 2 h after intake of the three diets (Fig. 1a).

NADPH oxidase

NADPH oxidases are likely to be the predominant source of ROS in the vasculature (Brandes and Kreuzer 2005; Forstermann 2008; Grienling 2004). Activation of the NADPH oxidase enzyme complex requires the assembly of the cytosolic regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac) with the membrane-bound cytochrome b558 (subunits $gp91^{phox}$ and $p22^{phox}$; Brandes and Kreuzer 2005).

We observed a decrease in fasting $p22^{phox}$ mRNA levels after intake of the Med and Med+CoQ diets compared to the SFA diet ($p=0.032$; Fig. 1b). At 2 h and 4 h after the SFA diet, we found higher postprandial $p22^{phox}$ and $p47^{phox}$ mRNA levels compared with the Med and Med+CoQ diets ($p=0.038$ and $p=0.003$, respectively; Fig. 1b,c). No significant differences were detected in $p47^{phox}$

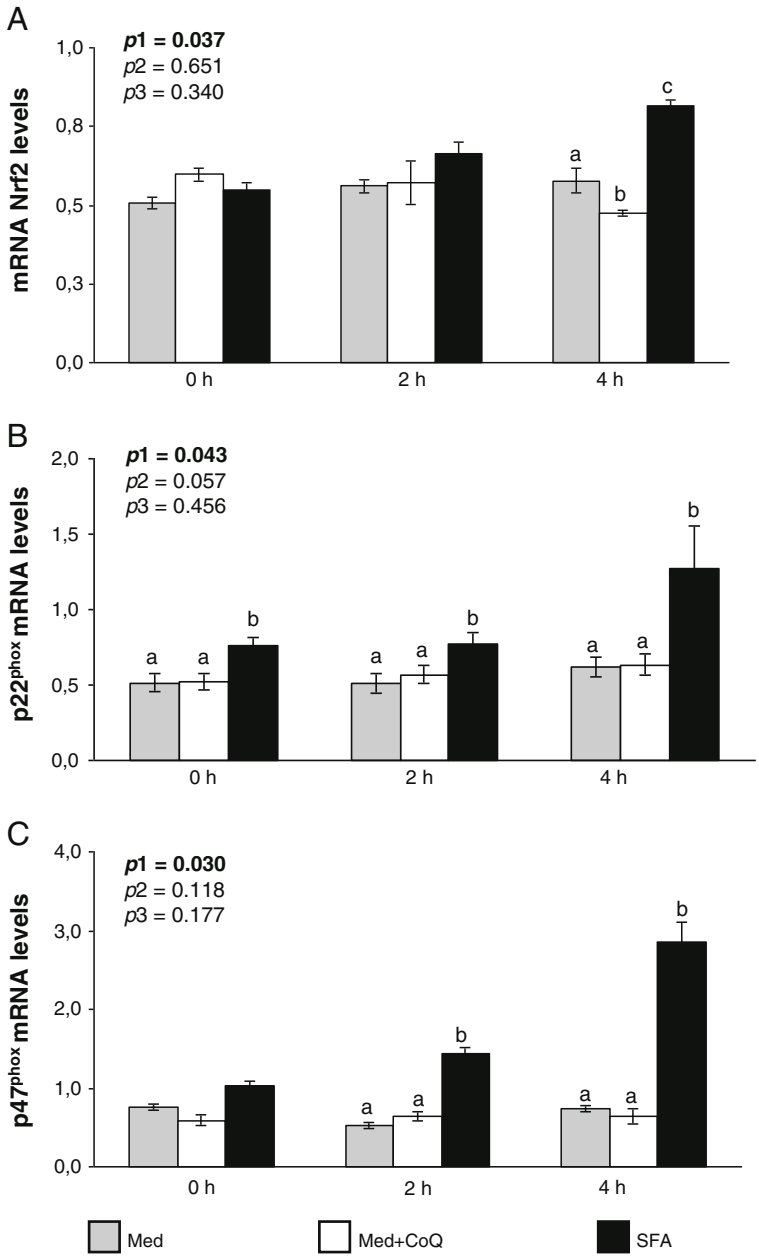

Fig. 1 Fasting and postprandial levels of Nrf2 mRNA (**a**), $p47^{phox}$ mRNA (**b**), and $p22^{phox}$ mRNA (**c**) in peripheral mononuclear cells according to the type of fat consumed. Data were analyzed using analysis of variance for repeated measures. All values represent the mean± standard errors (SE). *Bars with different superscript letters* depict statistically significantly differences ($p<0.05$). *p1* diet effect, *p2* time effect, *p3* diet x time interaction

mRNA levels in fasting after intake of any of the three diets (Fig. 1c).

SODs

SOD is a key antioxidant enzyme, scavenging the superoxide radical (O_2^-). SOD form the first line of defence against oxidative stress. We studied the expression of the two isoforms of human SOD: cytoplasmic SOD gene (SOD1 gene) and mitochondrial SOD gene (SOD2 gene). Fasting SOD2 mRNA levels were lower after participants consumed the Med and Med+CoQ diets than when they consumed the SFA diet ($p=0.007$; Fig. 2a). At 2 and 4 h after the SFA diet, we found higher postprandial SOD2 mRNA levels compared with the Med and Med+CoQ diets ($p=0.008$ and $p=0.003$, respectively). At 4 h after intake of the Med and Med+CoQ diets, we observed a greater postprandial decrease in SOD1 mRNA levels with respect to the SFA diet ($p=0.011$). However, no

significant differences were detected in SOD1 mRNA levels in fasting and at 2 h after intake of the three diets (Fig. 2b).

Gpx1

GPx enzyme catalyzes the reduction of H_2O_2 and scavenges organic hydroperoxides (de Haan et al. 1998). Fasting Gpx1 mRNA levels were greater after participants consumed the SFA diet than when they consumed the other two diet period (p=0.013; Fig. 2c). At 2 and 4 h after the Med+CoQ diet, we found lower postprandial Gpx1 mRNA levels compared with the SFA diet (p=0.013; Fig. 2c).

TrxR

TrxR is a ubiquitous oxidoreductase enzyme with antioxidant and redox regulatory roles. It also contributes to redox homeostasis and is involved in prevention, intervention, and repair of damage caused by H_2O_2-based oxidative stress (Nordberg and Arner 2001).

At 4 h after intake of the SFA diet, we found a greater postprandial increase in TrxR mRNA levels compared to the other diets (p=0.023). No significant differences were detected in TrxR mRNA levels in fasting and at 2 h after intake of the three diets (Fig. 2d).

Diet intake and cellular protein levels related to oxidative stress

Cytoplasmic and nuclear Nrf2 in PBMCs

In fasting and at 4 h after intake of the Med+CoQ diet, we observed higher cytoplasmic Nrf2 protein levels compared to the other diets (p=0.033 and p=0.011, respectively; Fig. 3a). In addition, at 4-h Med diet induced a significant increase of cytoplasmic Nrf2 protein levels compared to the SFA diet (p=0.026). However, both in fasting and at 4 h after intake of the Med+CoQ diet, we found lower nuclear Nrf2 protein levels compared to the SFA diet (p=0.001 and p= 0.019, respectively), with an intermediate effect for the Med diet (Fig. 3b).

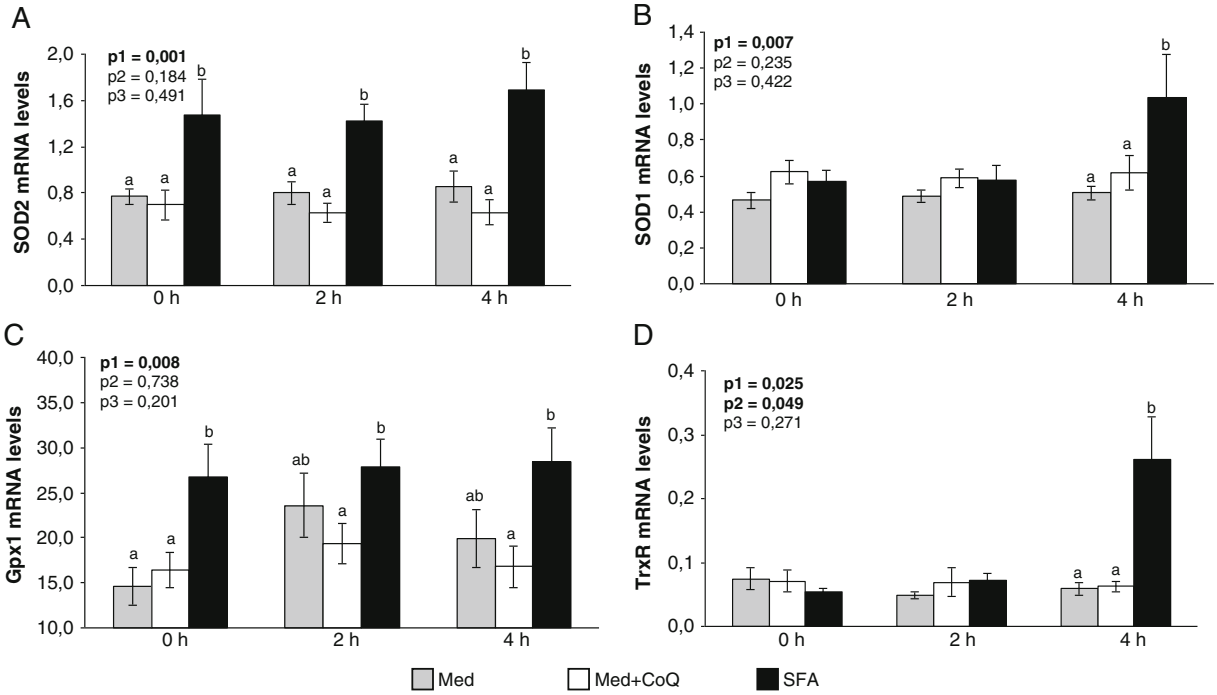

Fig. 2 Fasting and postprandial levels of SOD1 mRNA (**a**), SOD2 mRNA (**b**), Gpx1 mRNA (**c**), and TrxR mRNA (**d**) in peripheral mononuclear cells according to the type of fat consumed. Data were analyzed using analysis of variance for repeated measures. All values represent the mean±standard errors (SE). *Bars with different superscript letters* depict statistically significantly differences (p<0.05). *p1* diet effect, *p2* time effect, *p3* diet×time interaction

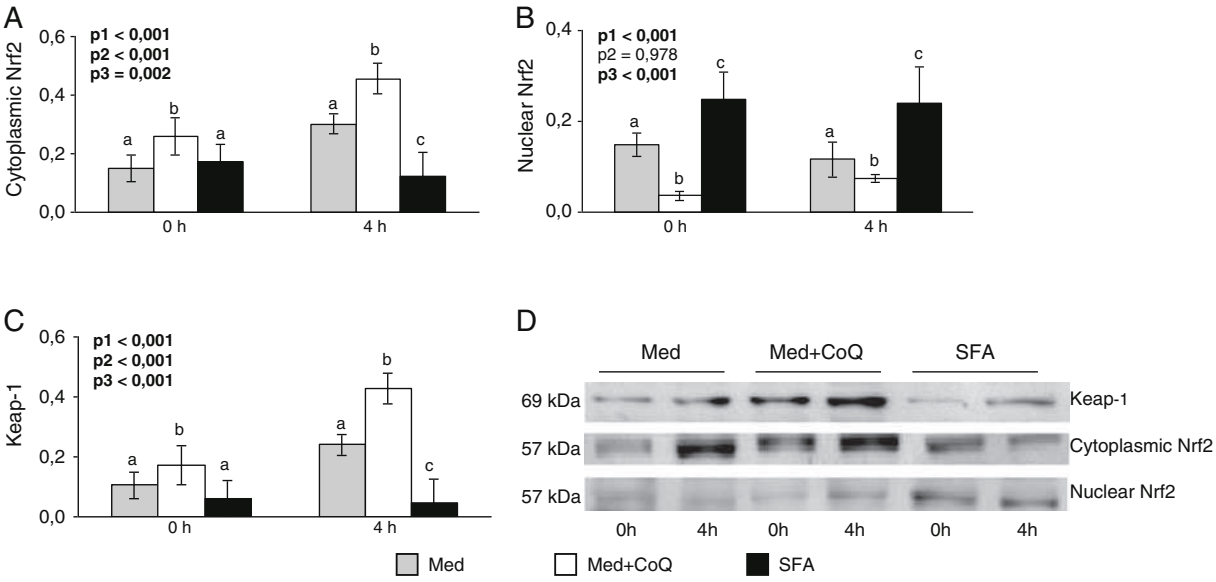

Fig. 3 Fasting and postprandial levels of cytoplasmic Nrf2 (**a**), nuclear Nrf2 (**b**), and Keap-1 (**c**) in peripheral mononuclear cells according to the type of fat consumed. Representative immunoblot of nuclear and cytoplasmatic Nrf2, Keap-1, and Actin. Depicted bands are different parts of the same blot stained for each antigen (**d**)

Keap-1 in PBMCs

Nrf2 activity is repressed by the cytosolic protein Keap-1, which has been proposed to act by sequestering and tethering the transcription factor in the cytoplasm (Nguyen et al. 2005). In fasting and at 4 h after intake of the Med+CoQ diet, we observed higher Keap-1 protein levels compared to the other diets (p=0.035 and p=0.003, respectively) (Fig. 3c). In addition, at 4 h Med diet induced a significant increase of postprandial Keap-1 protein levels compared to the SFA diet (p=0.017).

Discussion

The present study demonstrates that the consumption of a Med diet reduces the postprandial expression of genes that encoded proteins related to oxidative stress such as SOD (SOD1 and SOD2), TrxR, and NADPH-oxidase ($p22^{phox}$ and $p47^{phox}$ subunits) and increases the cytoplasmic Nrf2 protein levels in PBMCs. Moreover, the addition of CoQ had an additive effect on the Med diet since the participants that consumed this diet showed a greater postprandial decrease in gene expression of Nrf2 and GPx1 and a greater postprandial increase in cytoplasmic Nrf2 and Keap-1 protein levels in PBMCs with respect to the other diets.

In the same population of this study, we previously demonstrated that the Med diet improves the postprandial oxidative stress with a higher increase in capillary flow and plasma nitric oxide levels, a lower plasma lipid peroxidation products, nitrotyrosine and protein carbonyl levels, lower plasma antioxidant enzyme activities (GPx, catalase (CAT) and SOD), (Yubero-Serrano et al. 2010) and lower DNA damage in PBMCs (Gutierrez-Mariscal et al. 2011). Addition of exogenous CoQ in synergy with a Med diet had an additive effect reducing the postprandial oxidative stress in elderly men and women.

Moreover, few studies explored into the molecular effects that diet has on antioxidant gene expression and levels of proteins related with oxidative stress as Nrf2 and Keap-1 during the postprandial state, or into the effect of CoQ supplements in humans (Wang et al. 2010). While it is now widely accepted that oxidative damage plays an important role in the aging process (Lombard et al. 2005), the influence of age on the gene expression of antioxidant enzymes has not been widely studied; moreover, the few studies that have been performed have yielded conflicting results (Rao et al. 1990).

A central role in the defense against oxidative stress has been attributed to the transcription factor Nrf2. Nrf2 is localized in the cytoplasm where it interacts with the Keap-1, and is rapidly degraded by the ubiquitin–proteasome pathway (Zhang 2006). When the cell is exposed to oxidative stress, induction of Nrf2 occurs which dissociates Nrf2 from Keap-1. Stabilized Nrf2 then translocates to the nucleus and promotes the expression of genes encoding many antioxidant proteins and phase-II detoxifying enzymes such as CAT, SOD, GPx, and heme oxygenase-1 through binding specifically to the antioxidant-response element (ARE) found in the gene promoters (Xu et al. 2008; Kobayashi and Yamamoto 2005).

Our results show a greater increase in the cytoplasmic Nrf2 protein levels after intake of the Med+CoQ diet compared with the SFA diet, with an intermediate effect for the Med diet. These finding could be explained by the fact that the increase of Keap-1 protein levels obtained after intake of the Med+CoQ diet promotes its interaction with Nrf2 in the cytoplasm, which prevents the translocation of Nrf2 to the nucleus and does not allow the antioxidant genes expression. This hypothesis is corroborated with a greater postprandial decrease in the nuclear Nrf2 protein levels after intake of the Med+CoQ diet compared with the SFA diet, with an intermediate effect for the Med diet. Thus, after intake of the SFA diet, lower levels of Keap-1 in the cytoplasm and higher levels of nuclear Nrf2 protein may have favored the binding of Nrf2 to the ARE found in the antioxidant gene promoters and subsequent transcriptional gene activation. In fact, a postprandial rise in Nrf2, GPx1, SOD1, SOD2, and TrxR mRNA levels were observed for the SFA diet in our study.

SOD, GPx, and CAT are regarded as the first line of the antioxidant defense system enzymes against ROS. They protect cells against ROS produced during normal metabolism and after an oxidative insult. SOD metabolizes and protects the cells against O_2^--mediated lipid peroxidation, while CAT and GPx acts on H_2O_2 and/or ROOH by decomposing them, thereby neutralizing their toxicity. Also, peroxides, including lipid hydroperoxides and hydrogen peroxide can directly be reduced by TrxR (Zhong and Holmgren 2000; Bjornstedt et al. 1995). By this mechanism, TrxR could function as an alternative enzymatic pathway for the detoxification of lipid hydroperoxides, otherwise mainly managed by GPx. Any changes in one of these systems may break the equilibrium and cause cellular damage (Arsova-Sarafinovska et al. 2009). Previous studies have shown that the expression of SOD is upregulated by ROS (Mates et al. 1999). Thus, the decrease in SOD1 and SOD2 mRNA levels observed along the postprandial state of the Med and Med+CoQ diets with respect to the SFA diet could be explained on the basis of lower generation of superoxide in these diets. In addition, GPx1 mRNA levels showed the lowest postprandial levels in Med+CoQ diet with respect to the other diets. Presumably, CoQ supplementation contributed to a reduction in the production of H_2O_2 and in this way suppressed the GPx1 gene expression for which H_2O_2 serves as a substrate (Stachowska et al. 2005) since CoQ deficiency interferes with assembly or stability of the respiratory chain enzymes leading to unbalanced oxidative phosphorylation and enhanced ROS production (Quinzii et al. 2008). This phenomenon, together with the fact that there were observed an increase in TrxR mRNA levels after intake of the SFA diet compared with the Med and Med+CoQ diets could be due to the fact that the damage caused by oxidative stress is higher in the SFA diet than in the other diets.

NADPH oxidases are likely to be the predominant source of ROS in the vasculature (Brandes and Kreuzer 2005; Forstermann 2008; Grienling 2004). Activation of the NADPH oxidase enzyme complex requires the assembly of the cytosolic regulatory subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rac) with the membrane-bound cytochrome b558 ($gp91^{phox}$ and $p22^{phox}$; Brandes and Kreuzer 2005). High-fat diet had a significant increase in NADPH oxidase expression compared to low-fat diet in mice (Coate and Huggins 2010). Thus, we have observed that during the postprandial period, the intake of the SFA diet induced an increase in fasting and postprandial $p22^{phox}$ and postprandial $p47^{phox}$ mRNA levels, which could be due to an increased ROS production after the ingestion of this diet compared with the Med and Med+CoQ diets.

The present study has the advantage of a randomized crossover design in which all the participants have experienced the three diet periods, each individual acting as his/her own control and strengthening the fact that the effects observed are due to the influence of the type of diet. We acknowledge that our study has certain limitations, since ensuring adherence to dietary instructions is difficult in a feeding trial. However, adherence to the recommended dietary patterns was satisfactory, as

can be judged by the measurements of compliance. Another limitation of this study is that we did not study if supplementation with CoQ of the saturated fatty acid-rich diet may partly reduce the negative effects of this dietary model on postprandial oxidative stress.

A thorough nutrition status may be basic to understand the elderly total health. Therefore, our results support that consumption of a Med diet rich in olive oil (combined with vegetables, fruits, cereals, and a healthy lifestyle) contributes to redox homeostasis and is involved in prevention caused by H_2O_2-based oxidative stress. This protection is enhanced by exogenous CoQ supplementation, lowering of postprandial oxidative stress, modifying the postprandial antioxidant protein levels, and reducing the postprandial expression of antioxidant genes in PBMCs in elderly men and women. We can conclude that specific dietary intervention might be a new, interesting, and promising challenge in the treatment (and mainly prevention) of processes that lead to a rise in oxidative stress, such as cardiovascular, neurodegenerative diseases, and aging.

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Mediterranean Diet Supplemented With Coenzyme Q_{10} Modifies the Expression of Proinflammatory and Endoplasmic Reticulum Stress–Related Genes in Elderly Men and Women

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We have investigated whether the quality of dietary fat and supplementation with coenzyme Q_{10} (CoQ) modifies expression of genes related with inflammatory response and endoplasmic reticulum stress in elderly persons. Twenty participants received three diets for 4 weeks each: Mediterranean diet + CoQ (Med + CoQ), Mediterranean diet (Med), and saturated fatty acid–rich diet (SFA). After 12-hour fast, volunteers consumed a breakfast with a fat composition similar to that consumed in each of the diets. Med and Med + CoQ diets produced a lower fasting calreticulin, *IL-1b*, and *JNK-1* gene expression; a lower postprandial *p65, IKK-b, MMP-9, IL-1b, JNK-1, sXBP-1, and BiP/Grp78* gene expression; and a higher postprandial *IkB-a* gene expression compared with the SFA diet. Med + CoQ diet produced a lower postprandial decrease *p65 and IKK-b* gene expression compared with the other diets. Our results support the anti-inflammatory effect of Med diet and that exogenous CoQ supplementation in synergy with a Med diet modulates the inflammatory response and endoplasmic reticulum stress.

Key Words: CoQ_{10}—Mediterranean diet—Chronic inflammation—Endoplasmic reticulum stress—Gene expression.

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AGING is a biological process characterized by time-dependent, progressive physiological declines accompanied by the increased incidence of age-related diseases (1). A growing body of evidence points toward the oxidative damage caused by reactive oxygen species (ROS) as one of the primary determinant of aging (2). However, recent scientific studies have advanced the notion of chronic inflammation as other risk factor underlying aging and age-related diseases as neurodegenerative disorders, type 2 diabetes, atherosclerosis, and cardiovascular diseases (3–5). Addressing the central mechanisms underlying these pathologies will have implications for aging and should lead to new therapeutic approaches for treating these conditions (6).

One potential emerging mechanism involves the endoplasmic reticulum (ER), the organelle responsible for protein folding, maturation, quality control, and trafficking. When the ER becomes stressed due to the accumulation of newly synthesized unfolded proteins, the unfolded protein response is activated. A close examination of ER stress and unfolded protein response pathways has demonstrated many links to major inflammatory and stress signaling networks, including the activation of the JNK-AP1 and NF-κB-IKK pathways (7,8), as well as production of ROS and nitric oxide (9,10).

Nutritional intervention may influence the intrinsic rate of aging as well as the incidence of these age-associated diseases. Increasing evidence suggests that the quality of diet may also be important in modulating inflammation (11,12). Thus, consumption of a meal high in carbohydrates and fat results in multiple metabolic changes including oxidative and inflammatory stress and increase in insulin resistance, transient endothelial dysfunction, and platelet activation with impaired homeostasis. However, extensive scientific evidence shows that the Mediterranean diet

(Med diet) prevents the onset and progression of coronary heart disease (13), metabolic disorders, and other aging-related diseases (14,15) and has beneficial effects on selected cancers that are potentially more diet related (16,17). Common components of this diet include monounsaturated fatty acids (MUFA), α-tocopherol, phenolic compounds, phytoesterols, and other antioxidants so the leading hypothesis on the mechanism of this association is a decrease of oxidative stress due to the antioxidant capacity of this diet (16,18).

On the other hand, fasting is not the typical physiological state of the modern human being, which spends most the time in the postprandial state. For all these reasons, it is essential to know what changes are produced during the postprandial phase that is influenced by the quantity and quality of the fat ingested. With regard to the postprandial state, we have recently demonstrated the antioxidant effect of Med diet rich in olive oil and that exogenous coenzyme Q_{10} (CoQ: 2,3-dimethoxy-5-methy-6-decaprenyl-1, 4-benzoquinone) supplementation in synergy with a Med diet (Med + CoQ diet) has an additive effect, improving the postprandial oxidative stress in elderly men and women, with a higher increase in capillary flow, a lower plasma biomarker of oxidative stress levels, and a greater postprandial decrease in plasma antioxidant enzymatic activities with respect to a Western diet rich in saturated fat (SFA diet (19)).

According to these premises, the aim of this study was to determine whether diets with different fat quality influence on the postprandial expression of proinflammatory genes and genes related with ER stress and that this hypothetical improvement could be boosted by supplementation with a natural antioxidant, like CoQ, to a Mediterranean diet in peripheral blood mononuclear cells (PBMCs) of aged persons.

Materials and Methods

Participants and Recruitment

Volunteers were recruited using various methods including the use of general practitioner databases and poster and newspaper advertisements. A total of 63 persons were contacted among those willing to enter the study. All participants underwent a comprehensive medical history, physical examination, and clinical chemistry analysis before enrollment and gave their informed consent before joining the study. Inclusion and exclusion criteria were fulfilled by 20 patients (age ≥ 65 years; 10 men and 10 women). Clinical inclusion criteria were age 65 years or older, body mass index 20–40 kg/m^2, total cholesterol concentration less than or equal to 8.0 mmol/L, and nonsmokers. Clinical exclusion criteria were age less than 65 years, diabetes or other endocrine disorders, chronic inflammatory conditions, kidney or liver dysfunction, iron deficiency anemia (hemoglobin < 12 g/dL men, < 11 g/dL women), prescribed hypolipidemic and anti-inflammatory medication, fatty acid supplements including fish oil, consumers of high doses of antioxidant vitamins (A, C, E, β-carotene), highly trained or endurance athletes or those who participate in more than three periods of intense exercise per week, weight change greater than or equal to 3 kg within the last 3 months, smokers, and alcohol or drug abuse (based on clinical judgment). The study protocol was approved by the Human Investigation Review Committee of the Reina Sofia University Hospital, according to institutional and Good Clinical Practice guidelines.

Study Design

Participants were randomly assigned to receive, in a crossover design, three isocaloric diets for 4-week periods each. Three dietary periods were administered continuously (see Supplementary Figure 1). The three diets were as follows: (1) Mediterranean diet supplemented with coenzyme Q (Med + CoQ diet; 200 mg/day in capsules), containing 15% of energy as protein, 47% of energy as carbohydrate, and 38% of total energy as fat (24% MUFA [provided by virgin olive oil], 10% SFA [saturated fatty acid], and 4% PUFA [polyunsaturated fatty acid]); (2) Mediterranean diet not supplemented with CoQ (Med diet), with the same composition of the first diet, but supplemented by placebo capsules; and (3) Western diet rich in saturated fat (SFA diet), with 15% of energy as protein, 47% of energy as carbohydrate, and 38% of total energy as fat (12% MUFA, 22% SFA, and 4% PUFA).

The cholesterol intake was kept constant (<300 mg/day) during the three periods. Both the CoQ and the placebo capsules were specially produced by the same company (Kaneka Corporation, Osaka, Japan) and were identical in weight and external aspect. Patients taking capsules were unaware whether they were in the Med + CoQ or Med dietary period (see Supplementary Table 1). The composition of the experimental diets was calculated by using the U.S. Department of Agriculture (20) food tables and Spanish food composition tables for local foodstuffs (21).

Before the start of the intervention period, volunteers completed a 3-day weighed food diary and an extensive Food Frequency Questionnaires, which allowed identification of foods to be modified. At the start of the intervention period, each patient was provided with a handbook for the diet to which they had been randomized. Advice was given on foods to choose and those to avoid if eating outside home. They were also instructed to write down in the diary about any menu eaten out of the home and to call the monitoring study nurse reporting such event. At baseline, volunteers were provided with a supply of study foods to last for 2 weeks. They collected additional study foods every fortnight or when required. At these times, a 24-hour recall of the previous day's food intake and a short food-use questionnaire based on the study foods were completed to monitor and motivate volunteers to adhere to the dietary advice. A point system was used to assess the number of food

exchanges achieved in the 24-hour recall, and additional advice was given if either the 24-hour recall or food-use questionnaire showed inadequate intake of food exchange options. Volunteers were asked to complete 3-day weighed food diaries at baseline, Weeks 2, and 4. Weighed food intake over two weekdays and one weekend day was obtained using scales provided by the investigators. Fat foods were administered by dietitians in the intervention study. The dietary analysis software Dietsource version 2.0 was used. (Novartis S.A., Barcelona, Spain).

At the end of the dietary intervention period, the participants were given a fatty breakfast with the same fat composition as consumed in each of the diets. Patients presented at the clinical centers at 8 hours following a 12-hour fast (Time 0), abstained from alcohol intake during the preceding 7 days. After cannulation of a blood vessel, a fasting blood sample was taken before the test meal, which was then ingested within 20 minutes under supervision. The test meal reflected fatty acid composition of each subject after the chronic dietary intervention. Subsequent blood samples were drawn at 2 and 4 hours. Test meals provided an equal amount of fat (0.7 g/kg body weight), cholesterol (5 mg/kg of body weight), and vitamin A (60,000 IU/m^2 body surface area). The test meal provided 65% of energy as fat, 10% as protein, and 25% as carbohydrates. The composition of the breakfasts was as follow: Med with CoQ (400 mg in capsules) breakfast (12% SFA, 43% MUFA, and 10% PUFA), Med with placebo capsules breakfast (12% SFA, 43% MUFA, and 10% PUFA), and SFA-rich breakfast (38% SFA, 21% MUFA, and 6% PUFA).

Biochemical Determinations

Plasma samples.—Venous blood samples were obtained at the end of the each dietary intervention period on fasting state, after a 12-hour fast, before to breakfast ingest, and at 2 and 4 hours after the ingestion of the breakfast. Samples from the fasting and postprandial states were collected in tubes containing 1 g EDTA/L and were stored in containers with ice and kept in the dark. Particular care was taken to avoid exposure to air, light, and ambient temperature. Plasma was separated from whole blood by low-speed centrifugation at 1,500*g* for 15 minutes at 4°C within 1 hour of extraction.

Isolation of PBMCs.—PBMCs were isolated from 20 mL of venous blood in tubes containing 1 mg/mL of EDTA. The blood samples were diluted 1:1 in phosphate-buffered saline, and cells were separated in Ficoll gradient by centrifugation at 800*g* for 25 minutes at 20°C. The cells were collected and washed with cold phosphate-buffered saline two times and finally resuspended in Buffer A. This buffer contained 10 mM HEPES, 15 mM KCl, 2 mM $MgCl_2$, and 1 mM EDTA, and at the time of use, 1 mM phenylmethylsulfonyl fluoride and 1 mM Dithiothreitol were added. The cells thus obtained were stored at −80°C for further analysis.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction Analysis

RNA extraction.—Total RNA from PBMCs was extracted using the Trizol method according to the recommendations of the manufacturer (Tri Reagent; Sigma, St Louis, MO) and quantified in a NanoDrop 1000A Spectrophotometer. RNA integrity was verified on agarose gel electrophoresis and stored at −80°C. Next, because polymerase chain reaction can detect even a single molecule of DNA, RNA samples were digested with DNAse I (AMPD-1 KT; Sigma) before RT-PCR.

Biotrove OpenArray real-time PCR.—Each reaction was performed with 1 μL of a 1:5 (v/v) dilution of the first complementary DNA strand, synthesized from 1 μg of total RNA using the commercial kit iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instruction.

The reaction of real-time polymerase chain reaction was carried out using the platform OpenArray NT Cycler (Applied Biosystems employing Taqman probes). OpenArray subarrays were preloaded by Biotrove with the selected primer pairs. The individual primer pairs (synthesized by Sigma-Aldrich, St Louis, MO) were preloaded into BioTrove OpenArray plates. Each primer pair was spotted in duplicate. The primers that amplify genes of interest were selected from the database TaqMan Gene Expression Assays (Applied Biosystems), https://products.appliedbiosystems.com/ab/en/US/adirect/ab? Cmd = catNavigate2 & catID = 601267 in Assays search tab taking as search criteria: selection and homo sapiens Gene Expression Assays for each of the genes of interest.

Samples were loaded into OpenArray plates with the OpenArray NT Autoloader according to the manufacturer's protocols. Each subarray was loaded with 5.0 μL of master mix consisted of 1× LighCycler FastStart DNA Master SYBR Green Kit (Roche Applied Sciences, Indianapolis, IN), 1× SYBR Green I 80×, 0.5% glycerol, 0.2% Pluronic F-68, 1 mg/mL bovine serum albumin (New England Biolaboratories, Beverly, MA), 1 mM $MgCl_2$, 400 nM FP, 400 nM RP, 8% Formamide, 0.25× Rox, 1× TfR amplicon, and complementary DNA samples. The PCR OpenArray thermal cycling protocol consisted of 95°C for 10 minutes, followed by cycles of 10 seconds at 95°C, 10 seconds at 53°C, and 10 seconds at 72°C. All samples were tested in duplicate. The Biotrove OpenArray NT Cycler System Software (version 1.0.2) uses a proprietary calling algorithm that estimates the quality of each individual C_T value by calculating a C_T confidence value for the amplification reaction.

In our assay, C_T values with C_T confidence values below 700 were regarded as background signals. The remaining

positive amplification reactions were analyzed for amplicon specificity by studying the individual melting curves.

The same program allowed the selection of the most stable housekeeping gene in the samples processed for the relativization of the expression of genes of interest. Following this methodology, we analyzed the relative gene expression of these genes: *p65 (RelA)*, *IkB-α* (inhibitor of kB-subunit α), *IKK-β* (IkB kinase-subunit β), *MMP-9* (metalloproteinase-9), *IL-1β* (interleukin-1β), *JNK-1* (c-Jun N-terminal kinase-1), *sXBP-1* (x-box–binding protein-1), *CRT* (calreticulin), and *BiP/Grp78* (glucose-regulated protein 78 kDa).

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS 17.0 for Windows Inc., Chicago, IL) was used for the statistical comparisons. The Kolmogorov–Smirnov test did not show a significant departure from normality in the distribution of variance values. In order to evaluate data variation, Student's *t* test and an analysis of variance for repeated measures were performed, followed by Bonferroni's correction for multiple comparisons. We studied the statistical effects of the type of fat meal ingested, independent of time (represented by *p*1), the effect of time (represented by *p*2), and the interaction of both factors, indicative of the degree of the postprandial response in each group of participants with each fat meal (represented by *p*3). Differences were considered to be significant when $p < .05$. All data presented in text and tables are expressed as means ± *SE*.

Results

Metabolic Parameters Levels

The baseline characteristics of the 20 participants who completed the three dietary intervention periods showed that males had higher height, waist circumference, triglycerides, and apolipoprotein B than females. We did not find any other differences by gender (see Supplementary Table 2).

Moreover, we previously observed higher fasting plasma CoQ concentration ($p < .001$) after the intake of the Med + CoQ diet compared with the Med and SFA diets. At 2 and 4 hours after consumption of the Med + CoQ diet, we observed a greater postprandial increase in plasma CoQ levels compared with the Med and SFA diets ($p = .018$ and $p = .032$, respectively; see Supplementary Figure 2 (19)).

Diet Intake and Genes Related With Activation of Nuclear Factor Kappa B in PBMCs

The nuclear factor kappa B (NF-κB) transcription factor functions as homo- or heterodimers of the Rel family of proteins, which includes p50, p65, c-Rel, p52, and RelB. The most common combination of subunits is a heterodimer of the p50 and p65 proteins. Activation of NF-κB

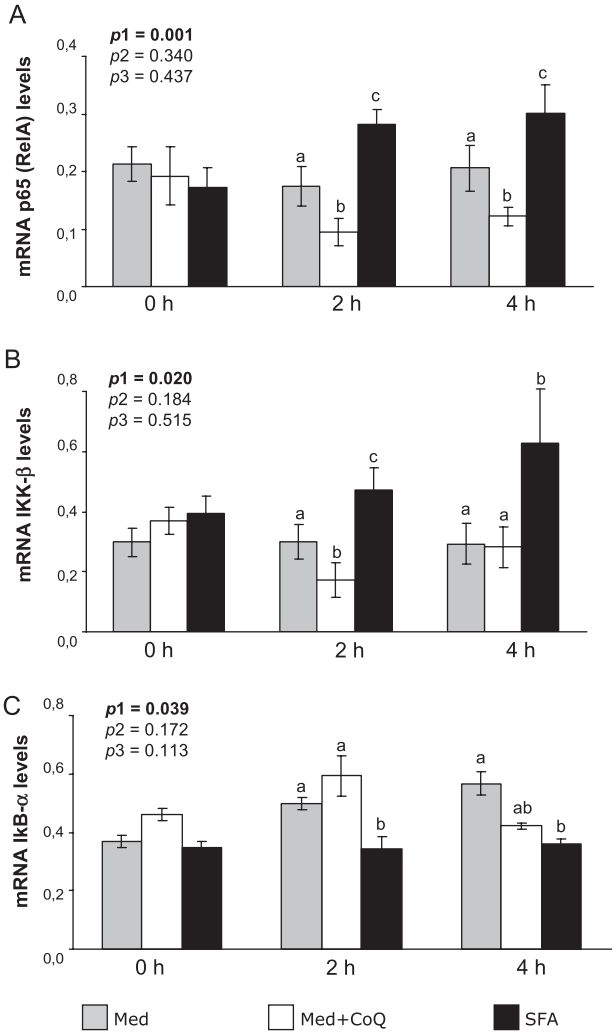

Figure 1. Fasting and postprandial levels of p65 (RelA) mRNA (**A**), IKK-β mRNA (**B**), and IkB-α mRNA (**C**) in peripheral mononuclear cells according to the type of fat consumed. Data were analyzed using analysis of variance for repeated measures. All values represent the mean ± *SE*s. Bars with different superscript letters depict statistically significantly differences ($p < .05$). *p*1: diet effect, *p*2: time effect, and *p*3: Diet × Time interaction.

dimers is the result of IKK-mediated phosphorylation-induced degradation of the IkB, which enables the NF-κB dimers to enter the nucleus and activate specific target gene expression (22).

At 2 and 4 hours after intake of the Med + CoQ diet, we found lower postprandial *p65 (RelA)* messenger RNA (mRNA) levels compared with the other diets ($p = .008$ and $p = .012$, respectively; Figure 1A). Furthermore, *p65 (RelA)* mRNA levels were lower after consumption of the Med diet compared with the SFA diet ($p = .033$).

At 2 hours after intake of the Med + CoQ diet, we observed a greater postprandial decrease in *IKK-β* mRNA levels compared with the other diets ($p = .010$; Figure 1B). Furthermore, *IKK-β* mRNA levels were lower after consumption of the Med diet compared with the SFA diet ($p = .034$). At 4 hours after intake of the Med and

Med + CoQ diets, we observed a greater postprandial decrease in *IKK-β* mRNA levels with respect to the SFA diet ($p = .011$).

At 2 hours after intake of the Med and Med + CoQ diets, we observed a greater postprandial increase in *IkB-α* mRNA levels compared with the SFA diet ($p = .028$). At 4 hours after the Med diet, we found higher postprandial *IkB-α* mRNA levels compared with the SFA diet ($p = .018$; Figure 1C).

No significant differences were detected in *p65 (RelA)*, *IKK-β*, and *IkB-α* mRNA levels in fasting after intake of any of the three diets (Figure 1A, B, and C, respectively).

Diet Intake and Genes Related With the Inflammatory Response in PBMCs

MMP-9 is a metalloproteinase, which is involved in several stages of atherosclerosis through remodeling of the extracellular matrix (23). We observed a decrease in fasting *MMP-9* mRNA levels after intake of the Med diet compared with the SFA diet ($p = .034$; Figure 2A). At 2 and 4 hours after the SFA diet, we found higher postprandial *MMP-9* mRNA levels compared with the Med and Med + CoQ diets ($p = .008$ and $p = .032$, respectively; Figure 2A).

IL-1 is an inflammatory cytokine that consists of two distinct ligands (IL-1α and IL-1β), and both subunits appear to play an essential role in many inflammatory response (24).

Fasting *IL-1β* mRNA levels were lower after participants consumed the Med and Med + CoQ diets than when they consumed the SFA diet ($p = .017$; Figure 2B). At 2 hours after intake of the Med + CoQ diet, we observed a greater postprandial decrease in *IL-1β* mRNA levels compared with the other diets ($p = .011$; Figure 1B). Furthermore, *IL-1β* mRNA levels were lower after consumption of the Med diet compared with the SFA diet ($p = .029$). At 4 hours after intake of the Med and Med + CoQ diets, we observed a greater postprandial decrease in *IL-1β* mRNA levels with respect to the SFA diet ($p = .015$; Figure 2B).

JNK is involved in inflammatory signals, changes in levels of ROS, and a variety of stress stimuli and consist of 10 isoforms derived from three genes: JNK-1 (4 isoforms), JNK-2 (4 isoforms), and JNK-3 (2 isoforms (25)).

Fasting *JNK-1* mRNA levels were lower after participants consumed the Med and Med + CoQ diets than when they consumed the SFA diet ($p = .037$; Figure 2C). At 2 and 4 hours after the SFA diet, we found higher postprandial *JNK-1* mRNA levels compared with the other diets ($p = .009$ and $p = .011$, respectively; Figure 2C).

Diet Intake and Genes Related With ER Stress in PBMCs

The transcription factor sXBP-1 has been identified as a key regulator of the mammalian unfolded protein response or ER stress response, which is activated by environmental stressors such as protein overload that require increased ER capacity (26). CRT, BiP/Grp78, calnexin, and other ER

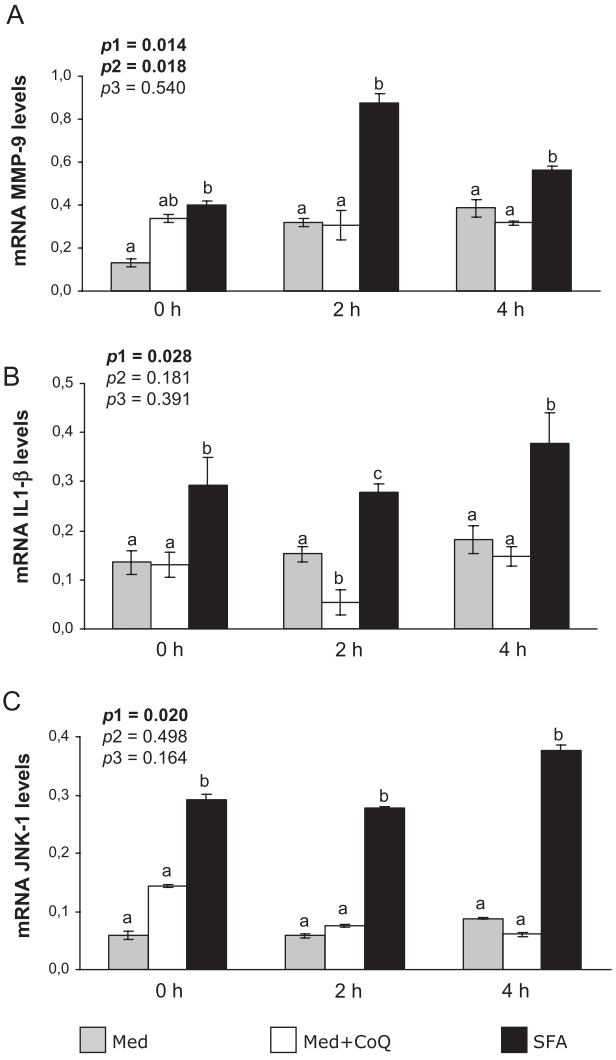

Figure 2. Fasting and postprandial levels of MMP-9 mRNA (**A**), IL1-β mRNA (**B**), and JNK-1 mRNA (**C**) in peripheral mononuclear cells according to the type of fat consumed. Data were analyzed using analysis of variance for repeated measures. All values represent the mean ± *SE*s. Bars with different superscript letters depict statistically significantly differences ($p < .05$). *p*1: diet effect, *p*2: time effect, and *p*3: Diet × Time interaction.

Ca2+ binding chaperons and folding enzymes are important component of protein folding and quality control (27).

At 2 and 4 hours after the Med and Med + CoQ diets, we found lower postprandial *sXBP-1* mRNA levels compared with the SFA diet ($p = .033$ and $p = .008$; Figure 3A). No significant differences were detected in *sXBP-1* mRNA levels in fasting after intake of any of the three diets (Figure 3A). In addition, fasting CRT mRNA levels were lower after participants consumed the Med and Med + CoQ diets than when they consumed the SFA diet ($p = .031$; Figure 3B).

At 2 hours after intake of the SFA diet, we found a greater postprandial increase in *BiP/Grp78* mRNA levels compared with the other diets ($p = .021$). No significant differences were detected in *BiP/Grp78* mRNA levels in fasting and at 4 hours after intake of the three diets (Figure 3C).

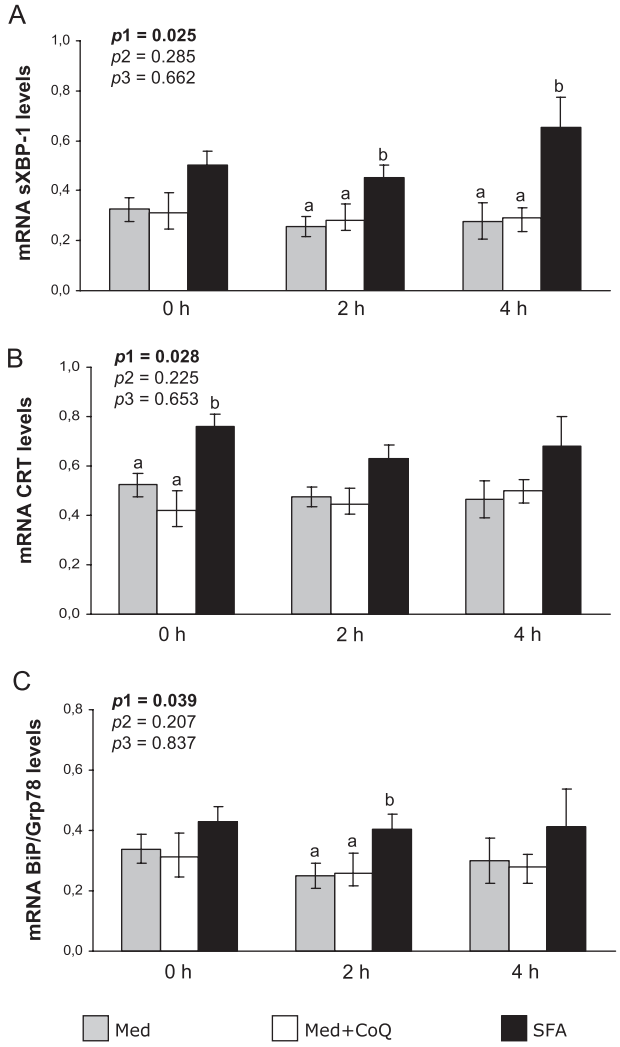

Figure 3. Fasting and postprandial levels of sXBP-1 mRNA (**A**), CRT mRNA (**B**), and BiP/Grp78 mRNA (**C**) in peripheral mononuclear cells according to the type of fat consumed. Data were analyzed using analysis of variance for repeated measures. All values represent the mean ± *SE*s. Bars with different superscript letters depict statistically significantly differences ($p < .05$). $p1$: diet effect, $p2$: time effect, and $p3$: Diet × Time interaction.

Discussion

The present study demonstrates that the consumption of a Med diet reduces the postprandial expression of genes related to both the activation of NF-κB as the inflammatory response such as *p65 (RelA)* and *IKK-β* and *MMP-9, IL1-β* and *JNK-1*, respectively, and increases the expression of *IkB-α* mRNA levels in PBMCs. Additionally, the consumption of a Med diet reduces the expression of genes related with ER stress (*sXBP-1, CRT,* and *BiP/Grp78*). Moreover, the addition of CoQ had an additive effect on the Med diet because the participants who consumed this diet showed a greater postprandial decrease in gene expression of *p65 (RelA)*, *IKK-β*, and *IL1-β* in PBMCs with respect to the other diets.

In the same population of this study, we previously demonstrated that the Med diet improves the postprandial oxidative stress with a higher increase in capillary flow and plasma nitric oxide levels, a lower plasma lipid peroxidation products, nitrotyrosine and protein carbonyl levels, lower plasma antioxidant enzyme activities (GPx, CAT, and SOD (19)), and lower DNA damage in PBMCs (28). Addition of exogenous CoQ in synergy with a Med diet had an additive effect, reducing the postprandial oxidative stress in elderly men and women.

The process of aging has been attributed to cellular free radical damage as well as a decrease in exogenous antioxidants. With the current understanding from human and animal studies, evidence supports that vitamins A and E supplementation may only provide life-span benefits when initiated early in life, and they may accumulate within our body, increasing their risk of toxicity (29–32). In addition, vitamins E and C have in combination shown long-term antiatherogenic effects, but their combined effect on clinical end points has been inconsistent (33).

Thus, interest in CoQ comes from the fact that it has a pivotal role as a redox link between flavoproteins and cytochromes in the mitochondrial respiratory chain (34) where additionally it plays very important antioxidant properties (35). CoQ is the only known bodily-synthesized lipophilic antioxidant, and as an endogenous compound, its toxicity risk may be lesser (36). Not only can reduced CoQ prevent lipid peroxidation chain reaction by itself it can also act by reducing (regenerating) other antioxidants such as α-tocopherol and ascorbate (37,38,39). Previous studies shown that lifelong supplementation of polyunsaturated fatty acid (PUFA) together with dietary CoQ increased life span and may also protect against the deleterious effects of PUFA diet when taken together in rats (29,35). Besides, CoQ can modulate proteins, decrease oxidative stress and cardiovascular risk, as well as control inflammation during aging in rats (40). In healthy humans, plasma oxidative damage may be partially prevented by CoQ supplementation (41), which has been replicated in other populations, like psoriasis (42) or coronary heart disease patients (43). However, whether CoQ added to a Mediterranean has an antioxidant additive effect has not been tested at present.

Accumulating evidence indicates that unresolved, low-grade chronic systemic inflammation plays a significant role in modulating the aging process and age-related diseases, such as metabolic syndrome, atherosclerosis, cancer, and osteoporosis (3–5). In fact, the NF-κB transcription factor can be viewed as the master regulator of the inflammatory process and can be activated by oxidative stimuli. In most cells, NF-κB (p50/p65) is present in an inactive form in the cytoplasm, bound to an inhibitor IkB. Oxidative and other stimuli such as the cytokines (IL-1β, IL-8, and others) result in the phosphorylation of IkB proteins by IKK, ubiquitination, followed by degradation via the 26 S proteasome. This allows active NF-κB to translocate to the nucleus, where it binds to its consensus sequences within the promoter regions of genes, thus activating transcription (44).

Aljada and colleagues (45) have demonstrated that consumption of a hypercaloric breakfast increased the nuclear

NF-κB activity, accompanied by a reduction in the cytoplasmic IkB-α expression in healthy participants. Furthermore, our group has already demonstrated that the Med diet decreases NF-κB activation in PBMCs when compared with butter- and walnut-enriched diets or a typical Western diet in healthy young people (46,47).

Consistently, our results shown a greater postprandial decrease in *p65 (RelA)* and *IKK-β* mRNA levels after intake of the Med + CoQ diet compared with the SFA diet, with an intermediate effect for the Med diet and greater postprandial increase in *IkB-α* mRNA levels after intake of the Med and Med + CoQ diets compared with the SFA diet.

The transcription factor NF-κB has been shown to regulate IL-1β, tumor necrosis factor-alpha, interleukin-6 (IL-6), and COX-2 expression (48,49). Thus, we observed, consistently, a greater increase in *IL-1β* mRNA levels after intake of the SFA diet compared with the Med and Med + CoQ diets.

Another interesting factor is MMP-9, which is involved in several stages of atherosclerosis through remodeling of the extracellular matrix. The expression of *MMP-9* in atherosclerotic plaques coincides with the production of free radicals (50). Moreover, MMP-9 might be a potential mediator of the NADPH oxidase–dependent ROS production in the atherosclerotic process, and previous studies suggested that NADPH oxidase activity increase significantly after consumption of a hypercaloric breakfast (45). In our study, we observed a reduction in *MMP-9* mRNA levels after the Med and Med + CoQ diets compared with the SFA diet, which indicate that this diet may influence not only the rate of atherosclerosis development but also promote the stability of the plaque (23). The mechanism responsible for rises in the levels of *MMP-9* with SFA diet could be the increase in ROS production, inducing the activation of the inflammatory response when this type of fat is consumed.

Activation of stress-kinase signaling has recently been recognized as an important pathophysiological mechanism in the development of diet-induced obesity, type 2 diabetes mellitus, and other aging-related pathologies. Hotamisligil and colleagues (51) revealed that mice deficient for the stress mediator JNK-1 are protected from the development of high-fat diet-induced obesity and glucose intolerance, as well as insulin resistance. The family of JNK kinases can not only be activated by cytokines but also by ER stress and hyperlipidemia (52). In our study, Med and Med + CoQ diets were associated with a lower expression of the *JNK-1* and genes related with ER stress as *sXBP-1, CRT,* and *BiP/Grp78*, which act to increase ROS levels that may elicit inflammatory responses, thereby providing yet another potential link between ER stress and inflammation (6).

The present study has the advantage of a randomized crossover design in which all the participants have experienced the three diet periods, each individual acting as his or her own control and strengthening the fact that the effects observed are due to the influence of the type of diet. We acknowledge that our study has certain limitations because ensuring adherence to dietary instructions is difficult in a feeding trial. However, adherence to the recommended dietary patterns was satisfactory, as can be judged by the measurements of compliance.

In conclusion, our results show that the anti-inflammatory effect of Med diet rich in olive oil and that exogenous CoQ supplementation, in synergy with a Med diet, has an additive effect modulating the inflammatory response and ER stress in elderly men and women and support that the consumption of a Med diet supplemented with CoQ is beneficial for healthy aging of individuals. We can conclude that specific dietary intervention might be a new, interesting, and promising challenge in the treatment (and mainly prevention) of processes that lead to a rise in chronic inflammation and oxidative stress, such as cardiovascular, neurodegenerative diseases, and aging.

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Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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J.L-M. and F.P-J designed research and conducted research; J.D-L., P.P-M., F.P-J., and J.L-M. provided materials or participants; J.D-L., N.D-C., L.G-G., O.R-Z., F.M.G-M., C.C-T., and E.M.Y-S collected and assembled the data; E.M.Y-S., L.G-G., F.M.G-M., and J.M.V. analyzed the data; E.M.Y-S. wrote the paper; J.M.V., F.M.T., J.D-L., and P.P-M provided significant advice and support in reviewing the drafting of the paper; J.L-M. and F.P-J. had primary responsibility for final content. All authors read an approved the final manuscript.

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Effects of the Mediterranean diet supplemented with coenzyme Q10 on metabolomic profiles in elderly men and women

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Keywords:	Coenzyme Q10, Hippurate, Mediterranean diet, Oxidative Stress, Phenylacetylglycine

Effects of the Mediterranean diet supplemented with coenzyme Q_{10} on metabolomic profiles in elderly men and women.

Mediterranean diet and CoQ_{10} in the elderly

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Abstract

Background: Characterization of the variations in the metabolomic profiles of elderly people is a necessary step to understand changes associated with aging. This study assessed whether diets with different fat quality and supplementation with Coenzyme Q_{10} (CoQ) affect the metabolomic profile in urine analyzed by ^{1}H-NMR spectroscopy from elderly people.

Methods: Ten participants received, in a cross-over design, 4 isocaloric diets for 4-week periods each: Mediterranean diet supplemented with CoQ (Med+CoQ diet); Mediterranean diet; Western diet rich in saturated fat (SFA diet); Low-fat, high-carbohydrate diet enriched in n-3 polyunsaturated fat.

Results: Multivariate analysis showed differences between diets when comparing Med+CoQ diet and SFA diet, with greater hippurate urine levels after Med+CoQ diet and higher phenylacetylglycine levels after SFA diet in women. Following consumption of Med+CoQ, hippurate excretion was positively correlated with CoQ and β-carotene plasma levels and inversely related to Nrf2, thioredoxin, superoxide dismutase 1 and $gp91^{phox}$ subunit of NADPH oxidase gene expression. After SFA consumption, phenylacetylglycine excretion was inversely related to CoQ plasma level and positively correlated with isoprostanes urinary level.

Conclusions: The association between hippurate excretion and antioxidant biomarkers along with the relationship between phenylacetylglycine excretion and oxidant biomarkers suggests that the long-term consumption of a Med+CoQ diet could be beneficial for healthy aging and a promising challenge in the prevention of processes related to chronic oxidative stress, such as cardiovascular and neurodegenerative disease.

Keywords: Coenzime $Q_{10,}$ Hippurate, Mediterranean diet, Oxidative stress, Phenylacetylglycine.

Introduction

Prospective studies suggest that a high degree of adherence to a Mediterranean diet is associated with a reduction of the incidence of major cardiovascular events (1), metabolic syndrome (2) and other aging-related diseases (3). Some of the beneficial effects of this dietary pattern are related to its antioxidant capacity, derived from a high consumption of fruits, vegetables and olive oil as the main source of fat (monounsaturared fatty acids (MUFA)) (4). Taking this into account, we have recently demonstrated that consumption of a Mediterranean diet rich in olive oil and supplemented with coenzyme Q_{10} (Med+CoQ diet) (CoQ: 2,3-dimethoxy-5-methy-6-decaprenyl-1,4-benzoquinone) reduces postprandial oxidative stress in elderly men and women with respect to a Western diet rich in saturated fatty acid (SFA diet) (5, 6).

CoQ is part of the mammalian mitochondrial electron transport chain and a potent lipid soluble antioxidant that is obtained through tissue synthesis and diet (7). Its beneficial effect has been reported in large preclinical and clinical studies regarding to cardiovascular disease (8), endothelial function (9) and other conditions linked to mitochondrial dysfunction (10).

It is commonly accepted that aging is a relentless complex process characterized by an increased occurrence of age-related disease (11). One of the main theories of aging is the “free-radical” theory, which postulates that an imbalanced situation between pro-oxidants compounds, such as reactive oxygen species and antioxidant capacity leads to increased oxidative stress and consequently, contribute to the age-related decline of an organism (12). In this regard, antioxidant supplementation interventions have been carried out suggesting that these therapies may ameliorate the antioxidant and inflammatory status (13, 14).

Recently, there has been an increase in interest in the application of metabolomics in human nutritional research. This science focuses on the interaction between products of metabolism in a biological sample and dietary intake of nutrients and non-nutrients, helping us to understand the regulatory roles of nutrition (15).

In this regard, several epidemiological studies have shown that urinary profile is subject to dietary influences (16), age, gender and hormonal status (17).

According to these premises, the aim of this study was to determine whether diets with different fat quality and supplementation with a natural antioxidant such as CoQ affect the metabolomic profile in urine from elderly people.

Methods

Participants and recruitment

A total of 63 persons were contacted among those willing to enter the study. Inclusion and exclusion criteria were fulfilled by 10 patients (age ≥65 years; 5 men and 5 women). The main cause was existence of several diseases. Complete inclusion and exclusion criteria were described in previous publications (6, 13). The study protocol was approved by the Human Investigation Review Committee of the Reina Sofia University Hospital according to institutional and Good Clinical Practice guidelines.

Study design

Participants were randomly assigned to receive, in a crossover design, four isocaloric diets for 4-week periods each (see Supplementary Figure 1). The four diets were as follows: (1) Mediterranean diet supplemented with CoQ (Med+CoQ diet; 200 mg/day in capsules), containing 15% of energy as protein, 47% of energy as carbohydrate and 38% of total energy as fat [24% MUFA (provided by virgin olive oil), 10% SFA, 4% polyunsaturated fatty acid (PUFA)], (2) Mediterranean diet not supplemented with CoQ (Med diet), with the same composition of the first diet but supplemented by placebo capsules, (3) Western diet rich in saturated fat (SFA diet) with 15% of energy as protein, 47% of energy as carbohydrate, and 38% of total energy as fat (12% MUFA, 22% SFA, 4% PUFA) and (4) Low-fat, high-carbohydrate diet enriched in n-3 PUFA (CHO-PUFA diet), with 15% of energy as protein, 55% as carbohydrate and 30% as fat (10% SFA, 12% MUFA and 8% PUFA with 2% a-linolenic acid) (see Supplementary Table 1).

The cholesterol intake was kept constant (<300 mg/ day) during the four periods. Both the CoQ and the placebo capsules were specially produced by the same company

(Kaneka Corporation, Osaka, Japan) and were identical in weight and external aspect. Patients taking capsules were unaware whether they were in the Med+CoQ or Med dietary period. The composition of the experimental diets was calculated by using the US Department of Agriculture (18) food tables and Spanish food composition tables for local foodstuffs (19). Before the start of the intervention period, volunteers completed a 3-day weighed food diary and an extensive Food Frequency Questionnaires, which allowed identification of foods to be modified. At the start of the intervention period, each patient was provided with a handbook for the diet to which they had been randomized. Advice was given on foods to choose and those to avoid if eating outside home. They were also instructed to write down in the diary about any menu eaten out of home and to call the monitoring study nurse reporting such event. At baseline, volunteers were provided with a supply of study foods to last for 2 weeks. They collected additional study foods every fortnight or when required. At these times, a 24-h recall of the previous day's food intake and a short food use questionnaire based on the study foods were completed to monitor and motivate volunteers to adhere to the dietary advice. A points system was used to assess the number of food exchanges achieved in the 24-h recall and additional advice was given if either the 24-h recall or food use questionnaire showed inadequate intake of food exchange options. Volunteers were asked to complete 3-day weighed food diaries at baseline, weeks 2 and 4.Weighed food intake over two weekdays and one weekend day was obtained using scales provided by the investigators. Fat foods were administered by dietitians in the intervention study. The dietary analysis software Dietsource version 2.0 was used (Novartis S.A., Barcelona, Spain).

Biofluid collection

First void urine samples were collected from participants at baseline (pre-intervention) and after a 12 h fast (time 0, post-intervention) and centrifuged at 1800g for 10 min at 4ºC. Aliquots (500 µL) of the urine were stored at -80ºC until analysis by Nuclear Magnetic Resonance (NMR) spectroscopy.

NMR spectroscopy

Urine samples were prepared by the addition of 250 µL phosphate buffer (0.2 M KH_2PO_4, 0.8 M K_2HPO_4) to 500 µL urine. Following centrifugation at 7155 g for 5 minutes, 50 µL D_2O and 10 µL TSP were added to 540 µL of the supernatant. Spectra were acquired on a 600 MHz NMR spectrometer (Varian, Palo Alto, CA 94304-1039, United States) using a Noesypresat pulse sequence at 25ºC. Spectra were acquired with 16 K data points and 128 scans over a spectra width of 8 kHz. Water suppression was achieved during the relaxation delay (2.5 s) and the mixing time (100 ms). All ^{1}H NMR urine spectra were referenced to TSP at 0.0 ppm, line broadened (0.3 Hz) and phase and baseline corrected using the processor on Chenomx NMR suite 5.1 (Chenomx Inc, Edmonton, Alberta, T5K 2J1). The spectra were integrated into spectral regions of 0.01 ppm. The water region (4 – 6 ppm) was excluded and the data was normalised to the sum of the spectral integral.

Plasma determinations and gene expression

Methodology for obtaining CoQ and β-carotene plasma levels, isoprostanes urinary levels and gene expression of transcription factor Nrf2 (Nrf2), thioredoxin (Trx), superoxide dismutase 1 (SOD1) and gp91^{phox} subunit of NADPH oxidase (gp91^{phox})

used for the correlations and gender comparison has been previously described and published (5, 6, 20).

Statistical analysis

The NMR spectral data were processed using SIMCA-P+ (version 12.0.1; Umetrics, Umea; Sweden), mean centered and pareto scaled ($1/\sqrt{SD}$). Principal component analysis (PCA) was applied to the urine dataset and the score plots were visually inspected for patterns and outliers. Differences between baseline and diet intervention were further analyzed by using partial least squares discriminant analysis (PLS-DA) which is a supervised technique that uses previous knowledge about the samples. The quality of the models formed by PCA and PLS-DA was assessed by interrogation of the R^2 and Q^2 parameters. The R^2 parameter is a representation of how much of the variation within the data set is explained by the components of the model, and the Q^2 parameter gives an indication of the prediction power of the model. Additionally, permutation testing was performed on each PLS-DA model (with 20 permutations) to assess the validity of the model. Permutation testing was performed in SIMCA-P+. Where robust PLS-DA models were built, the variable importance in the projection (VIP) and loadings plot were used to identify the regions of the spectra that had the largest discriminatory effect in the model. Variables with a VIP value >2 were considered important in discriminating between groups. Metabolites responsible for the peaks in these regions were assigned using in-house databases and the Chenomx Database. Assignments were confirmed using 2D TOCSY experiments.

The Statistical Package for the Social Sciences (SPSS 17.0 for Windows Inc., Chicago, IL, USA) was used for the statistical comparisons. The Kolmogorov–Smirnov test did not show a significant departure from normality in the distribution of variance values.

Hippurate, phenylacetylglycine, glycerol results and gender comparison were analyzed using Student's t test in order to evaluate data variation after dietary intervention. A study of the relationship among parameters was also carried out using Pearson's linear correlation coefficient. Differences were considered to be significant when $p<0.05$.

Results

Metabolomic analysis of urine samples

Urine samples, corresponding to baseline and 12 h fast after each dietary intervention, were collected from 10 participants (5 women and 5 men) aged ≥ 65. PCA of ^{1}H-NMR urine spectra resulted in a two component model with a R^2 of 0.31. Figure 1A shows the PCA score plot for the dataset. Visual inspection of the model showed grouping of samples according to gender. Pair wise comparison of samples using PCA according to diet did not reveal any pattern with all the participants and PLS models were not built. Following this, PCA analysis for women and men were conducted separately. Analysis for women displayed differences between diet interventions when comparing fasting state after Med+CoQ diet against SFA diet. PLS-DA analysis of these groups revealed one component model with a R^2X of 0.21, a R^2Y of 0.66 and a Q^2 of 0.17 (Figure 1B). Validation of the model was performed using permutation testing and resulted in a Q^2 intercept of (0.0, -0.1). No significant differences were found comparing the other diets. Analysis of the VIP plots revealed greater levels of hippurate excretion after consumption of Med+CoQ diet compared to SFA diet. Lower excretion in phenylacetylglycine levels was observed after Med+CoQ diet compared to SFA diet intervention (Table 1). However, visual inspection of PCA score plots for men revealed no patterns based on diets.

Correlation analysis between metabolites and other parameters

After consumption of Med+CoQ diet, we observed positive strong correlations between hippurate and CoQ and β-carotene plasma levels (Table 2). Nevertheless, we found a high inverse correlation between urinary levels of hippurate and Nrf2 gene expression and antioxidant enzymes gene expression as Thioredoxin (Trx), superoxide dismutase 1

(SOD1) and gp91^{phox} subunit of NADPH oxidase (Table 2). Similarly, after consumption of SFA diet, we observed a strong negative correlation between urinary levels of phenylacetylglycine and CoQ plasma levels and a great positive correlation between urinary levels of phenylacetylglycine and urinary isoprostanes (Table 2). Data from these parameters used for the correlations were previously analyzed and published (5, 6).

Gender comparison of oxidative stress biomarkers

Gender-related differences after Med+CoQ diet intervention are shown in Figure 2. We observed that levels of the antioxidants parameters CoQ and β-carotene are significantly greater in women compared to men. On the contrary, we found that oxidative stress biomarkers such as oxidized low-density lipoprotein (oxLDL) or lipid peroxides (LPO) after Med+CoQ are higher in men than in women (Figure 3). Data from these parameters used for the correlations were previously analyzed and published (5, 6).

Discussion

The process of aging has been attributed partly to cellular free radical damage as well as to decreased exogenous antioxidants. Thus, the present study demonstrates that the consumption of a Med diet supplemented with CoQ is associated with increased levels of excreted hippurate and decreased levels of phenylacetylglycine compared to a SFA-rich diet in postmenopausal women (≥ 65 years). We also observed that hippurate urine levels are strongly correlated with plasma antioxidant biomarkers and urine phenylacetylglycine levels are highly related to urinary oxidant biomarkers.

In the same population, we have previously demonstrated that Med+CoQ diet improves the postprandial OxS with a higher increase in capillary flow and plasma nitric oxide levels and lower plasma lipid peroxidation products, nitrotyrosine and protein carbonyl levels, and lower plasma antioxidant enzyme activities (5). Moreover, we have proved that this diet exerts a greater postprandial decrease in gene expression of genes related to oxidative stress (Nrf2, GPx1, SOD) (6), inflammatory response (p65, IKK- β, IL-1β) and endoplasmic reticulum stress (sXBP-1, CRT, Bip) (21) in peripheral blood mononuclear cells with respect to a SFA diet in elderly people.

Hippuric acid (N-benzoylglycine) is known to be catabolically synthesized by conjugation of benzoic acid with glycine from its precursor quinic acid, via the shikimate pathway in the gastrointestinal tract (22). This metabolite is considered as one of the final products of gut microbial metabolism in urine after intake of dietary polyphenols, in fact, several intervention studies have indicated that consumption of polyphenol-rich extracts (23) or tea (24) enhances its excretion. In this connection, it has been proposed that hippurate is not directly beneficial by itself but it could be considered as an indicator of antioxidant molecules synthesis by gut microflora (25), DNA repair enhancement and NF-kB inhibition (26). In accordance with these studies,

our strong correlation results suggest that hippurate is positively related to antioxidant compounds such as CoQ and β-carotene, and negatively associated with the gene expression of Nrf2 and several molecules related to OxS processes (Trx, SOD1, gp91^{phox}).

Little is known about the excretion of phenylacetylglycine in humans following dietary intervention. Herein, we show that phenylacetylglycine levels are inversely associated with CoQ levels and in contrast, highly positive correlated to urinary levels of isoprostanes, which are considered as biomarkers for monitoring oxidative status (27). Phenylacetylglycine is generated from phenylacetate via phase II detoxification mechanisms or from microbiota metabolism (28) and is often overexpressed in disease. It is one of the major metabolites excreted in spontaneous hypertensive rats (28) and it may act as a putative biomarker of phospholipidosis in rat models, a disorder characterized by the excess accumulation of phospholipids in tissues (29). In humans, urinary acylglycines are often used as diagnostic tool for mitochondrial fatty-acid oxidation disorders (30) and phenylacetylglycine levels are significantly higher in heart failure patients than in healthy controls (31, 32). Due to the important role of OxS in cardiovascular disease (33) and considering these studies, greater phenylacetylglycine excretion could be linked to an increase of OxS-related process. In fact, our correlation results show a great association between this metabolite and isoprostanes (urinary oxidative stress biomarker).

Our present data suggest that the benefit caused by Med+CoQ consumption is greater in elderly women compared to men. In this regard, the antioxidant compounds CoQ and β-carotene are higher in women than men, while lipid peroxides and oxidized low-density lipoprotein plasma levels in men are greater than women. This gender difference is interesting and should be retested in other studies since these women are

postmenopausal and therefore, they do not have the additional antioxidant benefits attributed to estrogen levels. Moreover, hippurate and phenylacetylglycine are microbial metabolites and differences in its levels could be due to gut microbial diversity between men and women. On the other hand, the fact that we only found differences when comparing Med+CoQ and SFA diet, may indicate that the antioxidant compounds present in Mediterranean diet, together with CoQ may produce a synergic antioxidant effect (the most antioxidant diet) when is compared to SFA diet (the most oxidant diet). The present study has the advantage of a randomized crossover design in which all the participants have experienced the four diet periods, each individual acting as his/her own control and strengthening the fact that the effects observed are due to the influence of the type of diet. Multiple studies have shown that a three week dietary period is enough for assessing its effects, and in longer periods, there is not influence of previous diets (34, 35). In our study, and to avoid any doubt, we extended the dietary periods to four weeks each, thus securing that the effects found were not provided by previous diets.

We are aware that the main limitations of the study are the low number of individuals and the subtle results obtained since changes in metabolomic profiles resulting from dietary intervention are not easy to detect because of the strong intra-individual variation (36).

An adequate nutritional status may be central to improve the health of elderly people. Therefore, our results show that the consumption of a Med diet supplemented with exogenous CoQ produces the excretion of urinary metabolites associated with an improvement of oxidative stress, while end-products excreted after the intake of a SFA diet are related to increased oxidative stress process in elderly people. Therefore, the consumption of a Med+CoQ diet could be beneficial for healthy aging of individuals

and specific dietary intervention might be a promising challenge in the prevention and treatment of processes that lead to a rise in chronic oxidative stress, such as cardiovascular, neurodegenerative diseases, and aging.

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Tables

Table 1. Metabolites identified as being discriminant between Med+CoQ and SFA diet.

Metabolite	Chemical shift (ppm)	VIP values	Med+CoQ	SFA	p-value
Hippurate	3.975	6.380	0.022 ± 0.006	0.014 ± 0.004	0.037
Hippurate	3.965	6.176	0.021 ± 0.006	0.013 ± 0.004	0.038
Hippurate	7.835	5.241	0.016 ± 0.005	0.009 ± 0.003	0.041
Hippurate	7.545	3.542	0.006 ± 0.002	0.004 ± 0.001	0.049
Phenylacetylglycine	7.435	2.963	0.003 ± 0.002	0.005 ± 0.002	0.045
Phenylacetylglycine	7.375	2.380	0.003 ± 0.001	0.005 ± 0.001	0.045
Un-assigned peak	3.725	2.413	0.007 ± 0.001	0.008 ± 0.001	0.084
Un-assigned peak	2.175	2.204	0.002 ± 0.001	0.003 ± 0.002	0.167
Un-assigned peak	3.635	2.134	0.006 ± 0.001	0.007 ± 0.002	0.160

Average intensities of metabolite bin regions fot the Med+CoQ diet and SFA diet are shown.

Values are means ± SD and are presented as arbitrary units. p-values are reported for paired t-test.

Table 2. Correlations between urinary levels of hippurate, phenylacetylglycine and other factors[a] after Med+CoQ diet intervention.

		CoQ	β-carotene	Nrf2	Trx	SOD1	gp91^{phox}
Hippurate	Pearson correlation	0.899*	0.928*	-0.978**	-0.976**	-0.913*	-0.898*
	p-value	0.038	0.023	0.004	0.004	0.030	0.039
		CoQ	Isoprostanes				
Phenylacetylglycine	Pearson correlation	-0.897*	0.952*				
	p-value	0.039	0.013				

[a]Pearson´s correlation: *p < 0.05; **p< 0.01. CoQ, coenzyme Q_{10} plasma levels; β-carotene, plasma levels; Nrf2, transcription factor Nrf2 gene expression; Trx, Thioredoxin gene expression; SOD1, superoxide dismutase 1 gene expression; gp91^{phox}, subunit of NADPH oxidase; Isoprostanes, urinary levels.

Figure captions

Figure 1. (A) PCA score plot (R^2 = 0.31, Q^2 = 0.17) derived from ^{1}H-NMR urine spectra collected pre- and post- dietary intervention. Open squares represent male samples and closed circles represent female samples. (B) PLS-DA score plot derived from ^{1}H-NMR urine spectra collected from women after Med+CoQ diet (open circles) and after SFA diet intervention (close squares). $R^2 = 0.21$, $Q^2 = 0.17$

Figure 2. Gender-related differences after Med+CoQ diet intervention of coenzyme Q_{10} (A) and β-carotene (B). Differences in the parameter levels between pre- and post-intervention are shown as Δ (value of post-intervention minus value of pre-intervention). Data were analyzed using unpaired t-test and differences were considered to be significant when $*p < 0.05$

Figure 3. Gender-related differences in fasting plasma levels after Med+CoQ diet intervention of oxidized low-density lipoprotein, oxLDL (A) and lipid peroxides, LPO (B). Data were analyzed using unpaired t-test and differences were considered to be significant when $*p < 0.05$

Figure 1

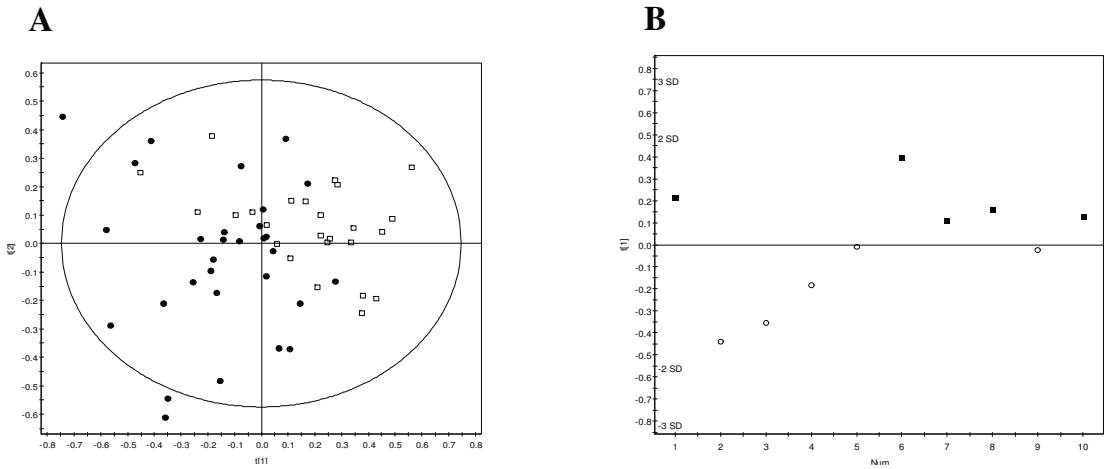


Figure 2

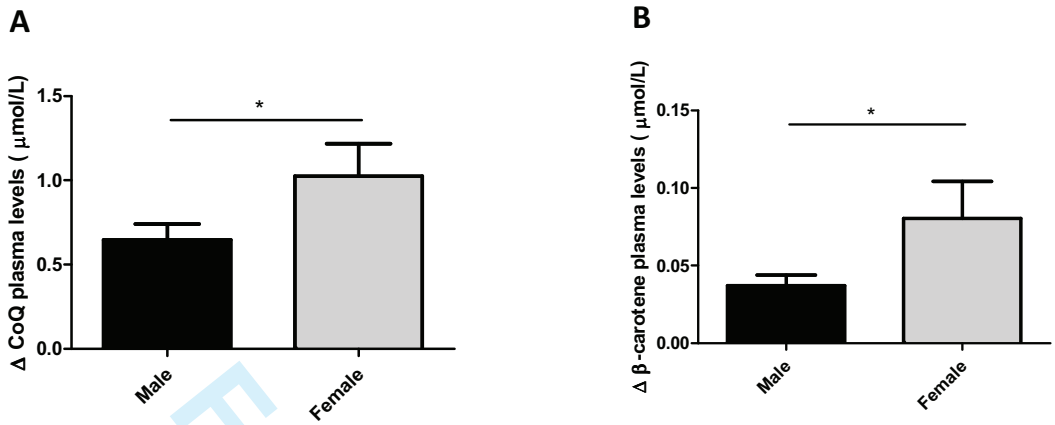
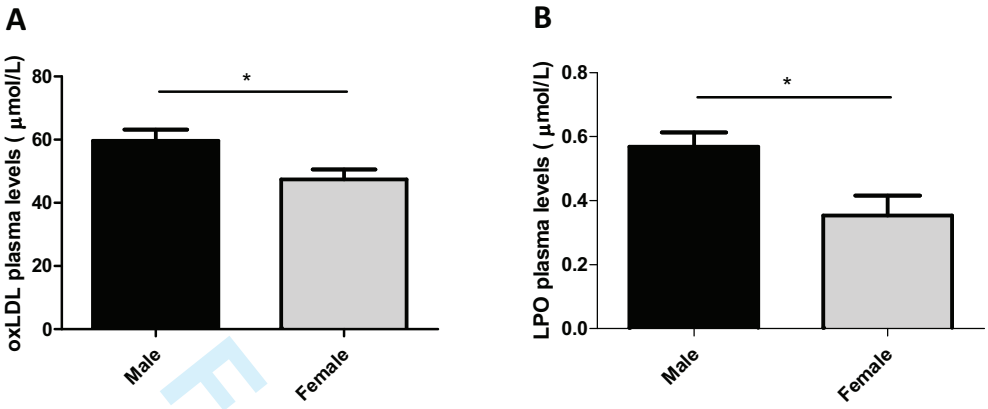
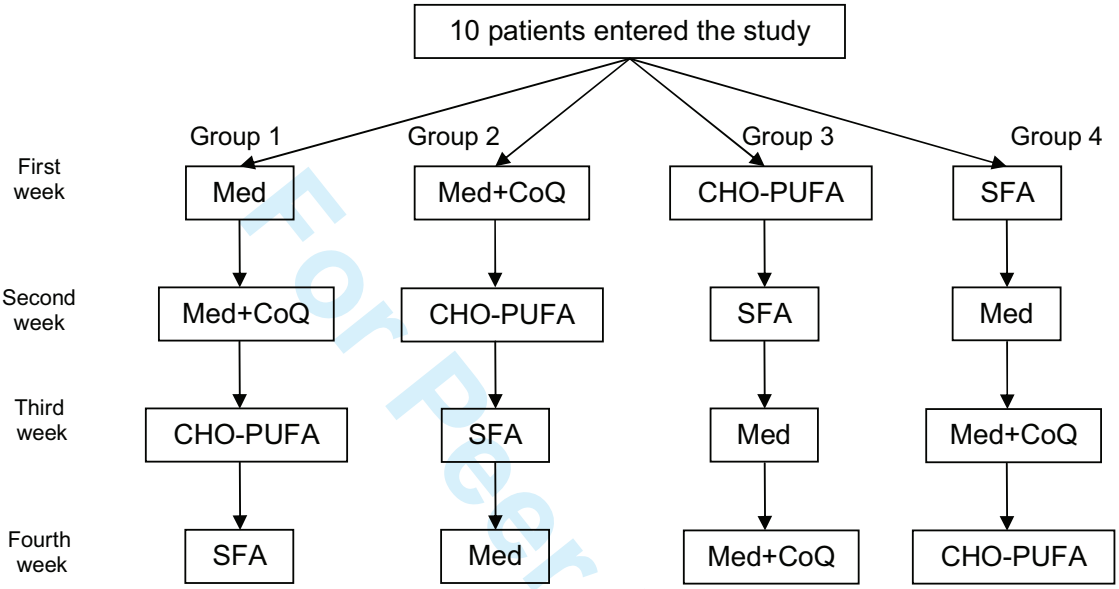


Figure 3





Supplementary Figure 1. Flow chart of subjects who participated in each 4-week feeding trial.

Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, Saturated Fatty Acid-rich diet; CHO-PUFA, Low-fat high carbohydrate diet enriched in n-3 polyunsaturated fatty acid.

Supplementary Table 1. Composition of diet at end of intervention period, alongside dietary targets[1]

	Diet			
	Med	Med+CoQ	CHO-PUFA	SFA
Target (MJ/d)				
%E from fat	38	38	28	38
%E from SFA	<10	<10	<10	22
%E from MUFA	24	24	12	12
%E from PUFA	4	4	8	4
End of intervention				
N	10	10	10	10
Energy (MJ/d)	8.22 ± 0.35	8.16 ± 0.32	8.31 ± 0.32	8.21 ± 0.31
%E from fat	39.11 ± 2.02	39.73 ± 2.65	28.63 ± 2.65	40.38 ± 2.03
%E from SFA	8.78 ± 0.61[a]	9.04 ± 0.71[a]	8.64 ± 0.71[a]	20.75 ± 0.87[b]
%E from MUFA	24.24 ± 0.92[a]	24.36 ± 1.47[a]	10.96 ± 1.47[b]	13.46 ± 0.71[b]
%E from PUFA	4.15 ± 0.23[a]	4.28 ± 0.29[a]	7.61 ± 0.29[b]	3.92 ± 0.25[a]
%E from CHO	44.64 ± 1.86	44.31 ± 2.29	53.71 ± 2.29	43.64 ± 1.73
%E from protein	16.33 ± 0.52	16.07 ± 0.64	17.77 ± 0.64	16.66 ± 0.53
Total α-tocopherol (mg/d)	18.91 ± 0.73[a]	18.95 ± 0.84[a]	5.25 ± 0.84[b]	6.97 ± 0.76[b]
Ascorbic acid (mg/d)	187.54 ± 5.83	181.17 ± 6.12	189.28 ± 6.12	195.62 ± 6.86
β-carotene (mg/d)	3.11 ± 0.92	3.13 ± 0.66	3.13 ± 0.66	3.21 ± 0.23
Total fibre (g/d)	27.93 ± 2.22	27.17 ± 2.38	30.27 ± 2.38	27.42 ± 2.34
Cholesterol (mg/d)	345.71 ± 35.93	345.86 ± 36.92	345.77 ± 36.92	378.18 ± 59.29

[1]Values are means ± SE $n = 10$. Means in a row with superscripts without a common letter differ, $p < 0.05$. Med diet, Mediterranean diet; Med+CoQ diet, Mediterranean diet supplemented with CoQ; SFA diet, SFA-rich diet; CHO-PUFA diet, low-fat high-carbohydrate diet enriched in n-3 PUFA; CHO, carbohydrate; SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid

AGE

Influence of endothelial dysfunction on telomere length in subjects with metabolic syndrome: LIPGENE study

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Abstract:	Previous evidences support that increased oxidative stress (OxS) may play an important role in metabolic syndrome (MetS) and both are closely linked to vascular dysfunction. This study determined whether there is a direct relationship between endothelial function and relative telomere length (RTL) in MetS subjects. In this cross-sectional study from the LIPGENE cohort, a total of 88 subjects (36 men and 52 women) were divided into four groups by quartiles of telomere length. We measured ischemic reactive hyperemia (IRH), total nitrite (NO) and protein carbonyl (PC) plasma levels, superoxide dismutase (SOD) and glutathione peroxidase (GPx) plasma activities. IRH and NO plasma levels were higher in subjects with longer RTL (quartile 3 and 4), while PC plasma levels and GPx and SOD plasma activities were lower in quartile 4 subjects (longest RTL). Additionally, MetS subjects with longer RTL had greater homeostatic model assessment-β (HOMAβ) level and lower triglycerides (TG) plasma levels. Our results suggest that endothelial dysfunction, associated by high levels of OxS, could be entailed in an increment of telomere attrition. Thus, further

	support of the molecular and cellular mechanisms involved in vascular dysfunction may contribute to the development of strategies to decelerate vascular aging or prevent cardiovascular disease.

Influence of endothelial dysfunction on telomere length in subjects with metabolic syndrome: LIPGENE study

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Abstract

Previous evidences support that increased oxidative stress (OxS) may play an important role in metabolic syndrome (MetS) and both are closely linked to vascular dysfunction. This study determined whether there is a direct relationship between endothelial function and relative telomere length (RTL) in MetS subjects. In this cross-sectional study from the LIPGENE cohort, a total of 88 subjects (36 men and 52 women) were divided into four groups by quartiles of telomere length. We measured ischemic reactive hyperemia (IRH), total nitrite (NO) and protein carbonyl (PC) plasma levels, superoxide dismutase (SOD) and glutathione peroxidase (GPx) plasma activities. IRH and NO plasma levels were higher in subjects with longer RTL (quartile 3 and 4), while PC plasma levels and GPx and SOD plasma activities were lower in quartile 4 subjects (longest RTL). Additionally, MetS subjects with longer RTL had greater homeostatic model assessment-β (HOMAβ) level and lower triglycerides (TG) plasma levels. Our results suggest that endothelial dysfunction, associated by high levels of OxS, could be entailed in an increment of telomere attrition. Thus, further support of the molecular and cellular mechanisms involved in vascular dysfunction may contribute to the development of strategies to decelerate vascular aging or prevent cardiovascular disease.

Keywords: endothelial dysfunction; LIPGENE study; metabolic syndrome; oxidative stress; telomeres

Introduction

According to the criteria defined by the Third Report of the National Cholesterol Education Program Adult Treatment Panel III (National Cholesterol Education Program (NCEP) Expert Panel on Detection E 2002), metabolic syndrome (MetS) refers to the clustering of three or more characteristics such as hyperglycemia, hypertriglyceridemia, low level of high-density lipoprotein cholesterol (HDL-C), hypertension and abdominal obesity (Duvnjak and Duvnjak 2009). One of the mechanisms underlying this pathology is oxidative stress (OxS) (Pena-Orihuela, Camargo et al. 2013). In fact, it has been recently published that the degree of OxS is influenced by the number of components of the metabolic syndrome (Yubero-Serrano, Delgado-Lista et al. 2013).

OxS is often defined as the imbalance between reactive oxygen species (ROS) and the antioxidant capacity of an organism (Jones 2006). In this regard, antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) play a substantial role in the first line of defense for the detoxification of products resulting from OxS (Ray and Husain 2002; Johnson and Giulivi 2005). Also, protein carbonyl (PC) is considered as a biomarker of severe OxS, characterized by an irreversible damage to the protein structure and function (Hopps and Caimi 2013).

It is established that an increase in OxS levels, coupled with an impairment in nitric oxide (NO) availability, may cause alteration of endothelial cells causing endothelial dysfunction (Ferroni, Basili et al. 2006). These findings would be reflected in decreased ischemic reactive hyperemia (IRH), which is a method for measuring changes in acute endothelial reactivity (Fernandez, Da Silva-Grigoletto et al. 2010).

Telomeres are DNA-protein complexes that cap and preserve the integrity of the chromosomal ends. They consist of several thousands of repetitive sequences of TTAGGG that are naturally shortened with each cell division due to the end replication problem

(Nakashima, Ozono et al. 2004; Saliques, Zeller et al. 2010). Progressive attrition of the telomere leads to a critical length that triggers the cell arrest phenomenon known as senescence (Khan, Chuturgoon et al. 2012), and due to the universal fact that telomere shorten with age, its study is crucial for understanding mechanisms of age-related diseases (Armanios 2013). Notably, it has been reported that telomere attrition and oxidative DNA damage in endothelial progenitor cells is higher in coronary artery disease patients with MetS than in those without MetS (Satoh, Ishikawa et al. 2008). In addition, endothelial damage and atherosclerosis have been shown to be associated with telomere shortening in white blood cells more tightly than chronological aging (Nakashima, Ozono et al. 2004). Nonetheless, there is still a lack of data in this field. It has not been described yet an explicit correlation between endothelial function (measured in terms of IRH) and telomere length in MetS subjects.

Taking this into account, the purpose of this study was to determine whether there is a direct relationship between endothelial function, through high OxS, and relative telomere length (RTL) in a cross-sectional study of MetS subjects of four groups divided by quartiles of telomere length.

Materials and Methods

Subjects and Design

This study was conducted within the framework of the LIPGENE study ('Diet, genomics and the metabolic syndrome: an integrated nutrition, agro-food, social and economic analysis'), a Framework VI Integrated Project funded by the European Union (Tierney, McMonagle et al. 2011)

Subject eligibility was determined using a modified version of the NCEP criteria for MetS, according to published criteria (2001).

Subjects were required to meet at least two of the following five criteria: waist circumference >102 cm (men) or >88 cm (women); fasting glucose 5.5–7.0 mmol/l; triglycerides ≥1.5 mmol/l; high-density lipoprotein cholesterol (HDL-C) <1.0 mmol/l (men) or <1.3 mmol/l (women); blood pressure (BP) ≥130/85 mmHg or treatment of previously diagnosed hypertension. We used a subgroup of pre-intervention data for 88 subjects (36 men and 52 women), which conformed to the LIPGENE inclusion and exclusion criteria ("Online Resource 1") (Perez-Martinez, Moreno-Conde et al. 2010).

All participants provided written informed consent and underwent a comprehensive medical history, physical examination and clinical chemistry analysis before enrollment. Participants displayed no signs of cardiac dysfunction or hepatic, renal and thyroid diseases and were requested to maintain their regular physical activity and lifestyle. Participants were also asked to record in a diary any event that could affect the outcome of the study, such as stress, changes in smoking habits and alcohol consumption or intake of foods not included in the experimental design. None of the participants showed evidence of high alcohol consumption or a family history of early-onset cardiovascular disease, nor were any active smokers. All patients were free from cardiovascular complications at the time of the enrollment. The study was carried out in the Lipid and Atherosclerosis Unit at the Reina Sofia University Hospital,

from February 2005 to April 2006. The experimental protocol was approved by the local ethics committee according to the Helsinki Declaration. The study was registered with the US National Library of Medicine Clinical Trials registry (NCT00429195).

Anthropometric measurements

After recording clinical histories and conducting physical examinations, we obtained the following anthropometric measurements for each individual: weight, height, body mass index and waist circumference. Weight was measured while the subject was wearing light indoor clothing, without shoes and after voiding. Height was obtained with a stadiometer graduated in millimeters. The subject was barefoot with the back and head in contact with the stadiometer in the Frankfurt horizontal plane. Body mass index was calculated by dividing weight (kg) by height squared (m2). Waist circumference (cm) was measured to the nearest 0.5cm with a tape measure at the umbilical scar level. A non-stretchable tape measure was used to measure waist circumference. The measurement was taken directly on the skin with the subject in a standing position with the abdomen relaxed, the arms at the sides and the feet together. We used the homeostatic model assessment index for insulin resistance ($HOMA_{IR}$: fasting insulin (mUl^{-1})/fasting glucose (mmol l^{-1})/22.5) and HOMAβ-cell function as the index of insulin secretory function derived from fasting plasma glucose and insulin concentrations, calculated as 20 x fasting insulin (mUl^{-1})/fasting glucose (mmol l^{-1})-3.5 (Matthews, Hosker et al. 1985). Insulin sensitivity was estimated by a quantitative insulin sensitivity check index (QUICKI) (1/[log insulin (mU l^{-1}) + log baseline glucose (mg dl^{-1})]) (Conwell, Trost et al. 2004). BP was measured using an automatic BP device. In accordance with the European Society of Hypertension Guidelines (Guidelines and Committee 2003), BP measurement was obtained with an appropriately sized cuff positioned at the heart level and

after the patient had been relaxed for at least 5min. The same arm was used for each measurement, and the average of two measurements was used for data processing.

Biochemical determinations

Plasma samples

Blood was collected in tubes containing ethylene diaminetetraacetic acid (EDTA) to yield a final concentration of 0.1% EDTA. Plasma was separated from red cells by centrifugation at 1500 x *g* for 15 min at 4 °C within 1 h of extraction. Plasma was immediately aliquoted and stored at -80 ºC until analysis.

Biochemical analysis

The lipid variables were analyzed with a modular autoanalyzer (DDPPII Hitachi; Roche, Basel, Switzerland) with the use of Boehringer-Mannheim reagents. Triglycerides (TG) in plasma was assayed by means of enzymatic procedures (Bucolo and David 1973). HDL-C was measured by analyzing the supernatant obtained following precipitation of a plasma aliquot in dextran sulfate-Mg^{2+}, as described by Warnick et al. (Warnick, Benderson et al. 1982). Plasma glucose concentrations were measured with an Architect-CG16000 analyzer (Abbott Diagnostics, Tokyo, Japan) by the hexokinase method. Plasma insulin concentrations were measured by chemoluminescence with an Architect-I2000 analyzer (Abbott Diagnostics, Tokyo, Japan). High-sensitivity C-reactive protein concentrations were measured according to Rifai et al. (Rifai, Tracy et al. 1999).

Relative telomere length measurement

DNA isolation

DNA was extracted from buffy coats using the Gentra Puregene Blood kit (Gentra Systems Inc., Minneapolis, MN, USA), and low yielding samples (<10 ng) were subjected to whole genome amplification using the REPLI-g kit (Qiagen Ltd. West Sussex, UK).

Quantitative PCR analysis of telomere length

RTL was determined using the Cawthon method where the measurements are performed by qPCR (Cawthon 2002). We estimated the relative ratio of telomere repeat copy number (T) normalized against a single copy gene copy number (Homo sapiens ribosomal protein L13a gene RPL13a; S) for all the samples. Results for each PCR were relativized to a standard curve built using a reference DNA sample. The standard curves for telomere and genomic PCRs consisted of eight standards of reference DNA (1-25 ng). The correlation between relative T/S ratios measured by qPCR and relative telomere DNA restriction fragment length by Southern blotting has been confirmed previously to be highly consistent (Cawthon 2002; Epel, Blackburn et al. 2004).

All PCRs were performed in duplicate with the use of iQ5-BIORAD thermal cycler and SensiFAST^{TM} SYBR Lo-ROX kit (Bioline). The thermal cycler profile for both amplicons began with a 95ºC incubation for 3 min to activate the polymerase, followed by 40 cycles of 95ºC for 5 s, 54ºC 15 s. The reaction mix composition was identical except for the oligonucleotide primers: 20 ng template DNA; 1X SensiFAST^{TM} SYBR Lo-ROX; 200 nM reverse primer; 200 nM forward primer. The primer sequences were (written 5'→ 3'):

RPL13aF, CCTGGAGGAGAAGAGGAAAGAGA;

RPL13aR, TTGAGGACCTCTGTGTATTTGTCAA

teloF, CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT;

teloR, GGCTTGCCTTACCCTTACCCTTACCC TTACCCTTACCCT (O'Callaghan and Fenech 2011).

Study of endothelial function using laser Doppler

The Laser-Doppler linear Periflux 5000 (Perimed S.A., Stockholm, Sweden) was used to measure IRH. We found an inter-study variability of 8.85% and an intra-study variability of 8.7%.

Briefly, capillary flow of the second finger of the dominant arm of the patient was assessed for one minute before (t0) and after applying four minutes (td) of ischemia to the arm using a sphygmomanometer. The ischemic reactive hyperemia was obtained via IRH = (AUC_{td} - AUC_{t0}) × 100 AUC_{t0}.

Determination of oxidative stress biomarkers

Nitric oxide (NO) is a free gas produced endogenously by a variety of mammalian cells. This molecule induces vasodilatation and inhibits platelet aggregation and adhesion to the vascular endothelium. Total nitrite (nitrite and nitrate) was used as an indicator of NO production and was assayed using the Griess method (Ricart-Jane, Llobera et al. 2002), with an intra-assay coefficient of variation of 6.1%, and an inter-assay coefficient of variation of 7.7%. The reaction was monitored at 540 nm (UV-1603 spectrophotometer, Shimadzu). Protein carbonyl (PC) content was carried out in plasma samples using the method of Levine (Levine, Garland et al. 1990). Intra-assay coefficient of variation of 4.7%, and inter-assay coefficient of variation of 8.0%. The carbonyls were evaluated in a spectrophotometer (UV-1603 Shimadzu, Kyoto, Japan) at 360 nm.

Antioxidant enzyme activities

Total superoxide dismutase (SOD; E.C: 1.15.1.1) activity was determined by colorimetric assay in plasma at wavelength of 525 nm according to the method described by McCord and Fridovich et al.(Nebot, Moutet et al. 1993), with an intra-assay coefficient of variation of

7.3%, and an inter-assay coefficient of variation of 8.7%. Glutathione peroxidase (GPx; E.C.: 1.11.1.9) activity was evaluated in plasma by the Flohé and Gunzler method (Flohe and Gunzler 1984; Zhu, Zhang et al. 2008), with an intra-assay coefficient of variation of 6.5% and an inter-assay coefficient of variation of 7.2%. The GPx assay is based on the oxidation of NADPH to NAD+, catalyzed by a limiting concentration of glutathione reductase, with maximum absorbance at 340 nm. The absorbance was evaluated in a Shimadzu UV-1603 spectrophotometer (Kyoto, Japan).

Statistical analysis

The Statistical Package for the Social Sciences (SPSS 17.0 for Windows Inc., Chicago, IL, USA) was used for the statistical comparisons. RTL was analyzed as quartiles after removal of experimental outliers. Telomere length was categorized into quartiles (shortest to longest) and analyzed as a categorical variable. This resulted in the following distribution of RTL, quartile 1: RTL ≤ 1.10, quartile 2: 1.10 < RTL ≤ 1.26, quartile 3: 1.26 < RTL ≤ 1.56, quartile 4: RTL > 1.56.

Univariate analysis of variance adjusted for age was performed to compare variables across quartiles of RTL with Bonferroni post hoc test used for multiple comparisons. Differences were considered to be significant when $p < 0.05$. All data presented in text and tables are expressed as the means ± standard error (SE).

Results

Biochemical and anthropometric characteristics and metabolic assessment

The anthropometric, biochemical, BP and metabolic parameters of the MetS subjects are shown in Table 1. Plasma TG was higher in the subjects with shortest RTL (quartile 1) compared to those in quartile 4 with largest RTL ($p = 0.031$).

Baseline HOMAβ was greater in the subjects grouped in the quartile 4 with largest RTL compared with the subjects in the quartiles 1, 2 and 3 ($p = 0.003$). We did not find significant differences with respect to the remaining parameters between the four groups of MetS subjects acording to the RTL (Table 1).

Study of endothelial function

NO plasma levels were higher in the quartile 3 (p = 0.012) and 4 (p = 0.016) than the quartile 1 (shortest RTL) (Figure 1a). In addition, IRH shows the same trend as the NO levels with greater percentage of change from baseline in the quartile 3 (longest RTL) (p = 0.015) and 4 (p = 0.011) than quartile 1 (Figure 1b).

Antioxidant enzymes activities and biomarker of oxidative stress

We found that GPx plasma activity of subjects grouped in the quartile 4 with longer RTL was lower compared to quartile 1 with shorter RTL (p = 0.003), with intermediate activity levels for quartile 2 and 3 (Figure 2a). Although differences of SOD plasma activity between quartiles were not statistically significant, there was a marked trend that shows an increase in the activity of the enzyme in the quartile 1 with respect to the other RTL groups.

PC plasma levels were higher in the subjects grouped in the quartile 1 with the shortest RTL compared with the subjects in quartile 4 (p =0.036; Figure 3).

Discussion

MetS comprises a cluster of cardiovascular risk factors (low HDL-C, elevated BP, fasting glucose and TG, and abdominal obesity) (Reaven 2002) leading to accelerated atherosclerosis and an increased risk of type 2 diabetes. Additionally, MetS is associated with major cardiovascular events and a high mortality rate (McNeill, Rosamond et al. 2005; Van Guilder, Hoetzer et al. 2006).

Although insulin resistance is considered to be at the core of the MetS in terms of molecular mechanisms, it is exponentially increasing the number of studies that point out the importance of OxS as a deep influence for MetS progression. In fact, in the same population of this study, we have previously demonstrated that MetS subjects with more MetS components (4 or 5 components) may have a higher OxS level (determined by SOD and GPx plasma activities, IRH and total nitrite plasma levels, among others parameters) (Yubero-Serrano, Delgado-Lista et al. 2013). So, the study of the redox state in early MetS patients may provide a starting point for understanding the pathways that contribute to both the development of MetS and its subsequent complications.

Taking this into account, the present cross-sectional study displays a gradual and inverse relationship between high OxS levels and endothelial dysfunction with regard to RTL in MetS patients showing that the shorter telomere length, the greater the degree of OxS, which leads to increased plasma activity of antioxidant enzymes (GPx, SOD), biomarker of OxS levels (PC) and decreased IRH and total NO levels.

It has been documented that increased production of ROS results in damage to the endothelium, and along with others mechanisms involved in vascular aging process such as activation of inflammatory mediators, impairment of the NO pathway, telomeres length and telomerase activity, all together contribute to the dysregulation of vascular homeostasis (Marin, Yubero-Serrano et al. 2013).

NO is an endogenous vasodilator that promotes the increase of blood flow, decreases vascular resistance, inhibits platelet aggregation and also acts protecting endothelial cells from senescence and apoptosis (Ferroni, Basili et al. 2006; Toda 2012). A strong relationship has been found between telomere attrition and cell senescence in HUVECs, suggesting that DNA damage manifested as telomere shortening is likely to play a central role in the development of endothelial dysfunction in the pathogenesis of vascular disease (Hastings, Qureshi et al. 2004). Thus, MetS study subjects with the shortest RTL have the lowest level of plasmatic NO , and in accordance with this, they showed the lowest IRH as well . Interestingly, those MetS subjects with the shortest RTL are the ones whose antioxidant enzyme activities are higher, where plasma GPx activity is increased and plasma SOD activity presents the same trend. A plausible explanation to these observations is the fact that in response to OxS and to prevent oxidative damage, cells attempt to strengthen their antioxidant arsenal as the first line of defense. For this purpose, antioxidant enzymes such as SOD, GPx and catalase increase their activity to balance the excessive generated OxS (Yubero-Serrano, Delgado-Lista et al. 2013). Furthermore, we also found an increase in PC plasma levels in the subjects with the shortest RTL (Fig. 3). PC are an indicator of severe OxS, characterized by an irreversible damage to the protein structure and function and frequently associated with obesity, insulin resistance, diabetes mellitus and MetS (Hopps and Caimi 2013). Our present observation is consistent with a study in coronary artery disease patients with MetS where telomere shortening and oxidative stress damage in endothelial progenitor cells was higher than in those without MetS. They suggest that telomere attrition in response to OxS may induce enhanced endothelial damage and contribute to increase the risk of subsequent cardiovascular events (Satoh, Ishikawa et al. 2008).

In this study, we measured telomere length in circulating leucocytes due to the fact that this method is less invasive than obtaining human vascular tissue from participants.

Although we did not study telomeres length in vascular cells, it has been demonstrated that circulating blood leucocyte DNA content is predictive of vascular telomere content and also is an accurate surrogate for vascular aging in population studies (Nakashima, Ozono et al. 2004; Wilson, Herbert et al. 2008).

Furthermore, systemic OxS is increased in subjects with MetS and is strongly associated with visceral fat accumulation (Fujita, Nishizawa et al. 2006; Yubero-Serrano, Delgado-Lista et al. 2013). In our study, we found that TG levels were higher and HOMAβ was lower in those people with shorter telomeres and increased OxS levels. The same findings have been shown in type diabetes mellitus patients where telomere length attrition was inversely correlated to TG levels and considerably explained by insulin resistance, which in turn, promoted an increase in ROS levels (Harte, da Silva et al. 2012).

We are aware that this cross-sectional study has some limitations since these findings do not prove a cause-and-effect relationship and the use of a small sample population. In this regard, larger and prospective studies are needed to elucidate which of the parameters studied herein has the strongest effect above the others.

In summary, our results show that there is a clear link among endothelial function, OxS and RTL in MetS subjects. High levels of OxS may induce endothelial dysfunction and may increase telomere attrition. Thus, further support of the molecular and cellular mechanisms involved in vascular dysfunction may contribute to the development of strategies to decelerate vascular aging or prevent cardiovascular disease.

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Figure captions

Fig. 1 Total nitrite levels in plasma (A) and ischemic reactive hyperemia (B) across quartiles of RTL. Data were analyzed using univariate analysis of variance adjusted for age. All values represent the means ± s.e. Differences were considered to be significant when $*p < 0.05$

Fig. 2 Glutathione peroxidase (GPx) plasma activity (A) and superoxide dismutase (SOD) plasma activity (B) across quartiles of RTL. Data were analyzed using univariate analysis of variance adjusted for age. All values represent the means ± s.e. Differences were considered to be significant when $*p < 0.05$

Fig. 3 Protein carbonyl plasma levels across quartiles of RTL. Data were analyzed using univariate analysis of variance adjusted for age. All values represent the means ± s.e. Differences were considered to be significant when $*p < 0.05$

Abstract

Previous evidences support that increased oxidative stress (OxS) may play an important role in metabolic syndrome (MetS) and both are closely linked to vascular dysfunction. This study determined whether there is a direct relationship between endothelial function and relative telomere length (RTL) in MetS subjects. In this cross-sectional study from the LIPGENE cohort, a total of 88 subjects (36 men and 52 women) were divided into four groups by quartiles of telomere length. We measured ischemic reactive hyperemia (IRH), total nitrite (NO) and protein carbonyl (PC) plasma levels, superoxide dismutase (SOD) and glutathione peroxidase (GPx) plasma activities. IRH and NO plasma levels were higher in subjects with longer RTL (quartile 3 and 4), while PC plasma levels and GPx and SOD plasma activities were lower in quartile 4 subjects (longest RTL). Additionally, MetS subjects with longer RTL had greater homeostatic model assessment-β (HOMAβ) level and lower triglycerides (TG) plasma levels. Our results suggest that endothelial dysfunction, associated by high levels of OxS, could be entailed in an increment of telomere attrition. Thus, further support of the molecular and cellular mechanisms involved in vascular dysfunction may contribute to the development of strategies to decelerate vascular aging or prevent cardiovascular disease.

table
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Table 1. Baseline characteristics of the study population according to quartiles of RTL [1,2,3].

	Quartile 1 (n = 22)	Quartile 2 (n = 24)	Quartile 3 (n = 20)	Quartile 4 (n = 22)	*p*
Age (years)	59.73 ± 1.87	59.71 ± 1.55	58.60 ± 1.60	56.59 ± 1.94	0.540
Sex (female/male)	14/8	14/10	12/8	12/10	
$HOMA_{IR}$	3.37 ± 0.07	3.97 ± 0.10	3.42 ± 0.11	4.32 ± 0.12	0.700
HOMAβ	89.94 ± 9.31 [a]	91.77 ± 8.93 [a]	94.21 ± 9.69 [a]	139.14 ± 9.49 [b]	0.003
Weight (kg)	87.78 ± 2.74	86.50 ± 2.63	89.66 ± 2.85	96.06 ± 2.79	0.940
Height (m)	1.57 ± 0.03	1.62 ± 0.02	1.54 ± 0.05	1.56 ± 0.04	0.129
BMI	34.28 ± 0.88	33.68 ± 0.84	34.28 ± 0.92	35.28 ± 0.90	0.800
Waist circumference (cm)	106.34 ± 2.18	103.54 ± 2.09	108.40 ± 2.27	109.71 ± 2.22	0.937
Insulin (mU/L)	12.22 ± 2.10	14.71 ± 2.05	12.69 ± 2.19	15.04 ± 2.14	0.657
Glucose (nmol/L)	6.29 ± 0.22	6.20 ± 0.22	5.93 ± 0.23	5.03 ± 0.23	0.143
TG (nmol/L)	1.89 ± 0.14 [a]	1.51 ± 0.13 [a, b]	1.55 ± 0.15 [a, b]	1.27 ± 0.14 [b]	0.031
LDL-C (nmol/L)	17.38 ± 8.63	34.02 ± 8.27	21.30 ± 8.78	26.16 ± 8.97	0.647
HDL-C (nmol/L)	1.11 ± 0.05	1.22 ± 0.05	1.08 ± 0.06	1.09 ± 0.06	0.325
hsCRP (mg/L)	6.63 ± 1.17	5.24 ± 1.12	7.09 ± 1.19	6.52 ± 1.16	0.573
SBP (mm Hg)	142 ± 3	144 ± 3	138 ± 3	142 ± 3	0.306
DBP (mm Hg)	87 ± 3	87 ± 2	88 ± 3	89 ± 3	0.900

[1] Data are means ± SE. $HOMA_{IR}$, homeostatic model assessment index for insulin resistance; HOMAβ, homeostatic model assessment index of insulin secretory function; BMI, body mass index; TG, triglycerides; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; hsCRP, high sensitivity C-reactive protein.

[2] Means in a column with different superscript letters are significantly different, $p < 0.05$ (Univariate analysis of variance adjusted for age).

[3] Quartile 1: RTL ≤ 1.10, quartile 2: 1.10 < RTL ≤ 1.26, quartile 3: 1.26 < RTL ≤ 1.56, quartile 4: RTL > 1.56.

Fig.1
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Fig. 1

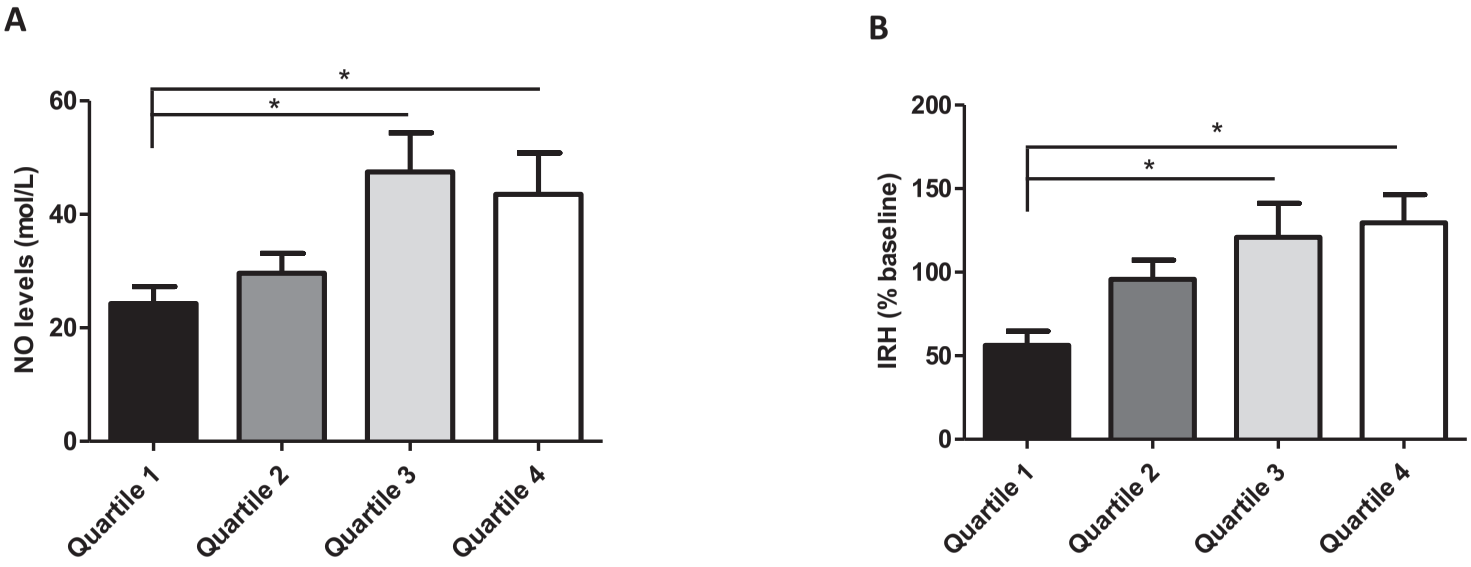

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Fig. 2

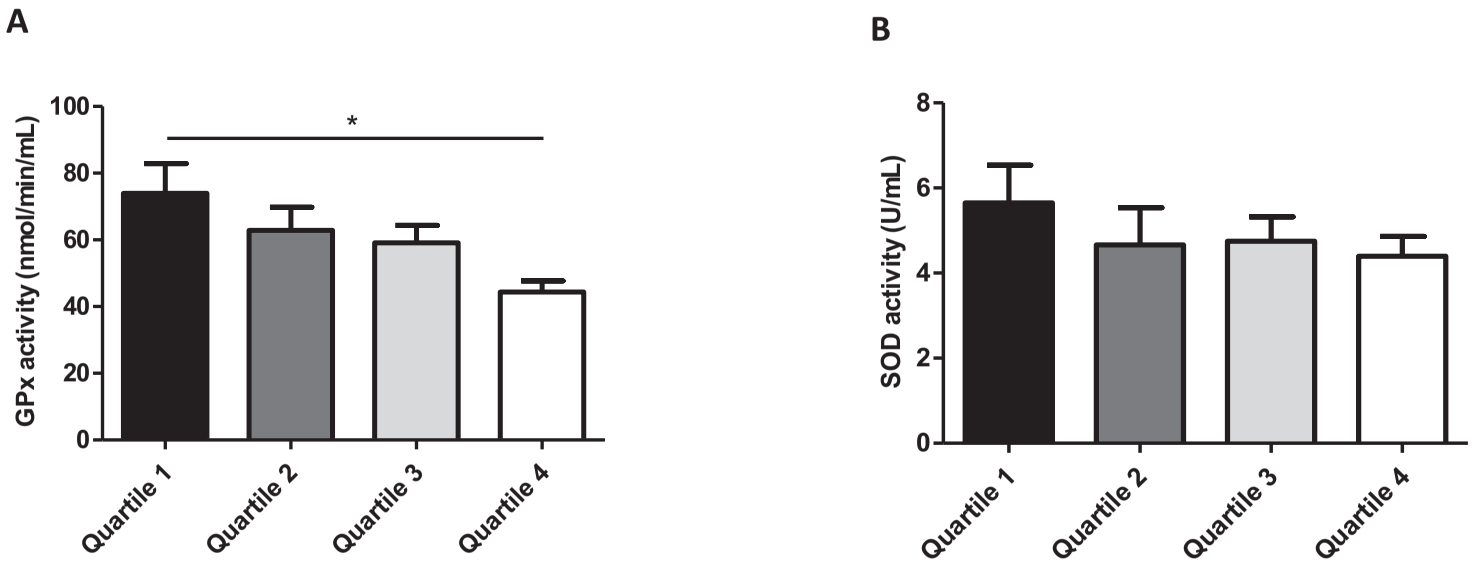

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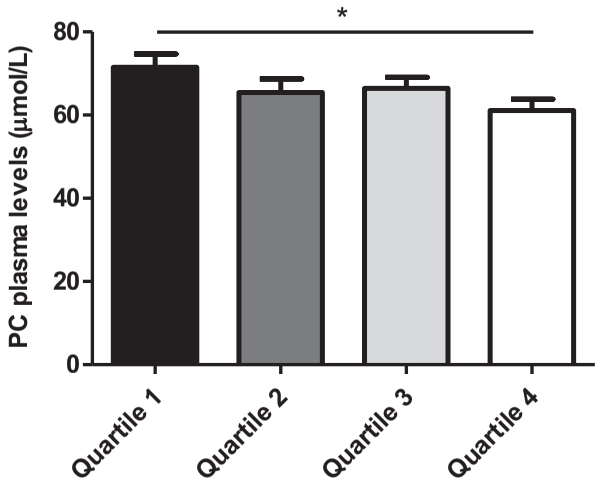
Fig.3



Supplementary Table 1. Inclusion and exclusion criteria of the LIPGENE study

Inclusion Criteria
Age: 35-70 years.
Gender: males and females (not pregnant or lactating).
Body mass index (BMI) 20-40 kg/m^2
Total cholesterol concentration equal to or <8.0 mmol/L.
Medications/nutritional supplements allowed, on conditions that the subjects adhere to the same regimen during the study: anti-hypertensive medication (including beta-blockers), oral contraceptives, hormone replacement therapy, multi-vitamin supplements, other non-fatty acid based nutritional supplements.
Smokers and non-smokers.
Regular consumers of alcohol, which is not excessive as defined by elevated liver enzymes (AST and ALT).
Ethnicity: intention to include white Europeans.
Exclusion criteria
Age: <35 or >70 years.
Diabetes or other endocrine disorders.
Chronic inflammatory conditions.
Kidney or liver dysfunction.
Iron deficiency anaemia (haemoglobin <12 g/dL men, <11g/dL women).
Prescribed hypolipidaemic medication.
Prescribed anti-inflammatory medication.
Fatty supplements including fish oils, evening primrose oil, etc.
Consumers of high doses of antioxidant vitamins (A, C, E, β-carotene).
Red rice yeast (Monascus purpureus) supplement usage.
High consumers of oily fish (>2 serving of oily fish per week).
Highly trained or endurance athletes or those who participate in more than three periods of intense exercise per week.
Volunteers planning to start a special diet or lose weight (e.g. the Slimfast Plan, Atkins Diet, etc.).
Weight change equal or >3 kg within the last 3 months.

Alcohol or drug abuse (based on clinical judgment).

Pregnant/lactating females/women planning a pregnancy in the next 12 months.

PREMIO A LA MEJOR PRESENTACIÓN ORAL EN LA SESIÓN ENFERMEDAD CARDIOVASCULAR. OBESIDAD Y SÍNDROME METABÓLICO. ENFERMEDADES HEPÁTICAS Y DIGESTIVAS. ENFERMEDADES RENALES Y NEFROUROLÓGICAS.

El Comité Científico de la IV Jornada de Jóvenes Investigadores del IMIBIC otorga este premio a:

Lorena González de la Guardia

Como reconocimiento al trabajo realizado:

"Effects of mediterranean diet supplemented with coenzyme q10 on metabolomic profile in elderly men and women"

En Córdoba, a 14 de mayo de 2013

Pérez Jiménez

Dr. Francisco Pérez Jiménez
Director Científico del IMIBIC

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A LA COMUNICACIÓN PRESENTADA EN EL XXVII CONGRESO NACIONAL S.E.A. BARCELONA 2014

a la comunicación póster:

INFLUENCIA DE LA DISFUNCIÓN ENDOTELIAL EN LA LONGITUD TELOMÉRICA EN SUJETOS CON SÍNDROME METABÓLICO: ESTUDIO LIPGENE

presentada por los autores:

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