Universitat de les Illes Balears

ciberobn
Centro de Investigación Biomédica en Red
Fisiopatología de la Obesidad y Nutrición
Grupo CB12/03/30038

TESIS DOCTORAL
2018

ACTIVIDAD FÍSICA Y ESTRÉS OXIDATIVO: EFECTOS SOBRE LA DINÁMICA MITOCONDRIAL Y MARCADORES DE INFLAMACIÓN

CARLA BUSQUETS CORTÉS

Universitat de les Illes Balears

ciberobn
Centro de Investigación Biomédica en Red
Fisiopatología de la Obesidad y Nutrición
Grupo CB12/03/30038

TESIS DOCTORAL
2018

Programa de Doctorado en Nutrición y Ciencias de los Alimentos (RD99/2011)

ACTIVIDAD FÍSICA Y ESTRÉS OXIDATIVO: EFECTOS SOBRE LA DINÁMICA MITOCONDRIAL Y MARCADORES DE INFLAMACIÓN

Carla Busquets Cortés

Director/a: Antoni Sureda Gomila
Director/a: Antoni Pons Biescas
Tutor/a: Antoni Sureda Gomila

Doctora por la Universitat de les Illes Balears

Con el beneplácito de los directores

Dr. Antoni Sureda Gomila
Profesor Titular de Universidad
Àrea de Bioquímica y Biologia Molecular
Dept. Biologia Fonamental i Ciències de la Salut
Universitat de les Illes Balears

Dr. Antoni Pons Biescas
Catedrático de Universidad
Àrea de Bioquímica y Biologia Molecular
Dept. Biologia Fonamental i Ciències de la Salut
Universitat de les Illes Balears

La interesada

Carla Busquets Cortés

A mis padres, Andrea, Carlos y Tra.

Universitat de les Illes Balears

Dr. Antoni Sureda Gomila, de la Universitat de les Illes Balears

DECLARO:

Que la tesi doctoral que porta per títol Actividad Física y Estrés oxidativo: Efectos sobre la Dinámica Mitocondrial y Marcadores de Inflamación, presentada per Carla Busquets Cortés per a l'obtenció del títol de doctora, ha estat dirigida sota la meva supervisió,

I perquè quedi constància d'això signo aquest document.

Signatura

Palma de Mallorca, de de 2018

Universitat de les Illes Balears

Dr. Antoni Pons Biescas, de la Universitat de les Illes Balears

DECLARO:

Que la tesi doctoral que porta per títol Actividad Física y Estrés oxidativo: Efectos sobre la Dinámica Mitocondrial y Marcadores de Inflamación, presentada per Carla Busquets Cortés per a l'obtenció del títol de doctora, ha estat dirigida sota la meva supervisió,

I perquè quedi constància d'això signo aquest document.

Signatura

Palma de Mallorca, de de 2018

Son nuestras elecciones las que muestran lo que somos,
mucho más que nuestras habilidades.

Albus Dumbledore

Agradecimientos

Hay ciertos momentos en la vida en los que nos toca decidir entre lo que es correcto y lo que es fácil. Llegados a este punto, y volviendo la vista atrás, no podría sentirme más agradecida por el entorno en el que he realizado mi tesis estos últimos cuatro años.

En primer lugar quiero dar las gracias a la Conselleria d´Educació, Cultura i Universitats del Govern Balear y al Fondo Social Europeo por la beca FPI-CAIB concedida, y que ha hecho posible el desarrollo de esta tesis. También agradecer al resto de miembros PAS-PDI de la Universidad, en especial a los de mi edificio Guillem Colom y los dels Serveis Científicotècnis, por el trato siempre ambale y por brindarme ayuda y consejo cuando lo he necesitado.

Als meus dos directors de tesi, gràcies per no escatimar cap esforç durant aquesta aventura doctoral: a nen **Toni Pons**, per encoratjar-me a unir-me al grup, ajudar-me tant amb els càlculs i per les encertades paraules en moments de *crisi*. A nen **Toni Sureda**, per estar a la meva disposició 24/7, per fer tanta via en corregir, i tractar-me com si fos una més de la família.

No me olvido de ti Silvia, por ser siempre tan amable y afectuosa conmigo. Y por supuesto, gracias al resto de profesores del departamento, **Magdalena, Ana, Pilar, Bel, Ádamo, Jordi**, cariñosos conmigo y fieles seguidores de mi humilde trayectoria operística. **Paco**, sempre entre noltros, més viu que mai.

A mis *compis* de labo i de *despatxet*, ese gran pilar en el que cualquier doctorando necesita apoyarse día tras día para escapar de las frustraciones y con quien celebrar los éxitos, ya sean académicos o personales.

- GMOT, llar dels millors expedients: **Lida**, *Lidish*, el meu primer *contacte* de confiança al labo durant es màster. Sempre tan científica i fidel, amb les idees tan clares i la roba tan obscura, gràcies per ajudar-me en TOT (a canvi d'uns descomptes per s'òpera!) i ser com una germana per jo. **Maria del Mar**, sempre has tengut paraules amables per jo i sé que amb tu, hi puc comptar. **Reyniel**, un placer compartir poyata con alguien tan agradable como tú (y tan aficionado a las series como yo).

- GMEIN, siempre tan unidos y amigables: **Marco**, *Marcus*, simpático conmigo *from the very beginning*, siempre dispuesto a organizar un buen partido de volley y machacar a los químicos. **Melanie,** *Melaniuska*, sempre tan dolça i afable, mai oblidaré lo bé que me vares cuidar quan vaig tenir febre al congrés de València. El genuí **Agustí,** *Agustineishon*, sempre dispost a fer un *coffee together* i fer sa xerradeta, gràcies per promoure el bon rollo a nes *despatxet.* **Bel**, *Beluski, Luski*, què te puc dir que no sàpigues ja. Gràcies per sa càlida acollida quan jo encara feia es màster, per totes ses aventures, converses, fotos, mojitos, camisetes iguals, frikades, *horapas* i confidències compartides, i per ser, en difinitiva, sa meva millor labo-amiga.

- NUCOX Team: **Xavi**, Xavi, Xavi... El meu jefe superior immediat. No oblidaré mai totes ses rialles, llonguets, *pegatinas*, motes, viatges en *autobús* (ascensor), dois, locures, pósters, articles, mostres, sushis i experiències en general que, sense adonar-mos, hem compartit. Gràcies! **Miquelot**, l'amo de Xile, pare del NOA i de qui vaig heredar es quadern de labo, gràcies per tenir-me sempre en compte. **Edu**: *my partner in crime*. Gracias por ser mi mano derecha e izquierda los últimos meses de labo, leyéndome siempre la mente y siguiéndome el rollo como si nada. **Marga**: benvinguda a sa nucox-family, gràcies per passar-me tots els treballs del CAP i ser tan bona companya de taula.

No me olvido de mi familia política desde hace más de 10 años, **Marisol, Manolo y Javier.** Gracias por estar pendientes de mí desde que os conozco y celebrar mi trabajo (por muy aburrido que yo os decía que era) y ayudarme a superar escollos que parecían insalvables. Y al todos mis **amigos, amigas y resto de miemros de mi familia**, que no nombraré aquí porque por suerte somos muchísimos y sería extenderme demasiado, pero que habéis tenido siempre buenas palabras conmigo, me habéis animado en todo momento y habéis creído en mí sin reparos, especialmente estos últimos meses: mil gracias!!

A mis padres **Xisco y María Ángeles** y a mi hermana **Andrea**, el motor de mi vida, gracias por apoyarme el 100% del tiempo en todas mis decisiones, por no escatimar ni un solo esfuerzo o recurso, por todos los *truis* que habéis llegado a hacer por mí, por ser mis mejores amigos y por ser un modelo a seguir en todos los aspectos de la vida.

Y ya para acabar, a mi compañero de viaje **Carlos**, gracias a quien estoy hoy aquí redactando estas líneas. Tu apoyo constante e incondicional, tu infinita paciencia, tus infalibles consejos y tu manera de ver y entender el mundo me han ayudado a crecer personal y profesionalmente, y no puedo explicar con simples palabras lo agradecida que te estoy. *Nobody but you, for all my life.*

Cabó

♥

Índice

Siglas y Acrónimos

$^1O_2$	Oxígeno singlete / *Singlet oxygen*
8-OHdG	8-Oxo-2'-desoxiguanosina / *8-Oxo-2'-deoxyguanosine*
DNA	Ácido desoxirribonucleico / *Deoxyribonucleic acid*
ADP	Adenosín difosfato / *Adenosine diphosphate*
ATP	Adenosín trifosfato / *Adenosine triphosphate*
AMPc	Adenosín monofosfato cíclico / *Cyclic adenosine monophosphate*
AMPK	Proteína quinasa activada por AMP / *AMP-activated protein kinase*
BMI	Índice de masa corporal / *Body Mass Index*
CAT	Catalasa / *Catalase*
COX	Ciclooxigenasa / *Cyclooxigenase*
COXIV	Subunidad IV de la citocromo C oxidasa / *Cytochrome c Oxidase Subunit IV*
CRP	Proteína C reactiva / *C Reactive Protein*
Cu	Cobre / *Copper*
CREB	Elemento de respuesta a AMPc / *cAMP response element-binding*
Cys	Cisteína / *Cysteine*
DCFH-DA	Diclorofluoresceína diacetato / *Dichloro-dihydro-fluorescein diacetate*
DHA	Ácido docosahexaenoico / *Docosahexaenoic acid*
DMSO	Dimetilsulfóxido / *Dimethyl sulfoxide*
Drp-1	Proteína relacionada con la dinamina 1 / *dynamin 1 related protein*
EDTA	Ácido etilendiaminotetraacético / *Ethylenediaminetetraacetic acid*
EPA	Ácido eicosapentaenoico / *Eicosapentaenoic acid*
Fe	Hierro / *Iron*
Fis1	Proteína de fisión 1 / *Fission protein 1*
GOX	Glucosa Oxidasa / Glucose Oxidase
GPx	Glutatión peroxidasa / *Glutathione peroxidase*
GRd	Glutatión reductasa / *Glutathione reductase*
GSH	Glutatión (reducido) / *Reduced Glutathione*

GSSG Glutatión oxidado / *Oxidized glutathione*

GTP Guanosín trifosfato / *guanosine triphosphate*

H_2O_2 Peróxido de hidrógeno / *Hydrogen peroxyde*

HClO Acido hipocloroso / *hypochlorous acid*

HIF-1 Factor de hipoxia inducible / *Hypoxia-inducible factors*

IκB Inhibidores de NFκB / *NF-κB inhibitors*

IL Interleuquina / *Interleukin*

IMC Índice de Masa Corporal / *Body Mass Index*

iNOS Óxido nítrico sintasa / *Nitric oxide synthase*

LC3 *Microtubule-associated protein 1A/1B-light chain 3*

LPS *Lipopolisacárido / Lipopolysaccharide*

MAPK Proteína quinasas activadas por mitógenos / *Mitogen-activated protein kinase*

MDA Malondialdehído / *Malondialdehyde*

Mtf Mitofusinas / *Mitofusins*

MitND5 NADH deshidrogenasa Subunidad 5 / *Mitochondrially encoded NADH dehydrogenase 5*

MPO Mieloperoxidasa / *Myeloperoxidase*

mtDNA DNA mitocondrial / *Mitocondrial DNA*

NADPH Nicotinamida adenina dinucleótido fosfato / *Nicotinamide adenine dinucleotide phosphate*

NFκB Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas / *Nuclear factor kappa-lightchain-enhancer of activated B cells*

Nitro-Tyr Nitrotirosinas / Nitrotyrosines

NK Células *Natural Killer* / *Natural killer cells*

NLRP3 Familia de receptores tipo Nod, que contiene el dominio pirina 3 / *Nod-like receptor family, pyrin domain-containing 3*

NO Óxido nítrico / *Nitric oxide*

NOS Óxido nítrico sintasa / *Nitric Oxide synthase*

NRF1 Factor nuclear de respiración 1 / *Nuclear respiration factor 1*

NRF2 Factor nuclear de respiración 2 (símbolo official: GABPA) / *nuclear respiratory factor 2 or GA binding protein transcription factor (official symbol: GABPA)*

Nrf-2 Factor nuclear eritroide 2 / *nuclear factor-erythroid-derived 2–like 2 (official symbol: NFE2L2)*

$OH^{\cdot}$ Radical hidroxilo / *hydroxyl radical*

$ONOO^{-}$ Peroxinitrito / *Peroxynitrite*

OPA1 Proteína de la atrofia óptica 1 / *Optic protein atrophy 1*

PAMPs Patrones moleculares asociados a patógenos / Pathogen-associated molecular patterns

PBMC Célula mononuclear de sangre periférica / *Peripheral blood mononuclear cell*

PGC-1α Coactivador del receptor activado por proliferadores peroxisomales 1 α / *Peroxisome proliferator-activated receptor gamma coactivator 1-alpha*

PCR Reacción en cadena de la polimerasa / *Polymerase chain reaction*

Pi Fosfato inorgánico / *inorganic phosphate*

PKC Proteína kinasa C / *protein kinase C*

PMA Acetato de forbol miristato / *Phorbol myristate acetate*

PPAR Receptores activados de proliferación de los peroxisomas / *Peroxisome proliferator-activated receptors*

PUFA Ácido graso poliinsaturado / *Polyunsaturated fatty acid*

Redox Óxido-reducción o reducción-oxidación / *reduction–oxidation*

ROS Especies reactivas de oxígeno / *Reactive oxygen species*

RONS Especies reactivas de oxígeno y de nitrógeno / *Reactive oxygen and nitrogen species*

RNS Especies reactivas de nitrógeno / *Reactive nitrogen species*

RT-PCR P CR a Tiempo Real / *Real Time PCR*

SIRT Sirtuina / *Sirtuine*

SLC-2A4 Miembro 4 de la familia 2 de transportadores de solutos / *Solute Carrier*

Family 2 Member 4)

SOD Superóxido dismutasa / *Superoxide dismutase*

TFAM Factor de transcripción mitocondrial A / *Mitochondrial transcription factor A*

TGF-β Factor transformante de crecimiento β / *Transforming growth factor beta*

TNF-α Factor de necrosis tumoral α / *Tumor necrosis factor α*

TLR Receptor tipo Toll / *Toll-like receptor*

Trx Tioredoxina / *Thioredoxin*

TrxR1 Tioredoxina reductasa / *Thioredoxin reductase*

UCP Proteína desacopladora / *Uncoupling protein*

XO Xantina oxidasa / *Xanthine Oxidase*

Zn Zinc / *Zinc*

ZYM Zymosan */ Zymosan*

ACTIVIDAD FÍSICA Y ESTRÉS OXIDATIVO: EFECTOS SOBRE LA DINÁMICA MITOCONDRIAL Y MARCADORES DE INFLAMACIÓN

Tesis doctoral, Carla Busquets Cortés, Departamento de Biología Fundamental y Ciencias de la Salud, Laboratorio de Ciencias de la Actividad física, Grupo de Investigación en Nutrición Comunitaria y Estrés Oxidativo, Universidad de las Islas Baleares, Palma de Mallorca

Resumen

La práctica de actividad física conduce a la generación de especies reactivas de oxígeno (ROS) con potencial para influir sobre numerosos procesos celulares. Estas ROS ejercen un efecto dual en función de su concentración, ya que niveles elevados se asocian con la aparición de estrés oxidativo y daño sobre biomoléculas y estructuras celulares, mientras que niveles más moderados actúan como mensajeros celulares modulando diversas vías de señalización. Sesiones de ejercicio agudo y de elevada intensidad se relacionan con elevadas tasas de generación de ROS que pueden sobrepasar la capacidad antioxidante del organismo. Contrariamente, la repetición de ejercicios de intensidad moderada se asocia con dosis fisiológicamente tolerables de ROS; a éstas se le atribuyen efectos beneficiosos dado que parecen estar implicadas en la regulación de cascadas de señalización celular que juegan un papel fundamental en la mediación de los beneficios derivados del entrenamiento. Por otra parte, se ha descrito que los ácidos grasos omega 3 ejercen importantes beneficios sobre el organismo al modular los mecanismos de defensa antioxidante y mediadores de inflamación. Así, el objetivo general de esta tesis se centra en evaluar y caracterizar la respuesta diferencial antioxidante, inflamatoria y mitocondrial, frente a diferentes dosis estímulo prooxidante, además valorar el uso de ácido docosahexaenoico (DHA) como suplemento nutricional en este contexto.

En futbolistas entrenados, el hecho de ingerir una bebida enriquecida con DHA durante ocho semanas incrementa en el contenido de este omega 3 en las membranas eritrocitarias. El entrenamiento y el ejercicio agudo promueven en estos sujetos la biogénesis y remodelación mitocondrial y potencia la capacidad de respiración mitocondrial en PBMCS. La suplementación de la dieta con DHA durante ocho semanas contribuye de manera sinérgica a los efectos del entrenamiento sobre la remodelación mitocondrial.

Un estilo de vida activo viene acompañado de menor peso, masa grasa, IMC, presión arterial diastólica y recuento neutrófilos y linfocitos en sujetos sanos sexagenarios en comparación con un estilo de vida sedentario. En sujetos activos en condiciones basales observamos una potenciación de la capacidad antioxidante endógena inducida por la práctica regular de actividades físicas moderadas. Además, se observa una atenuación de parámetros proinflamatorios y una potenciación de marcadores antiinflamatorios en sujetos activos respecto a sedentarios. Con todo, un estilo de vida activo ejerce efectos beneficiosos sobre la composición corporal, las defensas antioxidantes y el estado antiinflamatorio de las personas mayores.

La exposición continua de PBMCs y neutrófilos a concentraciones sostenidas de H_2O_2 producida por la enzima glucosa oxidasa permite emular una situación crónica de estrés oxidativo de manera *ex vivo.* Los neutrófilos presentan una mayor capacidad para eliminar el H_2O_2 que las PBMCs. Este hecho se traduce en menores niveles de H_2O_2 en el medio de cultivo de neutrófilos, lo que limita los efectos del H_2O_2 sobre de expresión génica y producción de ROS por parte de estas células. La exposición continua *ex vivo* de PBMCs y neutrófilos a concentraciones sostenidas de H_2O_2 estimula de manera hormética procesos implicados en la dinámica y biogénesis mitocondrial y la expresión de genes proinflamatorios en estas células. Un control de los de niveles de H_2O_2 en el organismo podría tener relevancia médica en enfermedades con estados inflamatorio y redox alterados como el síndrome metabólico.

En conclusión, la activación de las defensas antioxidantes, la inducción de daño oxidativo, junto con la modulación de la dinámica biogénesis mitocondrial y de los marcadores de inflamación, son procesos que se encuentran estrechamente relacionados en respuesta a diferentes grados estrés oxidativo derivado de la práctica de actividad física, el estilo de vida y la exposición exógena H_2O_2. La ingesta de DHA en dosis moderadas resulta efectiva potenciar de manera sinérgica las adaptaciones antioxidantes y mitocondriales.

ACTIVITAT FÍSICA Y ESTRÈS OXIDATIU: EFECTES SOBRE LA DINÀMICA MITOCONDRIAL Y MARCADORS D'INFLAMACIÓ

Tesi doctoral, Carla Busquets Cortés, Departament de Biologia Fonamental i Ciències de la Salut, Laboratori de Ciències de l'Activitat física, Grup d'investigación en Nutrició Comunitaria i Estrès Oxidatiu, Universitat de les Iles Balears, Palma de Mallorca.

Resum

La pràctica d'activitat física condueix a la generació d'espècies reactives d'oxigen (ROS) amb potencial per influir sobre nombrosos processos cel·lulars. Aquestes ROS exerceixen un efecte dual en funció de la seva concentració, ja que nivells elevats s'associen a l'aparició d'estrès oxidatiu i dany sobre biomolècules i estructures cel·lulars, mentre que nivells més moderats actuen com a missatgers cel·lulars i modulen diverses vies de senyalització. Sessions d'exercici agut i d'elevada intensitat es relacionen amb elevades taxes de generació de ROS que poden sobrepassar la capacitat antioxidant de l'organisme. Contràriament, la repetició d'exercicis d'intensitat moderada s'associa amb dosis fisiològicament tolerables de ROS; a aquestes se li atribueixen efectes beneficiosos atès que semblen estar implicades en la regulació de cascades de senyalització cel·lular que juguen un paper fonamental en la mediació dels beneficis derivats de l'entrenament. D'altra banda, s'ha descrit que els àcids grassos omega 3 exerceixen importants beneficis sobre l'organisme ja que modulen els mecanismes de defensa antioxidant i paràmetres inflamatoris. Així, l'objectiu general d'aquesta tesi es centra en avaluar i caracteritzar la resposta diferencial antioxidant, inflamatòria i mitocondrial, vers diferents dosis d'estímul prooxidant, a més de valorar l'ús d'àcid docosahexaenoic (DHA) com a suplement nutricional en aquest context.

El fet d'ingerir una beguda enriquida amb DHA durant vuit setmanes s'incrementa en el contingut d'aquest omega 3 en les membranes eritrocitàries a futbolistes entrenats. L'entrenament i l'exercici agut promouen en aquests subjectes la biogènesi i remodelació mitocondrial i potencia la capacitat de respiració mitocondrial a PBMCS. La suplementació de la dieta amb DHA durant vuit setmanes contribueix de manera sinèrgica a l'efecte de l'entrenament sobre la remodelació mitocondrial.

Un estil de vida actiu ve acompanyat per menor pes, massa grassa, IMC, pressió arterial diastòlica i recompte neutròfils i limfòcits, en subjectes sans sexagenaris, en comparació amb un estil de vida sedentari. A subjectes actius en condicions basals observem una potenciació de la capacitat antioxidant endògena induïda per la pràctica regular d'activitats físiques moderades. A més, s'observa una atenuació de paràmetres proinflamatoris i una potenciació de marcadors antiinflamatoris a subjectes actius respecte a subjectes sedentaris. Amb tot, un estil de vida actiu exerceix efectes beneficiosos sobre la composició corporal, les defenses antioxidants i l'estat antiinflamatori de la gent gran.

L'exposició contínua de PBMCs i neutròfils a concentracions sostingudes de H_2O_2 produïda per l'enzim glucosa oxidasa permet emular una situació crònica d'estrès oxidatiu de manera *ex vivo*. Els neutròfils presenten una major capacitat per eliminar l'H_2O_2 que les PBMCs. Aquest fet es tradueix en menors nivells d'H_2O_2 al medi de cultiu dels neutròfils, fet que limita els efectes de l'H_2O_2 sobre l'expressió gènica i la producció de ROS per part d'aquestes cèl·lules. L'exposició contínua *ex vivo* de PBMCs i neutròfils a concentracions sostingudes de H_2O_2 estimula de manera hormètica processos implicats en la dinàmica i biogènesi mitocondrial i l'expressió de gens proinflamatoris en aquestes cèl·lules. Un control dels de nivells d'H_2O_2 a l'organisme podria tenir rellevància mèdica en malalties amb estats inflamatori i redox alterats com la síndrome metabòlica.

En conclusió, l'activació de les defenses antioxidants, la inducció de dany oxidatiu, juntament amb la modulació de la dinàmica biogènesi mitocondrial i dels marcadors d'inflamació, són processos que es troben estretament relacionats en resposta a diferents graus d'estrès oxidatiu derivat de la pràctica d'activitat física, l'estil de vida i l'exposició exògena H_2O_2. La ingesta de DHA sota dosis moderades resulta efectiva potenciar de manera sinèrgica les adaptacions antioxidants i mitocondrials.

PHYSICAL ACTIVITY AND OXIDATIVE STRESS: EFFECTS ON MITOCHONDRIAL DYNAMICS AND INFLAMMATORY MARKERS
PhD thesis, Carla Busquets Cortés, Departamento de Biología Fundamental y Ciencias de la Salud, Laboratorio de Ciencias de la Actividad física, Grupo de Investigación en Nutrición Comunitaria y Estrés Oxidativo, University of Balearic Islands, Palma de Mallorca.

Summary

The practice of physical generates reactive oxygen species (ROS) with potential to influence on numerous cellular processes. ROS can exert a dual role depending on the concentration. High levels are associated to oxidative stress and damage to biomolecules and cellular structures, while moderate levels act as cellular messengers modulating various signalling pathways. Acute and high intensity exercise sessions are associated to high ROS generation rates that may overwhelm antioxidant defences. Conversely, the repetition of moderate intensity exercises is associated to tolerable doses of ROS that appear to be involved in the regulation of cell signalling cascades that play a fundamental role in mediating the training-derived benefits. On the other hand, it has been described that omega 3 fatty acids exert beneficial effects on the body by modulating the mechanisms of antioxidant defence and inflammation. Thus, the general aim of this thesis focuses on evaluating and characterizing the antioxidant, inflammatory and mitochondrial differential response against different doses of prooxidant stimulus. In addition we aimed to assess the use of docosahexaenoic acid (DHA) as a nutritional supplement in this context.

In trained football players, eight weeks of training and DHA consumption increases the content of this omega 3 in the erythrocyte membranes. Acute training and exercise promotes mitochondrial biogenesis and remodelling events in these subjects and enhances mitochondrial respiration capacity in PBMCs. Supplementation of the diet with DHA for eight weeks contributes synergistically to the effects of training on mitochondrial remodelling.

An active lifestyle is accompanied by lower weight, fat mass, BMI, diastolic blood pressure and neutrophils and lymphocytes counts in healthy sexagenarian compared to a sedentary lifestyle. In active subjects in basal conditions there is an improvement of the endogenous antioxidant capacity induced by the regular practice of moderate physical activities. In addition,

there is an attenuation of proinflammatory parameters and an enhancement of anti-inflammatory markers in active subjects compared to sedentary peers. Thus, an active lifestyle exerts beneficial effects on body composition, antioxidant defences and the anti-inflammatory status in the elderly.

The continuous exposure of PBMCs and neutrophils to sustained H_2O_2 produced by the enzyme glucose oxidase allows emulating an *ex vivo* situation of chronic oxidative stress. Neutrophils have a greater capacity to eliminate H_2O_2 than PBMCs. This fact is translated into lower levels of H_2O_2 in the culture medium of neutrophils, which limits the effects of H_2O_2 on gene expression and ROS production by these cells. Continuous *ex vivo* exposure of PBMCs and neutrophils to sustained concentrations of H_2O_2 stimulates in a hormetic way the processes involved in the mitochondrial dynamics and biogenesis and the expression of proinflammatory genes in these cells. A control of the levels of H_2O_2 in the organism could have medical relevance in diseases with altered inflammatory and redox states as the metabolic syndrome.

In conclusion, the activation of antioxidant defences, the induction of oxidative damage, together with the modulation of the mitochondrial biogenesis dynamics and the markers of inflammation, are processes that are closely related in response to different degrees of oxidative stress derived from the practice of physical activity, lifestyle and exogenous H_2O_2 exposure. The intake of DHA in moderate doses is effective to enhance synergistically antioxidant and mitochondrial adaptations.

Listado de artículos originales

La presente tesis se basa en los siguientes artículos originales:

Manuscript I. Training and acute exercise modulates mitocondrial dynamics in football player's blood mononuclear cells. **Carla Busquets-Cortés**, Xavier Capó, Miquel Martorell, Josep A. Tur, Antoni Sureda & Antoni Pons. *2017. European Journal of Applied Physiology*, *117*(10), 1977–1987. doi: 10.1007/s00421-017-3684-z. Epub 2017 Jul 26.

Manuscript II. Training Enhances Immune Cells Mitochondrial Biosynthesis, Fission, Fusion, and Their Antioxidant Capabilities Synergistically with Dietary Docosahexaenoic Supplementation. **Carla Busquets-Cortés**, Xavier Capó, Miquel Martorell, Josep A. Tur, Antoni Sureda &Antoni Pons. 2016. *Oxidative Medicine and Cellular Longevity*, *2016*, 2016:8950384. doi: http://doi.org/10.1155/2016/8950384

Manuscript III. Peripheral Blood Mononuclear Cells Antioxidant Adaptations to Regular Physical Activity in Elderly People**. Carla Busquets-Cortés**, Xavier Capó, Maria del Mar Bibiloni, Miquel Martorell, Miguel D. Ferrer, Emma Argelich, Cristina Bouzas, Sandra Carreres, Josep A. Tur, Antoni Pons & Antoni Sureda. Nutrients 2018, 10, 1555; doi:10.3390/nu10101555

Manuscript IV. Regular practice of moderate physical activity by older adults ameliorates their anti-inflammatory status. Miguel D Ferrer; Xavier Capó; Miquel Martorell; **Carla Busquets-Cortés**; Cristina Bouzas; Sandra Carreres; David Mateos; Antoni Sureda; Josep A. Tur & Antoni Pons. *2018. Nutrients, 10, 1780; doi:10.3390/nu10111780*

Manuscript V. Effects of hydrogen peroxide on inflammatory and redox gene expression in immune cells. **Carla Busquets-Cortés**, Xavier Capó, Miquel Martorell, Josep A. Tur, Antoni Sureda & Antoni Pons. *2018.* En vías de publicación.

Del trabajo de la presente tesis se derivan los siguientes artículos, también relacionados con la temática de la misma, que no forman parte del cuerpo de la tesis y que se incluyen como Anexo:

Manuscript VI. Effects of dietary almond- and olive oil-based docosahexaenoic acid- and Vitamin E-enriched beverage supplementation on athletic performance and oxidative stress markers. Xavier Capó, Miquel Martorell, **Carla Busquets-Cortés**, Joan Riera, Franchek Drobnic, Josep A. Tur, Antoni Sureda & Antoni Pons, (2016). *Food and Function*, 7(12), 4920–4934.
http://doi.org/10.1039/c6fo00758a

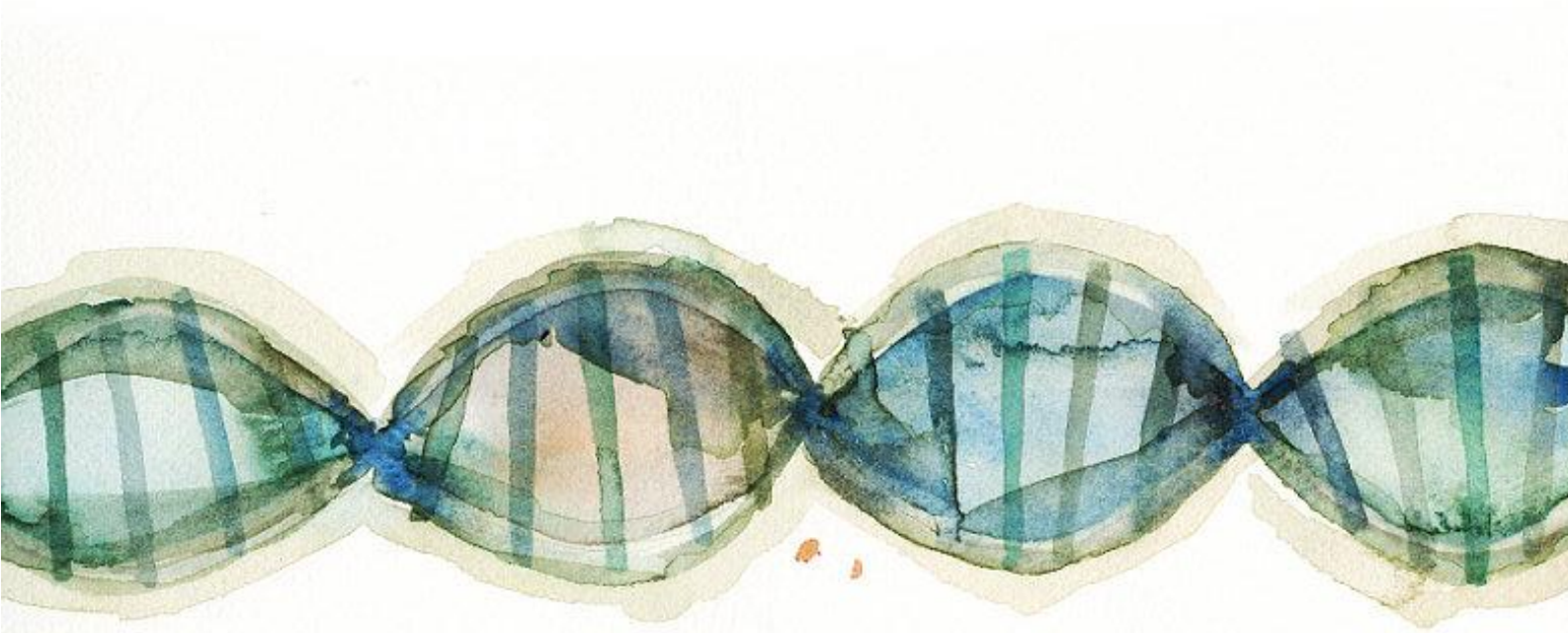
Manuscript VII. Resolvins as proresolving inflammatory mediators in cardiovascular disease. Xavier Capó, Miquel Martorell, **Carla Busquets-Cortés**, Silvia Tejada, Josep A. Tur, Antoni Pons, & Antoni Sureda. (2018). *European Journal of Medicinal Chemistry,* 153.
http://doi.org/10.1016/j.ejmech.2017.07.018

Manuscript VIII. Cyclooxygenase-2 inhibitors as a therapeutic target in inflammatory diseases. Miguel D. Ferrer, **Carla Busquests-Cortés**, Xavier Capó, Silvia Tejada, Josep A. Tur, Antoni Pons & Antoni Sureda. (2018). *Current Medicinal Chemistry*, 25, 1–15.
http://doi.org/10.2174/0929867325666180514112124

Agradecimientos a la financiación recibida

Esta tesis doctoral se ha llevado a cabo en el Grupo de Nutrición Comunitaria y Estrés Oxidativo (NUCOX), en el Laboratorio de Ciencias de la Actividad Física, en el Departamento de Biología Fundamental y Ciencias de la Salud de la Universitat de les Illes Balears (UIB) y en el Centro de Investigaciones Biomédicas en Red de Fisiopatología de la Obesidad y la Nutrición (CIBERobn) del Instituto de Salud Carlos III (CB12/03/30038). Esta tesis doctoral se enmarca en el Programa de Doctorado en Nutrición y Ciencias de los Alimentos (RD99) de la Universitat de les Illes Balears y ha sido posible gracias a los proyectos otorgados por el Ministerio Español de Sanidad y Consumo (Instituto de Salud Carlos III, Programa de Promoción de la Investigación Biomédica y Ciencias de la Salud, Proyectos 05/1276, 08/1259 y 11/01791, Red Predimed (RETIC RD06/0045/1004, PI14/00636), otorgados por el del Ministerio de Ciencia e Innovación (Acción Estratégica en Salud DPS2008-07033-C03-03), otorgados por el Gobierno de la Comunitat Autònoma de les Illes Balears (Ayudas a Grupos de Investigación Competitivos en el Sistema de Innovación de las Illes Balears ref. 32/2011), y acciones especiales AAEE35/2011 i AAEE 23/2012 de la Direcció General d'Universitats Recerca i Transferencia del Coneixement de la Conselleria d'Educació, Cultura i Universitats) y cofinanciado con fondos FEDER de la Unión Europea. La presente tesis no habría sido posible sin la beca-contrato predoctoral FPI/1648/2014 otorgada por la Conselleria d'Educació, Cultura i Universitats, Direcció General d'Universitats i Recerca, Govern de les Illes Balears y el Fondo Social Europe (FSE).

I. Introducción



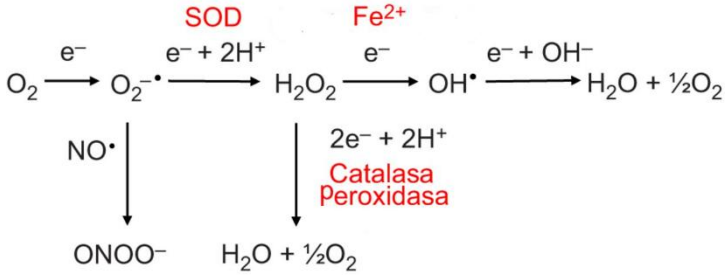
1. Estrés oxidativo

El estrés oxidativo como concepto en Biología redox fue formulado por primera vez en 1985 [1,2] y hace referencia a un estado fisiológico en el que los agentes oxidantes exceden la capacidad de los sistemas antioxidantes en el organismo como consecuencia de una pérdida del equilibrio entre la producción de radicales libres y la capacidad del cuerpo para contrarrestar sus efectos dañinos [3–6]. La regulación del estado reductor y oxidante es fundamental para la viabilidad, activación, proliferación y función de células, órganos y tejidos e incluye antioxidantes enzimáticos y no enzimáticos que generalmente son efectivos para mitigar los efectos dañinos de los radicales libres. La investigación actual sobre los interruptores redox moleculares que orquestan las respuestas al estrés oxidativo está en pleno auge. Entre los escollos inherentes a la metodología destaca el hecho de que los mecanismos moleculares subyacentes deben ser descifrados y elucidados en cada caso particular, cosa que resulta obvia para alcanzar un concepto global. La investigación del estrés oxidativo abarca no sólo la Química, sino también la Bioquímica, la Biología Celular, la Fisiología y la Medicina.

1.1 Especies reactivas de oxígeno

El oxígeno es un elemento esencial para la vida ya que actúa como aceptor final de electrones en la cadena respiratoria mitocondrial. En este proceso respiratorio la mayor parte del oxígeno se reduce a agua, pero se estima que alrededor de un 2% del oxígeno puede dar lugar a la formación de las especies reactivas de oxígeno (del inglés *Reactive Oxygen Species*, ROS)[7].

Diagrama 1. Reacciones de generación de los tres principales radicales libres de oxígeno: el ion superóxido, el peróxido de hidrógeno y el radical hidroxilo.



(Recurso: modificado de Tomanek, 2015)

Como puede verse en el diagrama 1, si el oxígeno molecular capta un electrón procedente de la cadena respiratoria mitocondrial [8] se genera el radical superóxido ($O_2^{\cdot-}$), siendo el radical más abundante y común a nivel celular. El $O_2^{\cdot-}$ en disolución acuosa, además de por la acción de la superóxido dismutasa (SOD), se puede dismutar de forma espontánea generando H_2O_2 [9,10]. Si ocurre que en el medio de reacción existen trazas de metales de transición -como el hierro o el cobre- que actúen como catalizadores, el $O_2^{\cdot-}$ y el H_2O_2 pueden combinarse y generar el radical hidroxilo ($OH^{\cdot}$). Otra forma reactiva del oxígeno es estado excitado de menor energía del oxígeno molecular: el oxígeno singlete ($^1O_2$), una especie electrofílica sumamente reactiva de corta vida media [11] capaz de difundir a través de las membranas celulares.

El $O_2^{\cdot-}$ es la base conjugada de un ácido débil, el radical hidroperóxido ($HOO^{\cdot}$) [12]. En las proximidades de la membrana, donde se produce dicho radical, el pH es mucho más bajo que en el citoplasma, por lo que predomina la forma ácida o $HOO^{\cdot}$. Debido a su carácter no iónico, el $HOO^{\cdot}$ posee la capacidad para penetrar la membrana celular y desencadenar procesos de peroxidación lipídica. El $HOO^{\cdot}$ es mucho más reactivo y más oxidante que el $O_2^{\cdot-}$, pero en disolución acuosa a pH fisiológico predomina la forma no protonada, es decir $O_2^{\cdot-}$. El $O_2^{\cdot-}$posee una vida media relativamente larga y muestra capacidad para difundir por la célula, incrementándose así el posible número de dianas de reacción posibles, además de mostrar la capacidad de reaccionar fácilmente con el óxido nítrico (NO) dando como producto el peroxinitrito ($OONO^-$) [13]. Los peroxinitritos son altamente reactivos y contribuyen al daño oxidativo generando productos tóxicos como el 4-hidroxinonenal por peroxidación lipídica, la oxidación de los grupos carbonilos de las proteínas y la nitración de los residuos tirosina de las proteínas (3-nitrotirosina), particularmente nocivos para las células [14]. El $OH^{\cdot}$ es una especie altamente reactiva con un elevado potencial oxidante, ya que reacciona con moléculas que se encuentren cercanas a su lugar de formación y que está implicado en reacciones de peroxidación lipídica de ácidos grasos no saturados.

Además de la radiación ultravioleta, que produce especies reactivas libres como el $^1O_2$, existen varios sistemas enzimáticos en distintos compartimentos celulares con capacidad para generar estas especies. Las mitocondrias, presentes en todas las células aerobias, son la principal fuente biológica de ROS.

El flujo de electrones a través de la cadena de transporte electrónico mitocondrial es un proceso imperfecto y parte del oxígeno consumido por las mitocondrias es reducido de forma incompleta por electrones que *escapan* de la cadena de transporte electrónico. El radical $O_2^{\cdot-}$ generado en las mitocondrias no atraviesa la membrana mitocondrial interna, por lo que se encuentra confinado en la matriz donde reacciona rápidamente con la enzima MnSOD y con el óxido nítrico dando lugar al H_2O_2 y al $OONO^-$, respectivamente. En la membrana celular se generan radicales $O_2^{\cdot-}$, H_2O_2, $^1O_2$ y ácido hipocloroso (HClO) mediante la acción de las enzimas NAD(P)H oxidasa -las cuales operan bajo el control de los factores de crecimiento y las citoquinas (22)-, mieloperoxidasa (MPO) y xantina oxidasa (XO) [15]. Además, otras enzimas como la lipooxigenasa (LOX) y la ciclooxigenasa (COX) también generan ROS durante la síntesis de leucotrienos, tromboxanos y prostaglandinas [16]. Los macrófagos y neutrófilos expresan la enzima NADPH oxidasa que cataliza la generación de $O_2^{\cdot-}$ a partir de oxígeno y NADPH [17]. De hecho, el proceso de fagocitosis por parte de neutrófilos y macrófagos una vez activados implica también la liberación de $O_2^{\cdot-}$ [18], que actúa en la defensa antibacteriana en un proceso denominado *oxidative burst* o estallido oxidativo.

El retículo endoplasmático es también una fuente de $O_2^{\cdot-}$ y H_2O_2 mediante la autooxidación de la flavoproteína NADPH citocromo P-450 reductasa y el citocromo P-450[19]. En el citoplasma se encuentran enzimas como la XO (metabolismo de las purinas) y la aldehído deshidrogenasa que durante sus ciclos catalíticos generan ROS y pequeñas moléculas solubles, como catecolaminas, flavinas y tioles, que mediante procesos de autooxidación reducen el oxígeno [20]. Las mitocondrias continuamente expuestas a elevados niveles de ROS sufren la oxidación de los complejos de la cadena de transporte electrónico, del mtDNA y de los lípidos, de manera que su función puede verse comprometida. El daño de estas moléculas empeora la función de la cadena de transporte electrónico, dando lugar a mayor producción de ROS [5,21].

1.2 Células sanguíneas y ROS

1.2.1 Células mononucleares de sangre periférica

Las células mononucleares de sangre periférica (PBMCs) son células sanguíneas que poseen un único núcleo, como los linfocitos y los monocitos. Los linfocitos se dividen en tres poblaciones celulares en base a su función y a sus componentes de membrana: linfocitos B, linfocitos T y células *natural killer* (NK). La producción de ROS en el sistema inmunitario se ha estudiado principalmente en células fagocíticas como macrófagos, derivados de los monocitos, y neutrófilos. Sin embargo, algunos estudios se han centrado también en la producción de ROS por parte de los PBMCs y la importancia de estas especies reactivas en la activación del linfocito frente a estímulos inmunitarios [22]. Tras la estimulación de PBMCs con agentes inmunogénicos como el forbol miristato acetato (PMA), zymosan (ZYM) o lipopolisacárido (LPS), así como con señales que estimulan los receptores de las células T (TCR), se produce un incremento en la generación de ROS por parte de estas células [23]. El metabolismo del ácido araquidónico, la mitocondria y la NAD(P)H oxidasa parecen ser las principales fuentes de ROS en el linfocito tras la activación de los receptores del TCR, siendo el H_2O_2 y el $O_2^{\cdot-}$ las principales ROS producidas en este proceso [24,25]. Las ROS generadas tras la estimulación de receptores de membrana parecen estar implicadas en procesos de señalización celular como la del factor nuclear kappa B (NFkB) (regulación de genes inflamatorios) o la vía de las MAPK (regulación del ciclo celular). Además, evidencias científicas han demostrado que la práctica de ejercicio vigoroso da como resultado un aumento transitorio del estrés oxidativo en PBMCs, detectable por elevados niveles de carbonilos en proteína en estas células acompañados por un aumento en peróxidos lipídicos plasmáticos [26,27]. Por tanto, se puede decir que las PBMCs constituyen un grupo heterogéneo de células con funciones diversas con capacidad para generar ROS, a la vez que están expuestas a ROS producidas por otras células. Durante los experimentos llevados a cabo en nuestro grupo de investigación durante los últimos años, y los propios que conforman la presente tesis, se ha trabajado con PBMCs, y se han elegido las ROS inducidos por ejercicio como el estímulo externo para valorar los efectos (daño-funcionalidad) sobre estas células y comprobar la influencia de situaciones fisiológicas como la actividad física sobre diferentes parámetros como la dinámica mitocondrial y marcadores de inflamación.

1.2.2 Neutrófilos

La función principal de los neutrófilos en la respuesta inmune innata es llevada a cabo a través de toda una serie de respuestas rápidas y coordinadas que culminan con la fagocitosis y eliminación de los patógenos. Para ello los neutrófilos contienen un potente *stock* de moléculas con capacidad antimicrobiana: agentes oxidantes, proteínasas y péptidos antimicrobianos, así como una elevada capacidad para producir especies reactivas [28]. Las principales fuentes de ROS en neutrófilos son la NADPH oxidasa, el metabolismo del ácido araquidónico, las óxido nítrico sintasas (NOS) y, en menor medida, la cadena de transporte electrónico, ya que el neutrófilo es un tipo celular que posee pocas mitocondrias. La NADPH oxidasa se encuentra latente cuando el neutrófilo está inactivo, pero se activa rápidamente tras estímulos bacterianos, y es la responsable del llamado *oxidative burst* en el que participan ROS como H_2O_2, $OH^{\cdot}$ i HClO. Durante la fagocitosis, el neutrófilo libera los compuestos antimicrobianos y las ROS en fagosomas, pero a pesar de esta estructuración celular, en condiciones patológicas los compuestos citotóxicos pueden ser liberados al espacio extracelular e inducir daño en los tejidos circundantes. Para hacer frente a esta elevada producción de especies reactivas, los neutrófilos poseen un elaborado sistema de oxidantes, tanto enzimáticas como no enzimáticas (vitaminas C, E y glutatión). Además, contienen cantidades importantes de ascorbato cuya función es la de preservar integridad celular y la del tejido circundante mediante la neutralización de los productos bactericidas producidos durante el *oxidative burst*.

1.3 ROS y hormesis

A pesar de la tradicional asociación de las ROS con la promoción de lesiones oxidativas, circunstancias patológicas e incluso con el propio fenómeno de envejecimiento, cabe destacar que estas moléculas resultan ser importantes elementos de comunicación entre las mitocondrias y el resto de la célula y actúan como señalizadores en varios procesos biológicos y fisiológicos [29–31]. De hecho, mientras niveles elevados de ROS se relacionan con eventos oxidantes, a niveles bajos/moderados se le asocian funciones como mensajeros activando vías sensibles al estado redox y promoviendo respuestas celulares encaminadas al mantenimiento de la homeostasis y a la adaptación para hacer frente al estrés oxidativo [6]. Este fenómeno de respuesta a dosis recibe el

nombre de *hormesis*, término proveniente del griego ὁρμάω, «hormáein», que significa estimular. La hormesis hace referencia a una respuesta adaptativa caracterizada por una dosis-respuesta bifásica (activación por dosis bajas-inhibición por dosis altas), que puede ser o bien directamente inducida, dependiendo del rango y amplitud del estímulo, o bien resultado de procesos biológicos compensatorios que aparecen tras una disrupción en la homeostasis celular o del organismo [32–34].

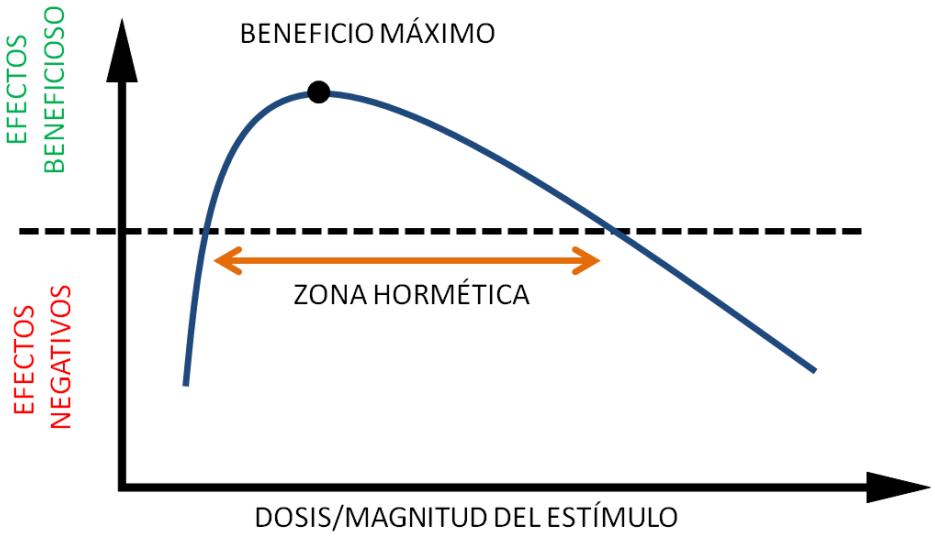


Figura 1. Fenómeno de la hormesis, caracterizado por una estimulación por dosis baja y una inhibición, o ausencia de efectos, para dosis altas, que resulta en una curva de respuesta a nuevas dosis en forma de J o de U invertida [35]***. (Imagen: elaboración propia).***

Este concepto de hormesis (Figura 1) resulta especialmente interesante desde el punto de vista biológico, ya que se trata de una respuesta adaptativa hacia bajos niveles de estrés o daño que produce una mejora de la eficiencia para algunos sistemas fisiológicos durante períodos de tiempo finitos. Bajo circunstancias específicas esto implica una respuesta continua a mensajes reguladores compensatorios, hasta que la condición de homeostasis se restablece [32,36]. Para recuperar la homeostasis debe existir una redistribución de los recursos del sistema en cuestión (células, tejido, etc.) que asegure que la reparación del daño es subsanada.

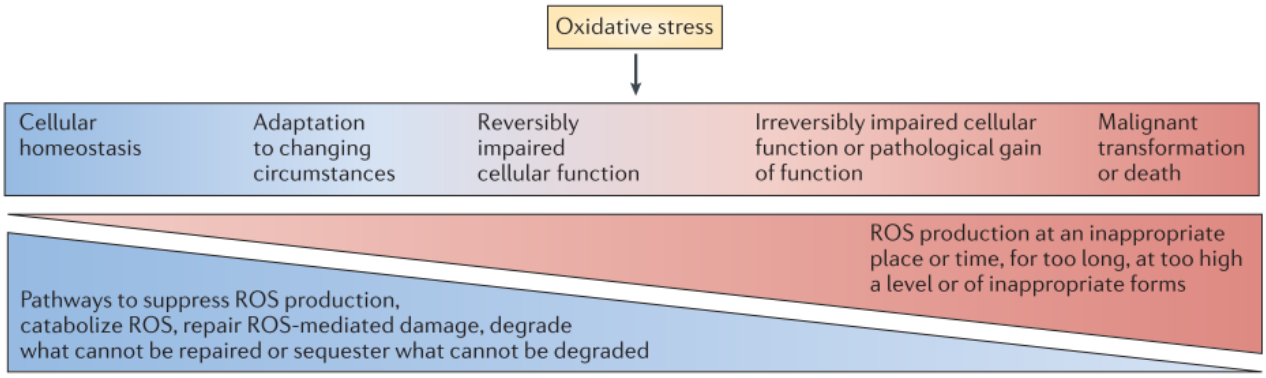


Figura 2. Espectro de acción atribuido a las ROS.
(Modificado de de Nathan and Cunningham-Bussel, 2013)[37]

Como puede observarse en la Figura 2, la restricción de la producción de ROS a ubicaciones subcelulares apropiadas y a períodos de tiempo, niveles y especies moleculares y definidas permite que las ROS contribuyan a la homeostasis y señalización celular (lado izquierdo). Cuando la producción de ROS escapa a estas restricciones (por ejemplo, niveles altos o producción sostenida), las macromoléculas son dañadas y aparece la situación de "estrés oxidativo". El daño mediado por ROS a menudo se puede revertir por reparación, reemplazo, degradación o secuestro de las macromoléculas deterioradas (centro). Sin embargo, el daño que excede la capacidad de reparación de la célula (lado derecho) puede conducir a la muerte célula. Cuando el daño al ADN resulta en mutagénesis, la consecuencia puede ser una transformación maligna [37].

En Biología y Bioquímica, el tipo de hormesis más relevante es aquel en el que la exposición a factores que median un estrés moderado en células o en organismos puede dar lugar a una adaptación que prevenga los daños derivados de un futuro estrés más intenso o prolongado. Recientemente se han descrito procesos de hormesis relacionados con orgánulos celulares concretos [38,39]. El que más relevancia ha adquirido es el denominado mitohormesis [40], en el cual un estrés moderado sobre la mitocondria desencadena una serie de respuestas que inducen un estado citoprotector derivado de cambios bioquímicos y metabólicos de este orgánulo [38]. Este término de mitohormesis se empleó inicialmente para describir el aumento agudo de la respiración mitocondrial cuando éstas eran están expuestas a toxinas como por ejemplo metales [41]. El mecanismo que parece subyacer a este fenómeno está relacionado con la respuesta coordinada entre el núcleo y la mitocondria. Ésta respuesta se puede

desencadenar bien por cambios en el potencial de membrana mitocondrial, que permiten el ensamblaje y reclutamiento de moléculas de señalización o la producción de ROS; o bien por modificación de los niveles de cofactores como el acetil-CoA o el par NAD+/NADH, implicados en la regulación de la actividad enzimática de proteínas como las sirtuinas, o por modificaciones en la propia estructura de la mitocondria y en su distribución. Las implicaciones de todos estos procesos en la biología humana están poco exploradas aún.

1.3.1 ROS: Efectos negativos

Los ácidos grasos poliinsaturados (PUFA), especialmente el ácido araquidónico [42], presentes en las membranas celulares, son especialmente susceptibles de ser atacados por los radicales libres, especialmente por el $OH^{\cdot}$, debido a que los hidrógenos de los dobles enlaces son particularmente reactivos [43,44]. Ello conduce a la formación y acumulación ubicua de productos de la oxidación lipídica [12,45], particularmente oxisteroles, hidroperóxidos y endoperóxidos. Los radicales lipídicos intermediarios que se forman durante la propagación son muy inestables y reaccionan rápidamente con otros compuestos, por tanto la peroxidación lipídica se suele determinar a través de los productos finales de reacción mayoritarios tales como los aldehídos α,β-insaturados (4-hidroxinonenal), di-aldehídos (malondialdehido, MDA) y ceto-aldehidos [46]. La estructura no cargada de estos aldehídos les permite migrar con relativa facilidad a través de membranas hidrófobicas y medios citosólicos hidrofílicos, aumentando así la distancia de migración con respecto al sitio de producción. Estos compuestos reaccionan con grupos nucleófilos en macromoléculas como proteínas y ácidos nucleicos, lo que puede derivar en daños estructurales a estas biomoléculas y pudiendo quedar comprometida su función. A modo de ejemplo, se han observado incrementos en el contenido de MDA atribuibles a la presencia excesiva de ROS en a) eritrocitos de mujeres con porfiria variegata, un desorden metabólico de la biosíntesis de los grupos hemo [22,47] b) en lesiones de la mucosa gástrica [48] c) en plasma de ciclistas semiprofesionales tres horas después de la carrera [49,50] d) en eritrocitos y PBMCs de futbolistas jóvenes tras una prueba de esfuerzo extenuante [25], entre otros. En este sentido, se ha hipotetizado que la activación de la XO contribuye a la generación de radicales libres durante la práctica de ejercicio [51].

Las proteínas también son moléculas diana de las ROS, principalmente H_2O_2, $OH^{\cdot}$ y NO, debido a su compleja estructura y elevado número de grupos funcionales oxidables. La modificación oxidativa post-traduccional de las proteínas puede ser catalizada en presencia o ausencia de metales. Las oxidaciones catalizadas por metales conllevan a la formación de grupos carbonilo en las cadenas laterales y se puede medir determinando los niveles de derivados carbonilos generados. Proteínas moderadamente carboniladas son marcadas para su degradación por el sistema proteasomal de la célula. Sin embargo, proteínas altamente carboniladas tienden a acumularse en forma de agregados de elevado peso molecular resistentes a la degradación, ejerciendo un impacto severo en la maquinaria celular [52]. Existen evidencias que apoyan el rol de la carbonilación de proteínas en la patogénesis de diversos trastornos neurodegenerativos humanos. [52–56]. Las nitrotirosinas (Nitro-Tyr) son producto de la nitración de tirosina mediado por especies reactivas del nitrógeno tales como el $OONO^-$ y el dióxido de nitrógeno, y la determinación de niveles de Nitro-Tyr también se usa como marcador del estrés oxidativo. La oxidación de las proteínas conduce a una modificación de la estructura de éstas, lo que en última instancia puede acabar modificando su función, llevando incluso la pérdida de la actividad enzimática en el caso de las proteínas dañadas sean enzimas.

La oxidación de DNA tiene lugar de manera continua en la célula incluso en ausencia de una situación de estrés oxidativo, y parece ser que estas lesiones oxidativas en las bases del DNA se van acumulando con la edad y forman parte del proceso de envejecimiento normal del ser humano [5,57]. La modificación de las bases de los nucleótidos, la aparición de roturas en una o las dos cadenas y la aparición de entrecruzamientos DNA-proteína son algunas de las modificaciones oxidativas que sufre el DNA. El producto más común del oxidación de las bases de DNA es la 8-hidroxi-7,8-dihidro-2,-deoxiguanosina (8-OHdG). La oxidación del DNA mitocondrial como consecuencia de un incremento en la producción mitocondrial de ROS parece estar implicada en el desarrollo diferentes patologías tales como el cáncer, la enfermedad del Parkinson, Esclerosis Lateral Amiotrófica o la enfermedad de Alzheimer [58].

1.3.2 ROS: señalización celular

Los desequilibrios que se generan a raíz de diferentes factores estresantes son el resultado de un desplazamiento temporal metabólico no perjudicial hacia el límite del rango fisiológico normal. Las células y tejidos poseen diversos mecanismos para restablecer el estado redox original. El estado redox intracelular está principalmente regulado por el glutatión reducido (GSH) endógeno y el sistema Tiorredoxina [59,60], los dos principales mecanismos antioxidantes dependientes del tiol en las células [61]. El GSH es un tripéptido con capacidad antioxidante que desempeña una serie de funciones clave en el control de los procesos de señalización, desintoxicación de ciertos xenobióticos y metales pesados, entre otras funciones. Dentro de las células, el GSH total existe libre y unido a proteínas. Dado que la enzima glutatión reductasa, que revierte el glutatión libre de su forma oxidada (GSSG), es constitutivamente activa e inducible ante el estrés oxidativo, el glutatión libre existe casi exclusivamente en su forma reducida [59]. En condiciones normales, se sabe que el par redox GSH está presente en células de mamíferos en el rango de concentración de 1-10 mM. En una célula en reposo, la relación molar GSH: GSSG supera los 100:1, mientras que en varios modelos de estrés oxidativo se ha demostrado que esta relación disminuye a valores de 10: 1 e incluso 1:1 [63]. El mantenimiento de la homeostasis redox se logra a través de mecanismos de retroalimentación que operan a diferentes niveles de complejidad. Además de modificaciones post-traduccionales diversas, una de las estrategias radica en la inducción por parte de las ROS de cascadas de señalización que conducen a la expresión de enzimas antioxidantes o derivan en un incremento de cisteína, lo que facilita el incremento en los niveles de glutatión.

Tradicionalmente el único papel destacado de las ROS era el de moléculas dañinas, causantes de oxidación de biomoléculas e inductoras de inactivación y disfunciones moleculares. Sin embargo, estudios realizados durante los últimos años [6,64–67] han descrito que las ROS son un componente imprescindible en numerosas funciones fisiológicas como la modulación de la respuesta antioxidante, regulación del tono vascular y de las funciones controladas por la concentración de oxígeno y potenciación de la transducción de señales a partir de receptores de membrana. Esta paradoja biológica es la base de mecanismos que resultan ser de vital importancia para la integridad de los organismos vivos y su envejecimiento. Las vías que regulan la homeostasis de las especies

reactivas son cruciales para mitigar la toxicidad de las ROS y proporcionar una fuerte evidencia sobre la especificidad en la señalización de ROS [68]. Las células de los mamíferos responden al estrés oxidativo utilizando respuestas protectoras de larga duración que suelen ser parte de programas de diferenciación global o mecanismos de muerte celular [68]. Las ROS pueden actuar a través de muchas vías de transducción de señales diferentes interaccionando con elementos señalizadores tales como calcio, tirosina quinasas/fosfatasa y fosfolipasas. La interacción de las especies reactivas con estas proteínas quinasas y fosfatasas resulta de vital importancia en los procesos reguladores ya que las cascadas de fosforilación están implicadas en numerosas funciones de transmisión de señales extracelulares desde la membrana plasmática hasta el núcleo. Si bien hay toda una serie de agentes químicos que pueden actuar como moléculas señalizadoras en respuesta al estrés oxidativo, uno de los más destacables es el H_2O_2, ya que se produce constantemente en la cadena respiratoria y es relativamente estable a pH fisiológico.

1.4 Peróxido de hidrógeno

El H_2O_2 se genera, de manera espontánea o catalizada por la enzima superóxido dismutasa, por la reducción del $O_2^{\cdot-}$[9]. Las mitocondrias son una fuente permanente de H_2O_2 [69–71]. En condiciones fisiológicas el H_2O_2 es relativamente estable y permeable a la membrana celular, de manera que puede difundir al citosol [72–77]. Las acuaporinas facilitan el paso del H_2O_2 a través de las membranas y desempeñan un papel funcional en la translocación de H_2O_2, por lo que también se las denomina peroxiporinas [78,79]. A pesar de tratarse de un agente citotóxico, el H_2O_2 no posee un potencial oxidante muy elevado en disolución acuosa [73], así que no es capaz de oxidar el DNA o los lípidos directamente. Por tanto, debido a sus propiedades fisicoquímicas, el H_2O_2 es capaz de servir como mensajero para transportar una señal redox desde el sitio de su generación hasta un sitio diana [6]. La regulación redox puede tener lugar a través del control de la actividad enzimática o a nivel transcripcional. El H_2O_2 modula la actividad de numerosos factores de transcripción en células de mamíferos, a destacar el factor nuclear eritroide 2 (Nrf-2), el factor de hipoxia inducible (HIF-1) y el NFκB, entre otros [80–82].

La regulación redox por parte del H_2O_2 viene determinada por la sensibilidad redox de las proteínas tirosina fosfatasas (PTP). Un ejemplo es la proteína fosfatasa 1 (PP1), la cual es inhibida por la oxidación de su centro de metal [83]. El *pool* de hierro lábil intracelular (no ligado a ferritina) es un determinante de la señalización redox inducida por H_2O_2 [84]. De todo ello se deduce que la especificidad y el ajuste fino se ejercen a través del control de fuentes y diana/sumideros de H_2O_2. En cuanto a las fuentes, el control preciso de las NADPH oxidasas por señales físicoquímicas resulta fundamental [85]. En cuanto a los sumideros, se ha centrado mucho interés en las peroxiredoxinas [86,87]. Por ejemplo, la peroxiredoxina-2 actúa como un receptor primario de H_2O_2 altamente sensible que transmite específicamente los equivalentes oxidativos a factores de transcripción regulados por el estado redox, como el STAT3 (del inglés, s*ignal transducer and activator of transcription 3*).

Estos mecanismos de transducción de señales redox reciente documentados están regulados por interruptores redox (del inglés, *redox switches*) [1,9,88,89], entre los que destacan por ejemplo el par Nrf2/Keap1 o NF-κB. De hecho, las diferentes vías de señalización reguladas por el H_2O_2 son activadas por diferentes concentraciones de este agente oxidante y, consecuentemente, tienen lugar mediante diferentes cinéticas [81,90–93]. El concepto de "estado estacionario de H_2O_2 de bajo nivel" (del inglés, *constitutive celular low level H_2O_2 steady-state*) está ganando reconocimiento según las últimas publicaciones, ya que la modulación y el mantenimiento de las funciones celulares mediante reacciones redox es la esencia del estrés oxidativo fisiológico, también llamado *oxidative eustress* o estrés beneficioso. Este estado fisiológico influye positivamente sobre multitud de procesos biológicos. Contrariamente, altos niveles localizados de H_2O_2 se producen en el llamado *oxidative distress*, con implicaciones negativas, siendo el ejemplo más característico *el oxidative burst* de los neutrófilos [37,94]. Concentraciones suprafisiológicas de H_2O_2 resultan muy nocivas para la célula, ya que al atravesar las membranas biológicas [81,95] puede oxidar diferentes grupos químicos como sulfhidrilos y sulfóxidos, además de promover la formación del radical hidroxilo en puntos alejados de su lugar de origen y tener la capacidad para inactivar ciertos enzimas [1,9,82,96–101]. De hecho, el aumento de las concentraciones de H_2O_2 intracelular se asocia con fenotipos patológicos e inestabilidad genética, llegando a un umbral tóxico que causa la muerte celular [94]. La diferenciación espaciotemporal y funcional de estos dos estados

extremos en células y tejidos constituye todo un desafío actualmente, y los estudios sobre los procesos horméticos de moléculas como el H_2O_2 están a la orden del día.

1.5 Defensas antioxidantes

Para evitar o neutralizar los efectos deletéreos que causaría un exceso de especies reactivas, el organismo presenta un potente sistema antioxidante, compuesto por mecanismos enzimáticos y no enzimáticos [102]. Los antioxidantes se encuentran presentes y funcionan tanto en el medio extracelular como en el interior de la célula, en este caso compartimentados entre los diferentes orgánulos y espacios celulares [4]. El mecanismo de estos sistemas radica en reducir la producción de ROS y/o aumentar su detoxificacion (o la de los productos de su reacción). Así, pueden operar regulando las concentraciones locales de oxígeno libre para prevenir la formación de radicales, inhibiendo sustancias prooxidantes, quelando iones metálicos, eliminando especies de oxígeno activadas o transformándolas en moléculas con menor potencial dañino, reparando el daño oxidativo y favoreciendo la eliminación de moléculas y orgánulos dañados.

1.5.1 Defensas antioxidantes enzimáticas

Las células poseen una batería de enzimas cuya función se basa en convertir las ROS en especies no reactivas o menos dañinas, y la reparación de los daños producidos por la oxidación. Los principales enzimas antioxidantes son la catalasa (CAT), la glutatión peroxidasa (GPx), la superóxido dismutasa (SOD), y la glutatión reductasa (GRd) [103,104]. La superoxido dismutasa constituye el primer *escudo* frente a $O_2^{\cdot-}$ y su función es la dismutacion de este radical a H_2O_2 y oxígeno. En mamíferos existen tres isoformas: CuZnSOD, localizada en el citoplasma y el espacio intermembrana de la mitocondria, requiere cobre-zinc como cofactor; MnSOD, en la matriz mitocondrial y precisa de manganeso; y ecSOD, que requiere cobre-zinc y se encuentra en el espacio extracelular [105]. La glutatión peroxidasa (GPX) es una enzima tetramérica que cataliza la reducción de H_2O_2 e hidroperóxidos (ROOH) a H_2O y alcoholes (ROH), respectivamente. En este caso el glutatión reducido (GSH) actúa como donador de electrones, siendo oxidado a disulfuro de glutatión (GSSG o glutatión oxidado) [100,106]. Cinco son las isoformas de GPx descritas que catalizan la

misma reacción pero difieren en la especificidad de sustratos (diferentes hidroperóxidos) y en la localización celular (citosólica, mitocondrial, etc.). Esta variedad optimiza la función de la GPx como defensa antioxidante. Debido a que la disponibilidad de GSH debe ser constante para una correcta función de GPx, la célula dispone de mecanismo de regeneración de GSH partir de GSSG. La glutatión reductasa (GRd) es la flavoenzima encargada de la reducción de GSSG a GSH, utilizando NADPH como cofactor [107]. La catalasa es un homotetrámero ampliamente distribuido por toda la célula [108]que cataliza, al igual que la GPx, la descomposición del H_2O_2 a H_2O. Sin embargo, no requiere de ningún otro sustrato para llevar a término la reacción. La catalasa utiliza hierro como cofactor, unido al centro activo de la enzima como parte de un grupo hemo. Aunque comparte sustrato con las GPx, la catalasa posee menor afinidad por el H_2O_2 a bajas concentraciones (GPx Km → 1 μM vs Catalasa Km → 1 mM) [109,110]. Además de estas enzimas antioxidantes principales, la célula cuenta con otras enzimas que contribuyen directa o indirectamente al equilibrio redox, como las peroxirredoxinas (Prx) y el sistema de la tioredoxina, compuesto por tioredoxina (Trx), tioredoxina reductasa (TrxR) y NADPH. Éstas son proteínas oxidoreductasasas presentes en el citoplasma (TRX1) y la mitocondria (TRX2) y que actúan como antioxidantes facilitando la reducción de otras proteínas a través de un intercambio tiol-disulfuro en la cisteína, usando FAD como cofactor [111].

1.5.2 Otras proteínas antioxidantes

Para mantener bajo control la producción continua de ROS por parte la cadena respiratoria, la propia mitocondria posee otras proteínas a las que se les ha atribuido capacidad antioxidante: las sirtuinas y las proteínas desacoplantes (UCPs), presentes en la membrana interna de las mitocondrias.

Las UCPs se encuentran presentes en diversas isoformas según el tejido en el que se encuentren. Las UCPs, como miembros de la familia de proteínas portadoras de aniones mitocondriales, desacoplan la fosforilación oxidativa de la síntesis de ATP disipando la energía en forma de calor y reduciendo el potencial de membrana mitocondrial en células de mamíferos. La disipación parcial del gradiente electroquímico de protones a través de la membrana contribuye a reducir la producción de ROS en la cadena de transporte de electrones. Además, se ha comprobado que los niveles de expresión de esta

proteína aumentan cuando hay un exceso en el suministro de ácidos grasos a las mitocondrias y se supera la capacidad de oxidación. Mediante la translocación de peróxidos de ácidos grasos desde la membrana interna hacia la externa, las UCPs juegan un papel en la protección de las mitocondrias frente al estrés oxidativo inducido por los lípidos [112]. En músculo esquelético se ha comprobado que la expresión de mRNA de UCP3 se eleva bruscamente a los 45 minutos de ejercicio, alcanzando de 7 a 8 veces el nivel de reposo a los 150 minutos, mientras que el aumento en el contenido de proteína UCP3 se eleva ~1.9 veces a los 120 minutos de ejercicio. Tanto el ARNm de UCP3 como la proteína UCP3 vuelven gradualmente a los niveles de reposo 24 horas después del ejercicio [113]. Por lo tanto, el ejercicio agudo promueve la expresión de UCP3, mientras que el entrenamiento de resistencia da como resultado una menor expresión de UCP3, y ésta menor expresión se correlaciona con una mayor eficiencia energética [114]. Estas funciones de las UCPs han sido ampliamente investigadas en músculo, por lo que resulta de sumo interés profundizar en otros tejidos o células menos estudiadas hasta el momento, como por ejemplo las células inmunitarias.

Por su parte, las sirtuinas son proteínas histonadesacetilasas de clase III presentes en siete isoformas (SIRT1-7). Concretamente, la sobreexpresión de SIRT3 induce un incremento de en la respiración y el desacoplamiento, a la vez que disminuye la formación de ROS [115]. A pesar de que los mecanismos mediante los cuales SIRT3 favorece el incremento de la respiración no están del todo elucidados, algunas evidencias apuntan a la desacetilación y activación de enzimas mitocondriales implicados en el metabolismo respiratorio y en el control del ciclo de Krebs, como por ejemplo la isocitrato deshidrogenasa, glutamato deshidrogenasa y acetilcoenzima A sintasa 2 [116,117].

2. Ejercicio

EL ejercicio es aquella actividad física que al ser planificada, estructurada y repetida mantiene o mejora las funciones del organismo. Dentro del ejercicio encontramos el ejercicio agudo y el ejercicio crónico, también denominado regular o entrenamiento. El ejercicio agudo se define como un episodio o una sesión de corta duración y de intensidad variable, mientras que el ejercicio crónico es una repetición frecuente de sesiones de intensidad moderada.

Durante y tras una sesión de ejercicio tienen lugar respuestas fisiológicas en el organismo para hacer frente a las elevadas demandas energéticas transitorias.

2.1 Generación de ROS asociada al ejercicio

Las principales fuentes de ROS durante el ejercicio son la cadena de transporte electrónico mitocondrial a nivel muscular, la XO y la NADPH de macrófagos y neutrófilos. La cadena de transporte electrónico mitocondrial produce ROS de manera continua en condiciones basales, en una proporción de alrededor del 2% del oxígeno consumido. Tradicionalmente se ha aceptado que por el mero hecho que durante la práctica del ejercicio se consume más oxígeno, la producción de ROS también será mayor. La oxidación del sustrato mitocondrial juega un papel importante en la determinación del origen y las tasas de producción de $O2^{\cdot -}$ y H_2O_2. Se conocen 11 sitios mitocondriales distintos por donde pueden *escapar* electrones hacia oxígeno y producir $O^{2.-}$ y/o H_2O_2[118]. Los cambios en el estado bioenergético durante la práctica de ejercicio físico influyen en la disponibilidad del sustrato mitocondrial y conducen a alteraciones en las tasas y las contribuciones de diferentes sitios de producción mitocondrial de $O_2^{\cdot -}$ / H_2O_2 [118–121].

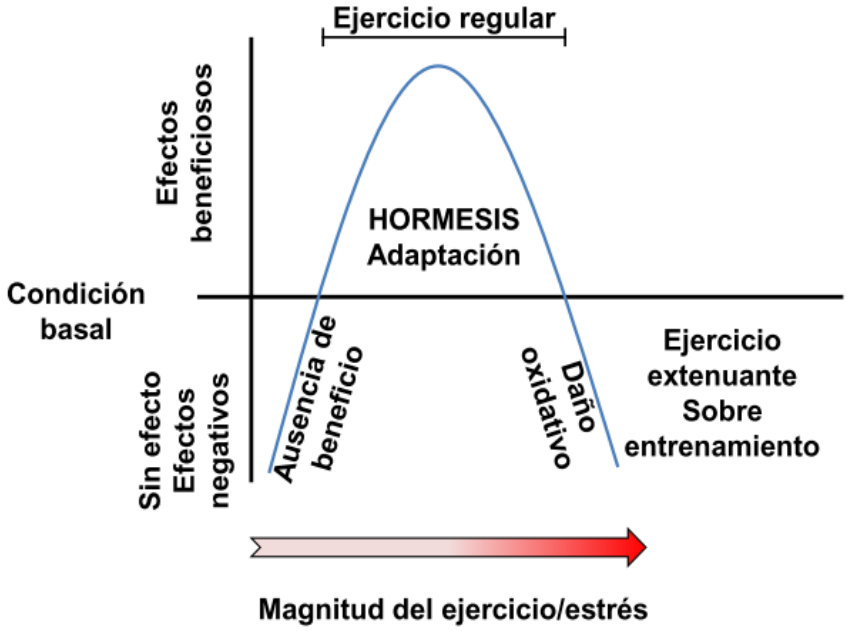


Figura 3. El fenómeno hormético aplicado a la práctica de ejercicio.
(Imagen: A. Sureda, 2018 para la SEBBM)

Como puede observarse en la Figura 3, la falta o muy leve práctica de ejercicio no conlleva a unos beneficios derivados del ejercicio, mientras que episodios de ejercicio extenuante o agotador comportan no solo fatiga muscular y lesiones a nivel tisular, sino que promueven la aparición de daño oxidativo. Sin embargo, la zona central de la curva representa la zona hormética o de adaptación; es decir, la práctica regular de ejercicio moderado, en otras palabras entrenamiento, promueve la aparición de efectos beneficiosos y adaptaciones dirigidas a sobrellevar futuros estímulos estresantes.

2.1.1 Ejercicio agudo

La práctica de un ejercicio agudo, sobre todo si es de elevada intensidad y extenuante, provoca daños estructurales a las células musculares y reacciones inflamatorias [4]. Algunos de estos efectos son atribuibles a una de una incrementada producción de ROS como resultado de la intensa actividad contráctil muscular. Para explicar el posible aumento de la producción de ROS se ha propuesto que las elevadas temperaturas que alcanza el interior del músculo durante la actividad física [123] provocan una desestabilización de la ubisemiquinona, lo que promueve un descenso en la transferencia electrónica a lo largo de la cadena a la vez que aumenta la transferencia directa del electrón al oxígeno [124]. Por otra parte, se ha descrito que durante la práctica de ejercicio exhaustivo, como respuesta inmune al daño que se produce en los tejidos, los neutrófilos y otra células fagocíticas también generan $O_2^{\cdot-}$, H_2O_2 y lisozima para fagocitar células dañadas [125,126]. Los neutrófilos participan de manera activa en la respuesta de fase aguda al ejercicio, experimentando incrementos en el número de células circulantes durante el proceso y produciendo grandes cantidades de ROS durante el *oxidative burst.* Este incremento en el potencial oxidativo del neutrófilo tras la realización de un ejercicio extenuante parece ir acompañado de un descenso en la actividad de las enzimas antioxidantes CAT, SOD y GPX, así como en los niveles de glutatión total y del ratio GSH:GSSG y de un incremento en los niveles intracelulares de ascorbato [127]. A pesar de esta disminución en las actividades enzimáticas tras una etapa ciclista de montaña, caracterizada por una marcada neutrofilia, no se ha observado la aparición de daño oxidativo en proteínas [27]. Por ello, aunque el neutrófilo podría estar aportando su capacidad antioxidante al plasma sanguíneo para protegerlo del posible daño oxidativo y quedando en cierta medida indefenso, resulta ser un modelo celular resistente a padecer estrés

oxidativo. Los PBMCs en cambio exhiben una respuesta al ejercicio antitética a la descrita para los neutrófilos: se produce un aumento en el número de linfocitos durante la práctica del ejercicio seguido de una bajada muy pronunciada hasta alcanzar niveles por debajo de los iniciales [128]. Las actividades de los enzimas antioxidantes tienden a aumentar tras un ejercicio intenso [129], aunque esta respuesta depende de la duración e intensidad del mismo. Así, se ha comprobado que tras una prueba de esfuerzo máximo de duración limitada se produce un incremento en el número de linfocitos mientras que las actividades catalasa y GPx experimentan un descenso. Por otro lado, tras un esfuerzo submáximo que se prolonga en el tiempo no se observa linfocitosis y la actividad GPx aumenta [164]. Se ha comprobado que un episodio de actividad física extenuante resulta estímulo suficiente como para incrementar la actividad SOD en diferentes tejidos incluyendo músculo esquelético y eritrocitos [130]. Se ha descrito que aumentos en las actividades y niveles proteicos de las enzimas antioxidantes que no llevan asociado un incremento en la expresión de dichos genes pueden atribuirse a regulaciones post-traduccionales de estas enzimas antioxidantes en PBMCs y eritrocitos [50,131]. Sin embargo la regulación de la actividad de la enzima varía en función de las diferentes isoformas (MnSOD, CuZnSOD) y de los diferentes tipos de ejercicio. La actividad de la GPx, en cambio, ha mostrado respuestas variables al ejercicio en función del tipo de músculo esquelético estudiado, ya que se han observado tanto aumentos asociados a la actividad física como situaciones en las que la actividad enzimática no varía con el ejercicio [132]

Aunque no todas las fuentes de ROS están completamente caracterizadas, de lo que no cabe duda es que el exceso de ROS producido durante la práctica de actividad física intensa o prolongada comporta procesos de oxidación de biomoléculas y la aparición de estrés oxidativo. Diversos estudios han puesto de manifiesto incrementos en los marcadores daño oxidativo en el DNA (8-OHdG) [133] y de peroxidación lipídica tras la práctica de actividad física intensa e incluso se han establecido relaciones entre los niveles MDA [134] y la intensidad de ejercicio. También se han documentado incrementos en los niveles de grupos carbonilos en proteínas después de ejercicios muy intensos como una carrera de maratón o una sesión de ejercicio de intensidad máxima [135–137]. El GSH es una de las principales defensas a la hora de hacer frente al estrés oxidativo generado por la actividad física. Durante la realización de una actividad física el GHS es oxidado en grandes cantidades a GSSG en el músculo

esquelético con el objetivo de neutralizar los efectos deletéreos de las ROS. Sin embargo, el ratio GSH:GSSG no llega a alterarse de manera significativa dado los eficientes mecanismos de regeneración de GHS y al importe de GSH desde el plasma mediante el ciclo del γ-glutamilo [141].

2.1.2 Entrenamiento

Las adaptaciones fenotípicas más remarcables que se producen en el músculo esquelético en respuesta al ejercicio crónico son el incremento de la vascularización (angiogénesis), la transformación del tipo de fibra hacia fibras musculares oxidativas y el aumento de contenido y de la función mitocondrial [139]. Así, el aumento de la capacidad metabólica provocado por el ejercicio contribuye a mejorar el rendimiento físico y la salud en general, previniendo así múltiples enfermedades crónicas, en gran medida a través de adaptaciones inducidas en el músculo esquelético [140]. El efecto del entrenamiento físico sobre las defensas antioxidantes y el estrés oxidativo se ha estudiado principalmente en el músculo [141–144]. Aunque menos estudiadas y utilizadas, las células sanguíneas resultan ser un modelo más que adecuado para el estudio de los efectos del ejercicio y el estrés oxidativo. Debido al incremento en el consumo de oxígeno asociado a la actividad física, los eritrocitos transportan mayores cantidades de oxígeno a los tejidos, hecho que les hace especialmente susceptibles de padecer estrés oxidativo [145]. A pesar de ser células que no tienen la capacidad de síntesis de nueva proteína, se ha puesto de manifiesto que los eritrocitos son capaces de adaptar sus defensas antioxidantes en respuesta al estrés oxidativo asociado al ejercicio mediante mecanismos de regulación post-traduccional [145].

El ejercicio crónico es aquel que se practica de manera frecuente o regular -sesiones repetidas- con una intensidad moderada. La práctica regular de una actividad física resulta beneficiosa para la salud y el bienestar de las personas de todas las edades que puedan practicarla. [146]. Se ha postulado que debido al hecho de que las respuestas adaptativas derivadas del ejercicio resultan de los efectos cumulativos de los episodios repetidos de actividad física, la señal inicial que desencadena la estimulación de la modulación a largo plazo debe tener lugar tras cada sesión individual [147–149]. Tras las diversas sesiones de entrenamiento, tienen lugar las adaptaciones fisiológicas que mejoran la función del organismo y lo preparan para futuras sesiones de ejercicio

[144,150]. Si bien un episodio aislado muy intenso puede conducir a la aparición de una situación de estrés oxidativo, tal y como se ha descrito con anterioridad, la práctica regular de actividad física moderada potencia los mecanismos antioxidantes y promueve una mayor resistencia a la aparición de dicho estrés. Además, es amplia la literatura que apoya que el entrenamiento regular ofrece una mayor protección frente a diversas enfermedades y que, en general, promueve mejoras en el estado de salud, además de conducir al organismo hacia un envejecimiento saludable [151–154]. El fenómeno de la hormesis, comentado anteriormente, juega un papel fundamental en la promoción de estos efectos positivos asociados a la práctica regular de deporte, ya que la presencia frecuente de estímulos tolerables de ROS favorece la aparición de fenómenos adaptativos como la expresión de enzimas antioxidantes y otros mecanismos de defensa [33,155,156]. Los efectos del entrenamiento sobre el estrés oxidativo y las defensas antioxidantes han sido estudiados mayoritariamente en músculo, por la implicación de este tejido a nivel mecánico en la práctica de ejercicio. Estudios con animales entrenados han puesto de manifiesto menores niveles de daño oxidativo tras la realización de una sesión de ejercicio extenuante en comparación con animales no entrenados [157]. También aumenta la actividad de aquellos enzimas implicados en la reparación del material genético, promoviéndose así una mayor resistencia a la aparición de lesiones en el DNA [3].

Referente a los enzimas antioxidantes se ha podido comprobar que individuos entrenados suelen poseer, por norma general, mayores actividades que los individuos no entrenados [158]. Se han descrito tanto incrementos como carencia de efectos de distintos tipos de entrenamiento de la actividad SOD [132]. Las discrepancias detectadas podrían ser debidas a las diferentes isoformas estudiadas, a la metodología empleada en la determinación, a los modelos de entrenamiento y a los diferentes tipos de fibras usadas. Se ha comprobado que con el entrenamiento la actividad CuZnSOD aumenta sin incrementos asociados de los niveles de proteína ni de la expresión génica, mientras que en el caso de la MnSOD el incremento en la actividad viene acompañado de un incremento en los niveles proteicos pero no de expresión. Todos estos resultados en su conjunto sugieren que la inducción por entrenamiento de ambas isoformas de la SOD está originada por mecanismos post-transcripcionales y que la modulación post-traduccional podría jugar un papel esencial en la activación de estas enzimas. Numerosos estudios han

puesto también de manifiesto la inducción de actividad GPx en músculo esquelético tras un periodo de entrenamiento [127,159–161], aunque esta activación también parece ser específica del tipo de fibra muscular. Se han obtenido resultados contradictorios con respecto a la respuesta al entrenamiento de la catalasa, ya que se han documentado tanto incrementos como descensos así como la carencia de efectos [162]. Aunque se desconoce la razón por la cual las diferentes enzimas e isoformas no responden de manera similar al entrenamiento, este patrón podría depender específicamente de la expresión génica así como de los umbrales requeridos para la inducción de la actividad o expresión. Hay que tener en cuenta que la síntesis *de novo* de una enzima requiere mucha energía y es un proceso fisiológicamente lento, por lo que este mecanismo no se pone en marcha hasta que la necesidad es máxima. La actividad SOD es relativamente alta en diferentes tejidos del organismo lo que parece indicar que la eliminación del anión superóxido no es un paso limitante la detoxificación de las ROS. Por el contrario la actividad glutatión GPx, que se encarga de eliminar los productos finales de la acción de las ROS, tales como peróxidos lipídicos, presentan actividad es relativamente menor. Este patrón podría ser atribuido al hecho de que la GPx muestra una mayor adaptación al entrenamiento que los enzimas antioxidantes CAT y SOD [132].

2.3 Respuesta inmune e inflamatoria asociada al ejercicio físico

2.3.1 Ejercicio agudo

El ejercicio físico intenso provoca dolor y daño muscular, sobre todo si se trata de ejercicios de muy larga duración [163]. La realización de una sesión de ejercicio físico agudo e intenso lleva asociada una secuencia de eventos similares a los que tendrían lugar durante una respuesta de fase aguda inducida por una infección, aunque en menor magnitud [164–166]. Los efectos de ejercicio de elevada intensidad sobre el sistema inmune tienen sus primeros indicios a nivel celular. Uno de los parámetros que aumenta durante la respuesta inmunitaria al ejercicio intenso es el número de leucocitos, llamado también leucocitosis. Concretamente, los neutrófilos circulantes no sólo incrementan en número en estas condiciones, sino que también se ve incrementada su capacidad para la desgranulación, la fagocitosis y el *oxidative burst*. Este proceso se conoce como *activación* y se puede comprobar detectando una aumentada producción de ROS por parte de neutrófilos aislados

tras el ejercicio [167]. Los neutrófilos, y posteriormente los monocitos, se infiltran rápidamente en el tejido muscular dañado tras la realización de ejercicio, emulando la respuesta de fase aguda a la infección. Por ello, parece que los neutrófilos contribuyen al daño muscular asociado al ejercicio, ya que su infiltración en el musculo y la posterior liberación de citoquinas inflamatorias, ROS y proteasas incrementan el mismo daño muscular [168]. La población de monocitos aumenta tras de la realización de un ejercicio agudo aunque su población se normaliza al par de horas tras haber finalizado el esfuerzo [169]. Como consecuencia del ejercicio físico agudo puede cambiar el fenotipo de los monocitos, cambiando el perfil de secreción de citoquinas y sus proteínas de superficie, ya que se movilizan preferentemente los monocitos CD14+ y CD16+ [170], los cuales tienen un perfil proinflamatorio más marcado. Tanto el ejercicio extenuante como el moderado tiene un potente efecto estimulador sobre la fagocitosis por parte de macrófagos, y ambas intensidades de ejercicio promueven un incremento de la producción de ROS [169]. Respecto a los linfocitos, está documentado que tiene lugar una linfocitosis, o aumento en el número de células circulantes, durante e inmediatamente después de la realización del ejercicio agudo, seguido de una reducción en el número de los mismos durante el periodo de recuperación [29,169,171]. Dichos cambios están estrechamente relacionados con la magnitud e intensidad del ejercicio y afecta tanto a linfocitos B como a linfocitos T, activando a estos últimos [172,173].

La reparación muscular tras de una lesión aguda se caracteriza por una fase proinflamatoria inicial durante la cual el músculo libera citoquinas, principalmente interleuquinas 1β y 6 (IL1β e IL6). Diferentes ensayos *in vitro* e *in vivo* han puesto de manifiesto incrementos tanto en la expresión como en la secreción de IL1 e IL6 asociados al ejercicio [174–176]. Entre los factores inductores de dicha producción de citoquinas destacan la fagocitosis de fragmentos de tejido y los peróxidos lipídicos generados por la acción de las ROS [167]. Como consecuencia del ejercicio físico agudo se ha comprobado que la IL-6 es la primera, y en mayor cantidad, citoquina liberada y es también la que tarda más en volver a valores basales, [175,177] ya que la principal fuente de IL-6 es el músculo durante la realización del ejercicio [177,178]. La presencia simultánea de niveles aumentados de IL6 y TNFα son indicadores de un proceso de inflamación [169]. En repuesta a un ejercicio de elevada intensidad, las células NK incrementan su número en la circulación además de aumentar su capacidad citotóxica [169,179,180]. En cambio, esta misma actividad citotóxica

se puede ver disminuida después de varias horas de ejercicio intenso pudiendo incrementar la susceptibilidad a padecer infecciones [181].

Sin embargo, para impedir una respuesta inflamatoria desmesurada y descontrolada durante la práctica de un ejercicio agudo, también tiene lugar la liberación de citoquinas con propiedades anti-inflamatorias como IL-1rα, IL-4, IL-10 y TGF-β, además de la secreción de receptores solubles de TNF-α y IL-2 que reducen la actividad inflamatoria de dichas citoquinas. Además, como consecuencia del ejercicio físico extenuante también tiene lugar la secreción de la hormona cortisol, un potente antiinflamatorio con capacidad no sólo de inhibir la síntesis de citoquinas proinflamatorias sino también de activar la liberación de diversas citoquinas antiinflamatorias [182]. Durante el ejercicio físico agudo tiene lugar la estimulación del sistema nervioso simpático y el eje hipotalámico-adrenal, hecho que incrementa los niveles circulantes de glucocorticoides y conduce a una inhibición de la repuesta inmunitaria [183].

2.3.2 Ejercicio regular

Aunque parece que el entrenamiento no provoca cambios significativos en el número de neutrófilos circulantes en condiciones basales [181,184], algunos estudios indican que sí pueden producirse descensos como consecuencia de la realización del ejercicio regular, contribuyendo así a los efectos antiinflamatorios de esta tipología de ejercicio [169]. También se ha indicado que sujetos entrenados presentan un menor porcentaje de monocitos con perfil más proinflamatorio CD14+ y CD16+ y una menor expresión de TLRs [169,181,185]. Además, el ejercicio físico practicado de manera regular disminuye la infiltración de macrófagos en los tejidos, lo que contribuye a reducir la inflamación sistémica[128]. Por todo ello, se ha postulado que el entrenamiento contribuye a la protección del organismo frente a patologías asociadas al propio proceso inflamatorio [166,186]. La práctica regular de ejercicio físico incrementa el gasto energético y reduce por tanto los niveles de tejido adiposo [151]. De hecho, se ha sugerido que la reducción de la grasa visceral, y por tanto la reducción del tejido adiposo, asociada a la práctica regular de ejercicio es uno de los factores que favorecen los efectos antiinflamatorios a nivel sistémico [169,184,186,187]. Este evento se debe principalmente al hecho de que el tejido adiposo es una fuente natural de citoquinas inflamatorias como TNF-α e IL-6 [188]. El ejercicio frecuente

promueve la secreción por parte del tejido adiposo de una adipoquina con efectos antiinflamatorios, anti-apoptóticos y antioxidantes: la adiponectina [189,190]. Por otra parte, se ha postulado que la IL-6 producida durante la contracción muscular podría desempeñar un rol trascendental en el metabolismo lipídico, convirtiéndose entonces en uno de los factores responsables de la perdida de tejido adiposo visceral y de otros depósitos de grasa [169]. Además, se ha descrito que la liberación de IL-6 con el ejercicio regular podría ser la responsable de los efectos antiinflamatorios a nivel sistémico, ya que su liberación precede a la liberación de otras citoquinas con carácter antiinflamatorio como IL-10 o IL-1rα. La práctica de ejercicio moderado frecuente regular reduce los niveles basales de varios marcadores de inflamación sistémica como el TNF-α, proteína C reactiva, e IL-8, entre otros, además de los receptores solubles de TNF-α e IL-6, y produce un incremento de los niveles de citoquinas antiinflamatorias como la IL-10, IL-12, IL-4 y TFG-β1, entre otras [184,190,191] Además, el ejercicio físico parece que puede inhibir la síntesis de TNF-α por parte del sistema inmunitario [192,193]. Por tanto, parece evidente que la intensidad (elevada, moderada, leve) y duración (minutos/día, semana, mes) de la actividad física realizada influye directamente en los efectos antiinflamatorios sobre el organismo, sin olvidar por supuesto las características del individuo (edad, sexo, nivel físico, estado de salud, patologías asociadas, etc.).

3. Mitocondrias

3.1 Retículo mitocondrial

La visión de las mitocondrias como orgánulos aislados ha sido profundamente cuestionada en las últimas décadas en base al descubrimiento de que estos orgánulos conforman en las células eucariotas una red o retículo tubular integrado, parcialmente interconectado y dinámico [205] que se extiende por todo el volumen citosólico, excluyendo el núcleo [195]. Este nuevo concepto reticular de la mitocondria sugiere que la arquitectura y morfología de la red es bastante diversa, flexible, capaz de ajustarse en una escala de tiempo de minutos, dependiendo de la condición fisiológica, y es muy variable entre los diferentes tipos celulares. En la mayoría de las situaciones, una célula contiene

clústeres en red de diferentes tamaños junto con numerosas mitocondrias individuales, lo que representa un estado intermedio entre el estado completamente interconectado y el estado completamente fragmentado [194,196]. Esta constelación de mitocondrias interconectadas juega un papel clave en músculo esquelético, donde ha sido más estudiada de momento, ya que contribuye a distribuir el potencial de membrana mitocondrial a través de la célula rápidamente a grandes regiones de las células musculares [140,143,194]. Resulta pues de sumo interés conocer las características arquitectónicas mitocondriales en otros tipos celulares, como por ejemplo las células del sistema inmunitario.

3.2 Función, dinámica y biogénesis mitocondrial

El correcto funcionamiento e integridad estructural de las mitocondrias es indispensable para el mantenimiento bioenergético celular, hecho que se consigue mediante el control de la biogénesis mitocondrial. La biogénesis mitocondrial es un evento clave en el funcionamiento celular constituido por dos procesos distintos e intrínsecamente ligados: la proliferación mitocondrial, que consiste en el aumento del número de mitocondrias por célula, y la diferenciación mitocondrial, mediante la cual el orgánulo adquiere las características estructurales y capacidades funcionales apropiadas para el desarrollo de las funciones específicas en los distintos tipos celulares [197–201]

Este proceso engloba tanto la proliferación, o aumento del número de mitocondrias, como la diferenciación, o aumento de las capacidades funcionales de las mitocondrias preexistentes. La biogénesis mitocondrial es un proceso complejo que implica la expresión coordinada de más de 1000 genes codificados tanto en el genoma mitocondrial como en el nuclear. La expresión de estos genes se adapta, para el mantenimiento de la morfología mitocondrial, a la demanda energética y a condiciones fisiológicas cambiantes [202]. Por ejemplo, la biogénesis mitocondrial aumenta en el músculo en respuesta al ejercicio o en el tejido adiposo durante la termogénesis adaptativa [203].

Existen varios elementos que actúan como coordinadores de los genomas nuclear y mitocondrial, con el fin de regular la biogénesis y la actividad de las mitocondrias en función de la demanda energética celular [204]. Entre ellos destacan los coactivadores del receptor activado por proliferadores

peroxisomales γ (PGCs), los factores nucleares de respiración (NRFs), el factor de transcripción mitocondrial A (TFAM)[204,205]. Dentro de la familia de los PGCs destaca el PGC1α, un coactivador transcripcional que regula la expresión de genes implicados en el metabolismo energético. PGC1α también es considerado el principal inductor y regulador de la biogénesis mitocondrial en diversos tejidos [206]. Los PGC1s no presentan actividad histona acil transferasa que les permita remodelar la cromatina y ejercer un control directo sobre la transcripción génica. De hecho, los PGCs actúan como plataformas de unión con otras proteínas que sí poseen dicha actividad transferasa y favorecen el ensamblaje de maquinaria de transcripción. Además, la actividad de los PGCs puede ser modulada a través de modificaciones post-traduccionales como la fosforilación inducida por la quinasa activada por AMP (AMPK) o la desacetilación inducida por la sirtuina 1 [207]. En este sentido, PGC1α se identifica como un coactivador transcripcional capaz de regular un amplio abanico de aspectos relacionados con la biogénesis mitocondrial y el metabolismo, ya que interacciona y coactiva directamente a los NRFs. Los NRFs son factores de transcripción que regulan la expresión de genes implicados en la progresión del ciclo celular y la síntesis de proteínas, así como en la biogénesis mitocondrial. La isoforma NRF1 activa la expresión de genes que codifican para componentes de sistema de fosforilación oxidativa, transportadores mitocondriales y proteínas ribosomales mitocondriales, además de regular la expresión de TFAM. NRF2 juega un papel directo en el control de la expresión de las subunidades del complejo IV de la cadena de transporte de electrones, de proteínas implicadas en el transporte de maquinaria mitocondrial y de factores iniciales de la traducción mitocondrial, así como también de TFAM. TFAM es un factor de transcripción codificado en el genoma nuclear cuya función es la estimulación de la transcripción del mtDNA. Se une de manera específica a regiones situadas corriente arriba (o *upstream*) de los promotores de ambas cadenas, desde donde permite el reclutamiento de la RNA polimerasa, permitiendo el inicio y la estabilización de la transcripción. Además, TFAM se une de manera inespecífica al genoma mitocondrial, permitiendo su empaquetamiento y estabilización [199,208].

Actualmente la descripción cuantitativa de esta compleja estructura como es el retículo mitocondrial no está todavía establecida, pero se cree que es el resultado del proceso, o conjunto de procesos, denominado dinámica mitocondrial, regido por su constante movimiento intracelular a lo largo de los

filamentos del citoesqueleto y por la capacidad de fusionarse y dividirse en diferentes posiciones en una escala de minutos u horas [209,210]. A diferencia de otros orgánulos de la célula, las mitocondrias no son generadas *de novo*, sino que proliferan por crecimiento y división de orgánulos preexistentes. Cabe destacar que las mitocondrias, como ya se ha dicho anteriormente, no son orgánulos estáticos, sino que cambian continuamente de morfología y de localización para mantener un correcto funcionamiento tanto en condiciones normales como en respuesta al estrés. Estas acciones dinámicas incluyen tanto procesos de fusión como de fisión mitocondrial [211–213] y permiten el remodelado del retículo mitocondrial. Estos eventos posibilitan la transmisión de moléculas de señalización y el intercambio de metabolitos dentro de la célula y participan en una amplia variedad de procesos biológicos, incluyendo el desarrollo embrionario, el metabolismo, la apoptosis y la autofagia [214].

3.2.1 Fusión y fisión mitocondrial

El proceso de fusión permite la unión de dos mitocondrias, con la consiguiente mezcla y unificación del contenido de ambas. La fusión atenúa o mitiga el estrés mediante la unión y cooperación del contenido de mitocondrias parcialmente dañadas, ya que permite complementar el ADN mitocondrial dañado [215]. Desde un punto de vista mecanístico, la fusión es un procedimiento complejo, fuertemente ligado a la hidrólisis de GTP [216], en el que se produce la unión de dos mitocondrias *vecinas*. Los principales reguladores de la fusión mitocondrial en el humano son las proteínas mitofusinas (Mtf) y la proteína de la atrofia óptica 1 (OPA1). Las Mtf se presentan en dos isoformas (Mtf1 y Mtf2) y son proteínas integrales de la membrana mitocondrial externa con actividad GTPasa, con sus dominios N-terminal (dominio GTPasa) y C-terminal orientados hacia el citosol. Por su parte, en su extremo C−terminal, existe una estructura proteica en espiral que actúa como anclaje a la membrana externa de mitocondrias adyacentes [196,217] y posterior fusión mitocondrial [218,219]. OPA1 se localiza en el espacio intermembrana y asociada a la membrana mitocondrial mediante hélices transmembrana en su extremo N− terminal [220]. OPA1 es una proteína sumamente compleja, que sufre una importante regulación postranscripcional y que se distribuye de un modo ubicuo en el organismo, expresándose más en ciertos órganos y tejidos [221]. OPA1 participa en el remodelado de las crestas mitocondriales y el acercamiento y fusión de la membrana mitocondrial interna

[221,222]. Para que la fusión mitocondrial se lleve a cabo, es necesario que el potencial de membrana esté intacto [216]. Por tanto, las mitocondrias despolarizadas no pueden entrar en fusión y quedan excluidas del ciclo fusión−fisión [223], activándose entonces el proceso degradativo de mitofagia.

La fisión constituye el mecanismo celular que permite producir mitocondrias nuevas a partir de otras ya existentes, durante la citoquinesis celular [224]. Así, se obtienen orgánulos morfológica y funcionalmente diferentes entre sí, logrando que el contenido mitocondrial pase a las células hijas de manera correcta durante la división celular [225,226]. Hay varios estudios que concluyen que este proceso puede tener también un papel importante en la eliminación de las mitocondrias dañadas, ya que serviría para marcarlas para la posterior mitofagia [223,227] y en la prevención de la elongación mitocondrial excesiva, la cual produciría fenómenos de senescencia celular [228]. En mamíferos la fisión mitocondrial está regulada, entre otras, por las actividades de la proteína relacionada con la dinamina 1 (Drp-1) y la proteína de fisión 1 (Fis1) [229]. Drp-1 posee homología de secuencia con las dinaminas, GTPasas que regulan el tráfico vesicular y la endocitosis. El mecanismo molecular preciso de Drp-1 en este proceso es aún materia de debate. Sin embargo, uno de los modelos postula que esta proteína actúa como mecano-enzima que participa activamente en el corte de membranas por constricción. Drp-1 es una proteína principalmente citoplasmática, pero con una fracción que se localiza en puntos específicos de la membrana mitocondrial externa que representan futuros sitios de fisión. Drp-1 carece de una secuencia de destino mitocondrial, por lo que se recluta a la membrana a través de Fis1, una proteína adaptadora que participa en el ensamblaje de complejos de fisión de alta masa molecular [230]. Es importante aclarar que el proceso de fisión mitocondrial ocurre habitualmente en todas las células en condiciones normales. Sin embargo, la fisión mitocondrial también se ha asociado a condiciones de estrés metabólico, así como a la autofagia y la apoptosis.

3.4 Ejercicio y dinámica mitocondrial

Presumiblemente, el retículo mitocondrial está constantemente sujeto a procesos de fusión (unión de mitocondrias separadas que conducen a la formación de redes continuas) y la fisión (división de mitocondrias en unidades individuales) para mantener el contenido mitocondrial y la homeostasis

estructural y, por lo tanto, la calidad mitocondrial [140]. Las mitocondrias, que oxidan los sustratos de nutrientes para generar ATP, son las principales responsables de satisfacer las demandas energéticas que supone la práctica de ejercicio prolongado. La eficiencia con la que las mitocondrias satisfacen estas demandas y, por lo tanto, alimentan la función del músculo esquelético, está determinada por su función general, es decir, contenido, estructura y respiración [108,178]. La contracción muscular inicia una serie de eventos moleculares, bioquímicos y fisiológicos que promueven la biogénesis mitocondrial (Figura 5). La biogénesis mitocondrial es un proceso complejo que requiere la síntesis, importación e incorporación de proteínas y lípidos al retículo mitocondrial existente, así como la replicación del mtDNA [231]. En respuesta a estímulos como la actividad contráctil, varias quinasas y fosfatasas son activadas en la célula, generándose múltiples eventos de transducción de señales. Estas señales modulan la expresión de genes nucleares que codifican para proteínas mitocondriales. Las proteínas destinadas a la mitocondria son dirigidas al compartimiento mitocondrial mediante maquinaria de importación de proteínas. Una vez dentro de la matriz mitocondrial, Tfam es dirigido hacia el genoma mitocondrial, donde desempeña un rol clave en la regulación de la transcripción y replicación del mtDNA. Como puede observarse en la Figura 5, el ejercicio estimula la actividad y expresión de PGC1α, que promueve la biogénesis mitocondrial, principalmente coordinando las transcripciones en el genoma nuclear (a través de la interacción con NRF1/2) y el genoma mitocondrial (a través de la transcripción del gen TFAM) [140]. Las mitocondrias nuevas y sanas (verde) se fusionan con la masa o retículo mitocondrial preexistente. En cambio, las mitocondrias antiguas y/o dañadas (rojo) se separan por fisión de dicho retículo. El ejercicio promueve tanto la fusión (a través de OPA1 y MTF1/2) [232] y la fisión (a través de y DRP1). Las mitocondrias dañadas, probablemente debido a la pérdida del potencial de membrana mitocondrial, pueden ser reconocidas por la maquinaria de autofagia a través de las proteínas de autofagia como LC3 (microtubule-associated protein 1A/1B-light chain 3), entre muchas otras. Al mismo tiempo, el ejercicio conduce a la activación secuencial de otras moléculas que promueven la formación del llamado fagóforo, que engloba a las mitocondrias dañadas para formar un autofagosoma. La fusión del autofagosoma con el lisosoma completa el ciclo de vida mitocondrial.

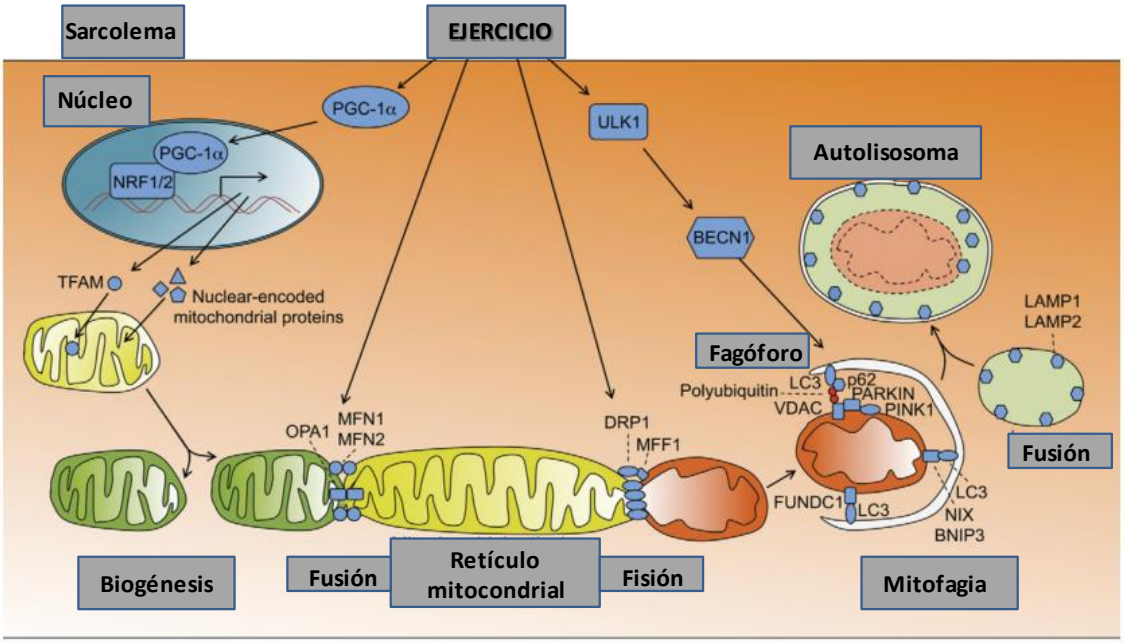


Figura 5. Dinámica y biogénesis mitocondrial inducida por el ejercicio físico en músculo esquelético. MFF: receptor de Drp1. Las siglas VDAC, LC3, p662, PARKIN, PINK, NIX, LAMP1/2 y BNIP3 hacen referencia a moléculas específicas implicadas en el proceso de mitofagia que no resultan relevantes en la presente tesis. (Imagen: Adaptado de Drake et al, 2015).

Como puede verse en la Figura 5, el ejercicio juega un papel en la cantidad y calidad mitocondrial global [39,232] a través de la síntesis e incorporación de nuevas proteínas y mtDNA en el retículo existente (biogénesis) [139,147], y separación (fisión) y unión estructural (fusión) [212], así como degradación de mitocondrias dañadas y disfuncionales (mitofagia) [233]. El cambio de color mitocondrial en la Figura 5 denota la calidad mitocondrial: del verde, indicativo de buena calidad, al rojo, de baja calidad. Por tanto, estos procesos, inducibles por el ejercicio, son la base de un mejor rendimiento físico, así como de otros beneficios para la salud, como resultado del entrenamiento físico. Este conjunto de respuestas ha sido ampliamente estudiado y descrito principalmente en músculo esquelético. Dado que las células del sistema inmunitario también desempeñan un rol fundamental en los procesos inmunes y en las adaptaciones derivadas del ejercicio regular, resulta de sumo interés intentar encontrar patrones mitocondriales similares en leucocitos, de más fácil obtención que las biopsias musculares.

4. Envejecimiento y estilo de vida

El envejecimiento es un fenómeno fisiológico normal y multifactorial caracterizado por un deterioro progresivo generalizado de las diferentes funciones en el organismo [234]. Todo ello resulta en una mayor vulnerabilidad al estrés ambiental y un creciente riesgo de enfermedad y muerte [234–236]. Entre los mecanismos que se proponen a día de hoy para explicar el proceso de envejecimiento destacan las alteraciones en el ADN y en la expresión de genes, el estrés oxidativo, el inflamosoma, la genotoxicidad y la aparición de telómeros más cortos [237]. Con todo, a esta disminución de la capacidad homeostática también se le asocia una disminución general de la eficacia de diversos mecanismos implicados en la prevención del daño a las macromoléculas y la reparación o sustitución de aquellas deterioradas [238]. Uno de los principales problemas asociados al envejecimiento es la aparición de numerosas afecciones patológicas como la diabetes y otros trastornos metabólicos, las enfermedades cardiovasculares, los trastornos neurodegenerativos o varios tipos de cáncer [239]. La teoría del envejecimiento basada en radicales libres mitocondriales [5,57] especula y propone que el daño oxidativo progresivo y acumulado en las mitocondrias y el ADN mitocondrial causado por las ROS, producidas como subproductos durante el metabolismo normal, es una de las causas del envejecimiento. Los radicales libres, tanto los que se generan en la propia mitocondria como los que le llegan desde el citosol, van dañando inexorablemente a la célula y la función mitocondrial va disminuyendo paulatinamente. Como consecuencia, la cadena de transporte electrónico y la fosforilación oxidativa se hacen ineficientes, disminuyendo la producción de ATP. En esta situación, la mitocondria se hace más vulnerable al ataque de los ROS, disminuyendo todavía más su eficiencia [240]. A medida que los niveles intracelulares y extracelulares de estrés oxidativo aumentan durante el envejecimiento o en diversas enfermedades, también lo hace la cantidad de biomoléculas dañadas, ya que los mecanismos de reparación también son objetivos de daño oxidativo y, por lo tanto, se vuelven gradualmente ineficaces con el tiempo. El daño oxidativo afecta a la replicación y la transcripción de mtDNA, resultando en una disminución en función mitocondrial. Ello a su vez conduce a una mayor producción de ROS y un mayor daño al ADN mitocondrial, puediendo derivar en apoptosis o muerte celular programada [57,241,242].

Con todo, con el aumento de la esperanza de vida, aumenta notablemente el costo socio-sanitario asociado al envejecimiento y se convierte en un problema en numerosos países. En este sentido, es fundamental el desarrollo de estrategias encaminadas a la mejora de la calidad de vida de las personas mayores, reducir el costo económico asociado al envejecimiento [243] y a lograr el llamado envejecimiento saludable (del inglés, *healthy ageing*). De manera resumida, se pueden distinguir tres componentes en la definición de envejecimiento saludable: alcanzar la edad anciana con una baja probabilidad de padecer enfermedades o invalidez, con elevada capacidad funcional física y cognitiva y con mantenimiento de una vida activa en la sociedad.

4.1 Envejecimiento, inflamación y ejercicio físico

La inflamación sistémica crónica de bajo grado es una manifestación común del envejecimiento. Aumentos de dos a cuatro veces en los niveles circulantes de citoquinas proinflamatorias como la IL6 y el TNFα, y proteínas de fase aguda como la proteína C reactiva (CRP) [244], son típicos en los ancianos en comparación con los jóvenes, incluso en ausencia de enfermedad crónica [249]. Son múltiples los mecanismos que probablemente contribuyen a la inflamación asociada a la edad (del inglés, *inflammaging* [246,247]). Durante el envejecimiento se producen importantes disminuciones en la función inmunológica que promueven procesos inflamatorios; pero este estado inflamatorio crónico de bajo grado en los ancianos también es claramente una consecuencia de enfermedades crónicas relacionadas con la edad (obesidad, inactividad física, patologías cardiovasculares, diabetes, enfermedad renal crónica, osteoartritis y la enfermedad de Alzheimer)[138,248–250]. Por ello, resulta complejo descifrar si estas condiciones son causa o consecuencia de la inflamación crónica en ancianos [251].

Existe numerosa información en la literatura que determina que los estilos de vida físicamente activos promueven la salud y el envejecimiento saludable [153,154,252,253] y corrobora los efectos beneficiosos del ejercicio sobre diversos parámetros fisiológicos y psicológicos en adultos y ancianos [254,255]. Las diversas respuestas fisiológicas del cuerpo al ejercicio aeróbico ocurren en los sistemas musculoesquelético, cardiovascular, respiratorio, endocrino e inmune. Estas reacciones están orquestadas por factores neuronales y endocrinos e influyen en la respuesta general del cuerpo al ejercicio [179,256].

Teniendo en cuenta los beneficios documentados de la práctica regular de ejercicio físico, parece imprescindible incluir la actividad física regular como un punto clave para el manejo y la prevención de enfermedades crónicas y como factor promotor del envejecimiento saludable [153,239]. La asociación entre la grasa corporal, los marcadores inflamatorios y el estrés oxidativo ha sido objeto de estudio previo [257–259]. El tejido adiposo es un órgano complejo con más funciones además de la de almacenamiento de energía, incluida la secreción de varias adipocinas, como TNF-α, IL-6, CRP y resistina, entre otras. De hecho, se ha propuesto que el tejido adiposo contribuye al estado inflamatorio sistémico en sujetos con sobrepeso y obesos, lo que se correlaciona con un aumento del estrés oxidativo en dichas patologías [258]. Por el contrario, el hecho de llevar un estilo de vida más activo y una rutina de actividades diarias se asocia con valores significativamente menores de peso, IMC y grasa corporal cuando se compara con sujetos sedentarios [151,260]. Este hecho contribuye a una atenuación del estado inflamatorio en participantes ancianos, debido a la disminución de linfocitos circulantes [261], y a una mejorada capacidad antioxidante [262,263]. Por lo tanto, resulta de sumo interés profundizar en estrategias y rutinas de ejercicio físico en adultos mayores para promover un envejecimiento saludable caracterizado por un estado antioxidante y una función mitocondrial óptimos.

5. Ácidos grasos omega 3

Los ácidos eicosapentaenoico (EPA, C20:5n3) y docosahexanoico (DHA, C22:6n3), incluidos en el grupo de las ácidos grasos (AG) poliinsaturados (PUFA) omega 3 esenciales, forman parte de las membranas celulares [264], condicionando la estructura de éstas y modulando la actividad de enzimas y receptores celulares, entre otras funciones [265–267]. Diversos estudios señalan que los AG omega 3 ejercen efectos antiinflamatorios [268–270]. Este efecto se atribuye, entre otros, al hecho de que la incorporación en la dieta de alimentos ricos en omega 3 reduce por competición la concentración de ácido araquidónico (omega-6) [271] y promueve aumentos de prostaglandina 2 en plasma, [272] la cual desempeñar un papel en la resolución de la inflamación. De hecho, un consumo mayor de omega-6 respecto a los omega-3 puede desencadenar la producción de prostaglandinas y eicosanoides de carácter proinflamatorio, aumentando el riesgo de padecer enfermedades inflamatorias crónicas. Otros estudios [268] apuntan a una inhibición, mediada por DHA, de la

producción de ROS inducida por IL-1α, lo que contribuiría a las acciones antiinflamatorias de los omega 3 a nivel endotelial.

Por otra parte, se ha descrito recientemente que los ácidos grasos poliinsaturados omega-3 ejercen efectos antioxidantes y antiinflamatorios través del factor nuclear derivado de eritrocitos 2 (Nrf-2). Estos hallazgos muestran que el DHA y el EPA pueden inducir la expresión de la lipooxigenasa 1 y la catalasa a través de Nrf2, a la vez que promueven un incremento en los niveles de GSH, protegiendo así a estas células del estrés oxidativo [273]. También se ha comprobado que el DHA regula al alza las enzimas antioxidantes en las células endoteliales de la vena umbilical humana [274] y puede prevenir la citotoxicidad inducida por el H_2O_2 en adipocitos 3T3 [275].

5.1 Omega 3 y ejercicio

La suplementación de la dieta con alimentos ricos en DHA y EPA, como el aceite de pescado, promueve un incremento del contenido de estos omega 3 en las membrana de los eritrocitos [150,272], hecho que incrementa la plasticidad de los eritrocitos. Esta característica adquirida de los glóbulos rojos para deformarse conlleva una mayor capacidad de transporte y liberación tisular de oxígeno [276]. La suplementación de la dieta con AG omega 3 reduce el ritmo cardiaco, disminuye la presión arterial, reduce el consumo de oxígeno [277] y facilita el flujo de lactato y protones desde la célula muscular al espacio intersticial [278] , eventos que pueden reducir el grado de fatiga en atletas [169]. Además, al consumo de EPA y el DHA se le atribuye la capacidad de atenuar la respuesta infamatoria asociada a la actividad física extenuante relacionada con el daño muscular que afecta de manera negativa al rendimiento[279]. Asimismo, los omega-3 proporcionan una mejora en el perfil lipídico, específicamente en las fracciones de triglicéridos y colesterol circulantes, a la vez que promueven un incremento de la fluidez de las membranas [280,281]. Estos efectos son similares a los que se atribuyen a la práctica regular de ejercicio aeróbico [282]. Los PUFA y sus metabolitos pueden actuar a nivel nuclear afectando la transcripción de una variedad de genes, no necesariamente relacionados con el metabolismo lipídico, en conjunción con factores de transcripción y receptores nucleares [283]. Al mismo tiempo las vías activadas por los receptores activados por proliferadores de peroxisomas (PPARs) reducen el estrés oxidativo mediante la expresión de proteínas

desacopladoras mitocondriales, disminuyendo la producción de ROS mitocondriales. Por otra parte, se ha descrito que los ácidos grasos dietéticos mejoran la función mitocondrial y promueven la fusión mitocondrial tanto en experimentos *in vivo* e *in vitro* [284].

Con todo, resulta de interés profundizar en el estudio de los efectos antioxidantes e inflamatorios de los omega 3 y su interacción con el ejercicio, con el objetivo de caracterizar dosis efectivas que optimicen y promuevan los efectos beneficiosos de los mismos sin que se inhiba la respuesta celular, a la vez potencie los mecanismos endógenos adaptativos.

II. Objetivos y planteamiento experimental

1. Hipótesis

El cuerpo humano está constantemente expuesto a multitud de agentes que inducen la producción de especies reactivas de oxígeno (ROS) [6,285]. La interrupción del control redox y el desequilibrio en favor de las especies prooxidantes conduce a la exposición de las células a niveles elevados e incontrolados de subproductos oxidantes del metabolismo oxidativo [286]. Este hecho ha sido ampliamente interpretado como inductor de la aparición de estrés oxidativo y daño oxidativo en células y tejidos [80,287]. Numerosos estudios demuestran que el ejercicio físico es fuente natural de ROS [67] con potencial para influir sobre numerosos procesos celulares a la vez que generar daño sobre diferentes biomoléculas y estructuras celulares [288]. Diversas evidencias apuntan a que la respuesta celular a este estímulo difiere en función de la persistencia e intensidad del mismo [32,35]. A este fenómeno de respuesta dual se le conoce con el nombre de hormesis [65], y viene definido por una estimulación por dosis bajas y una inhibición para dosis altas. Se ha establecido que la señalización redox se caracteriza por modificaciones reversibles de oxidorreducción que sugieren que las ROS producidas a niveles bajos a moderados pueden ejercer resultados beneficiosos [289,290]. En este sentido, dosis fisiológicamente tolerables de ROS están implicadas en la regulación de cascadas de señalización celular que ejercen efectos terapéuticos y protectores contra enfermedades [67] y juegan un papel fundamental en la mediación de los beneficios derivados del ejercicio [33,291,292]. Por tanto, las especies reactivas derivadas de la práctica de ejercicio parecen actuar como un arma de doble filo según la intensidad y duración del estímulo que las genera.

Una amplia variedad de situaciones fisiológicas y patológicas, incluyendo el envejecimiento, patologías inherentes al síndrome metabólico y la práctica de ejercicio, se encuentran asociadas a una aumentada producción de estos agentes oxidantes [5,65]. La práctica de sesiones únicas o aisladas de ejercicio de elevada intensidad promueve incrementos puntuales de ROS, hecho que induce la aparición de cambios fisiológicos transitorios para hacer frente a las demandas metabólicas y energéticas circunstanciales. Si los niveles o la constancia de estas especies son lo suficientemente elevados, la célula queda expuesta a un ambiente prooxidante en el que se pierde el equilibrio homeostático y las biomoléculas son más susceptibles de ser dañadas de manera irreversible, pudiendo quedar comprometida su función (proteínas mal

plegadas, peroxidación lipídica, disfunción mitocondrial, modificaciones estructurales y daño sobre el DNA, entre otras). En cambio, cuando la actividad física se lleva a cabo de manera regular, continuada y moderada (durante un periodo de entrenamiento), los estímulos a los que la célula se debe enfrentar son de menor magnitud respecto a los generados durante la práctica de ejercicio agudo. Así, se ha sugerido que la producción leve de ROS inducida por el entrenamiento desempeña, al menos parcialmente, un papel en la orquestación de la respuesta antioxidante, en la reparación del ADN y en mejora de la calidad del retículo mitocondrial, entre otras funciones [156,159,293]. Todo ello conlleva a la aparición de adaptaciones celulares beneficiosas, dirigidas a enfrentar subsiguientes situaciones de estrés oxidativo, pudiendo resultar en una disminución de la incidencia de enfermedades relacionadas con el estrés oxidativo y conduciendo a un envejecimiento saludable.

Así pues, no parece ser sólo la tipología de ROS (iones de oxígeno, radicales libres, peróxidos, etc.) sino también la dosis del factor estresante (niveles y tasas de producción) la que representa una variable clave que inclina la balanza hacia la generación de efectos beneficiosos o deletéreos en el organismo. Resulta pues de sumo interés desgranar las variables (tiempo de exposición, dosis, tipo de ROS) que actúan en el proceso de señalización celular durante situaciones con carácter prooxidante como la práctica de ejercicio y los efectos que ejerce este estímulo sobre la funcionalidad celular. Todo ello para poder así determinar y promover aquellas situaciones o estrategias que potencian una cierta producción de ROS que resulte beneficioso para el organismo y la salud, promoviendo adaptaciones que eviten posibles daños de una excesiva y/o subsiguente producción de estas especies reactivas. Así, resulta interesante profundizar en efectos de las ROS sobre las defensas antioxidantes endógenas, la producción de citoquinas pro y antiinflamatorias, el daño oxidativo y el retículo mitocondrial. Se hace patente la necesidad de disponer de situaciones fisiológicas en las que el desequilibrio oxidativo sea resultado de diferentes dosis de estímulo y de diferentes tasas de producción y/o de eliminación de ROS, como por ejemplo agudo, entrenamiento, estilo de vida y estrés oxidativo inducido *ex vivo,* para poder evaluar el estado redox, mitocondrial, inflamatorio y antioxidante de la célula y posibles adaptaciones fisiológicas. Asimismo, la suplementación de la dieta con ácidos grasos poliinsaturados con características prooxidantes altera el balance oxidativo, siendo otro posible modelo para estudiar los efectos de las ROS. El consumo de suplementos dietéticos ha sido

tradicionalmente considerado como una herramienta para la mejora de una variedad de funciones en el organismo y que contribuyen a mantener un buen estado de salud. Sin embargo, a pesar de las evidencias científicas que apoyan la utilidad de muchos de estos suplementos, hay mucha controversia con respecto a este tema. En este sentido, se precisan criterios para la ingesta y consumo de suplementos exógenos, como los ácidos grasos omega 3, que permitan establecer dosis efectivas que optimicen y promuevan los efectos beneficiosos de los mismos sin que se inhiba la respuesta celular, a la vez potencie los mecanismos endógenos adaptativos. Resulta preciso valorar cómo afecta la suplementación crónica con un ácido graso poliinsaturado omega 3, como el DHA, al balance oxidativo y sobre procesos de dinámica mitocondrial tras la realización de una actividad física aguda e intensa y tras un periodo de entrenamiento.

El músculo esquelético es el modelo de estudio por antonomasia en ciencias de la actividad física por sus múltiples propiedades (plasticidad, excitabilidad, tamaño), pero su obtención se debe realizar mediante técnicas de extracción invasivas como son las biopsias musculares. La presente tesis propone el uso de células mononucleares de sangre periférica (PBMCs) y neutrófilos como modelo celular de estudio, ya que su obtención mediante extracción de sangre rápida, sencilla y resulta menos invasiva, reduciendo la afección al paciente. Además, las PBMCS y los neutrófilos se pueden aislar y purificar siguiendo un sencillo protocolo de purificación por gradiente de densidad.

La hipótesis es que el estrés oxidativo derivado de la práctica de diferentes modalidades de ejercicio físico, agudo o crónico, actúa como estímulo para modular la respuesta antioxidante, inflamatoria y mitocondrial en células inmunitarias, con efectos favorables sobre la generación de daño oxidativo, las defensas antioxidantes, la función mitocondrial y el estado inflamatorio. Además, hipotetizamos que la dinámica mitocondrial y la respuesta antioxidante tras la práctica de actividad física se ve influenciada de manera beneficiosa tras una suplementación de la dieta con ácidos grasos poliinsaturados omega 3, concretamente el DHA.

2. Objetivos

El objetivo general de esta tesis radica en evaluar y caracterizar la respuesta diferencial antioxidante, inflamatoria y mitocondrial, frente a diferentes dosis de ejercicio físico, como estímulo con carácter prooxidante, y valorar el uso de ácido docosahexaenoico como suplemento nutricional en este contexto.

El objetivo general puede, a su vez, fraccionarse en los siguientes objetivos específicos:

I. Investigar el efecto de 8 semanas de entrenamiento junto con la suplementación de la dieta con una bebida funcional enriquecida con DHA sobre la dinámica de mitocondrial y el estado antioxidante en células mononucleares de sangre periférica.

II. Diferenciar los efectos de 8 semanas de entrenamiento y una sesión de ejercicio agudo sobre la dinámica mitocondrial y el estado antioxidante en células mononucleares de sangre periférica.

III. Poner de manifiesto que tanto el ejercicio físico practicado de forma aguda como de forma regular son un buen modelo fisiológico para estudiar los mecanismos de respuesta mitocondrial y pro-antioxidante y su modulación por nutrientes como los ácidos grasos poliinsaturados omega 3.

IV. Evaluar la composición corporal, la hipertensión y el perfil lipídico, así como el estado inflamatorio, en adultos mayores y su asociación con la práctica regular de actividades físicas y un estilo de vida activo.

V. Evaluar el estado antioxidante y mitocondrial en células mononucleares de sangre periférica, junto con el daño oxidativo en plasma, según el grado de actividades físicas diarias y el estilo de vida.

VI. Emular una situación crónica de estrés oxidativo *ex vivo* mediante la exposición de células mononucleares de sangre periférica y neutrófilos a peróxido de hidrógeno producido por la enzima glucosa oxidasa durante 2 horas, con el fin de determinar respuestas diferenciales en la producción de citoquinas pro/antiinflamatorias, especies reactivas de oxígeno, y cambios en la expresión génica de proteínas

antioxidantes, mitocondriales, pro/antiinflamatorias e implicadas en la respuesta inmunitaria.

VII. Establecer una metodología *ex vivo* de exposición continua a peróxido de hidrógeno que permita rastrear respuestas celulares diferenciales a estímulos externos para determinar sensibilidades y capacidades de respuesta disímiles en células del sistema inmunitario.

3. Planteamiento experimental

Para la consecución de los objetivos anteriormente planteados se diseñan, planean y llevan a cabo una serie de experimentos utilizando como modelo experimental células mononucleares de sangre periférica (PBMCs) y neutrófilos, junto con el plasma. La tipología de estímulos oxidantes seleccionados incluye ejercicio agudo (prueba de esfuerzo extenuante), ejercicio crónico (8 semanas de entrenamiento), situación de estrés oxidativo emulada *ex vivo* (mediante exposición a peróxido de hidrógeno) y estilo de vida. La tipología de pacientes incluidos en los diferentes proyectos son futbolistas jóvenes profesionales (hombres), adultos mayores de 55 años con síndrome metabólico (ambos sexos) y adultos mayores de 55 años sanos (ambos sexos). El nutriente introducido en la dieta como modulador de las funciones celulares y moleculares de estudio es DHA, incluido en el grupo de los ácidos grasos omega 3. Los participantes fueron debidamente informados de los objetivos y posibles riesgos asociados a los experimentos y todos participaron de manera voluntaria, firmando siempre el consentimiento informado. En todos los experimentos se siguieron y cumplieron los requisitos de la Declaración de Helsinki sobre la experimentación con seres humanos y los protocolos fueron aprobados por la Comisión de Ética de Investigación clínica de las Islas Baleares.

Experimento 1. Ejercicio agudo, entrenamiento y DHA: estado antioxidante y dinámica mitocondrial

Efectos diferenciales del ejercicio agudo y crónico en células mononucleares de sangre periférica en futbolistas profesionales jóvenes tras ocho semanas de dieta suplementada con DHA o placebo sobre el estado antioxidante y la dinámica mitocondrial.

Para corroborar los objetivos 1, 2 y 3 desarrollamos un diseño experimental fundamentado en una intervención nutricional consistente en una suplementación de la dieta con DHA durante ocho semanas de entrenamiento y una prueba de esfuerzo al final de dicho período (ClinicalTrial.gov NCT02177383) (Figura 6). Un total de quince futbolistas federados de entre 16 y 35 años del equipo filial del Real Club Deportivo Mallorca, al inicio de la temporada deportiva 2011-2012, fueron seleccionados para participar en el estudio. Los sujetos fueron distribuidos en dos grupos, experimental (N=9) y placebo (N=6), y entrenaron normalmente durante 8 semanas a la vez que consumieron 1 litro al día (5 días/semana) de una bebida de almendra enriquecida con DHA (1.14 g/día) o una bebida placebo, respectivamente. Ambas bebidas, experimental y placebo, fueron elaboradas (Liquats Vegetals S.A., Girona, Spain) con almendras (3%), y presentan la misma composición, con la única diferencia que una parte del aceite de oliva de la bebida experimental se sustituyó por una cantidad equivalente de DHA (0.2%, DHA-S Market, Market Biosciences Corporation, Columbia, EEUU), con lo que la bebida experimental estaba enriquecida con DHA. Dicho enriquecimiento se realizó mediante triglicéridos ricos en DHA, con baja presencia de ALA y EPA. Ambas bebidas presentaban elevado contenido en vitamina E (0.4 mg/mL), con el objetivo de evitar la oxidación antes de su consumo y equilibrar la mayor ingesta de ácidos grasos poliinsaturados (prooxidantes) que implicaba la intervención nutricional.

Tras las 8 semanas de intervención nutricional los futbolistas llevaron a cabo un ejercicio de intensidad elevada con objeto de evaluar los efectos de una actividad física aguda. Para ello, los participantes realizaron una sesión de actividad física intensa y controlada durante 2 horas, de manera que durante más del 50% del tiempo de entrenamiento los deportistas se ejercitasen a tasas superiores al 70% de su VO_2máx. El entrenamiento consistió en la ejecución del test de capacidad máxima Léger-Boucher, para la determinación indirecta la

VO_2máx individual, y la realización de ejercicios tácticos de simulación de situaciones que tienen lugar durante un partido de futbol y que suponen de una elevada intensidad física.

En total se practicaron tres extracciones de sangre venosa del brazo: una en condiciones basales al inicio del estudio (muestra basal) y otras dos tras las 8 semanas de estudio, una en condiciones de reposo (muestra pre-ejercicio) y otra muestra 2 horas después de finalizar la sesión de actividad física aguda e intensa programada (muestra post-ejercicio). La muestra pre-ejercicio fue extraída tras un día de descanso tras el entrenamiento, para asegurar así la recuperación completa de los jugadores.

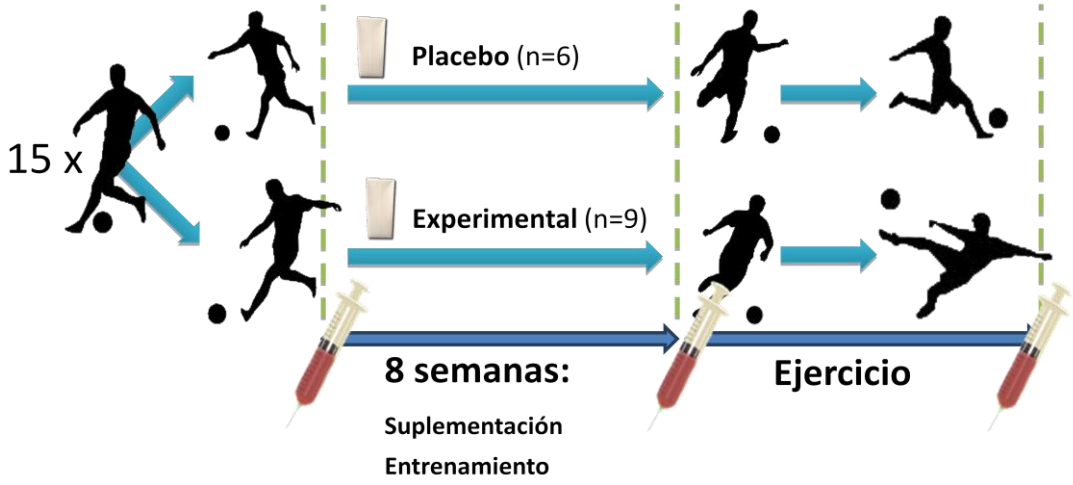


Figura 6. Intervención deportivo-nutricional.

El recuento celular de eritrocitos y PBMCs (linfocitos y monocitos) se determinó mediante citometría de flujo. En el laboratorio se fraccionaron las muestras sanguíneas para la obtención de PBMCs y eritrocitos. En eritrocitos se determinó la composición de ácidos grasos de membrana mediante extracción por método de Folch, derivatización con Meth-Prep II y separación por cromatografía de gases. La cuantificación se llevó a cabo en referencia a un patrón estándar interno C17:0. En PBMCs, purificadas mediante separación diferencial en gradiente de densidad con *ficoll*, se determinaron, mediante western blot, los niveles de proteínas antioxidantes (UCP2, UCP3, MnSOD, COXIV) y proteínas implicadas en la dinámica mitocondrial (PGC1α, NRF1, Tfam, Mfn 1, Mfn 2, OPA1, OMA1, FIS1. También se evaluaron marcadores de daño oxidativo en lípidos (MDA) y proteínas (carbonilos mediante Dot-Blot). Mediante Real-Time PCR se determinó la expresión relativa de los mRNA de

COXIV, MitND5 y PGC1α. La producción de peróxido de hidrógeno (H_2O_2), como forma de ROS, se cuantificó mediante fluorimetría. Para ello, las PBMCS se incubaron con diclorofluoresceína diacetato (DCFH-DA), una sonda apolar con capacidad para atravesar la membrana plasmática. Al estimular las células con PMA (10 ng/mL) tiene lugar la formación de H_2O_2, el cual oxida la sonda desacetilada (DCFH) dando lugar a la forma fluorescent (DCF). Por ello, con este método se cuantifica el H_2O_2 intracelular y es un indicativo del estrés oxidativo celular. Por último, se comprobó el nivel de activación de NFκB mediante técnica de ELISA (*Enzyme-linked Immunosorbent Assay*), usando anticuerpos primarios que se unen específicamente a un epítopo únicamente accesible cuando NF-κB está activado, es decir, cuando no se encuentra inhibido por los inhibidores de NF-κB (IκB) y es libre para entrar al núcleo dónde puede activar la expresión de los genes específicos que tienen cerca sitios de unión de ADN para NFκB.

Los resultados de este estudio se recogen en los manuscritos I y II.

Experimento 2. Estilo de vida: estado antioxidante e inflamatorio.

Respuesta antioxidante, inflamatoria y daño oxidativo en relación con el estilo de vida en pacientes sanos sexagenarios según el grado de actividades físicas diarias y el estilo de vida.

Para la consecución de los objetivos 4 y 5 se seleccionaron 61 hombres>55 años y 66 mujeres>60 años sin enfermedad cardiovascular previamente documentada- incluidos en un estudio transversal dirigido a identificar factores de riesgo cardiovascular en la población anciana de las Islas Baleares (proyecto PHYSMED [294]), y se agruparon según su estilo de vida. Los individuos fueron categorizados según terciles de equivalente metabólicos (METs, definidos como la cantidad de oxígeno consumido en reposo) obtenidos mediante el test *Minnesota Leisure-time Physical Activity Questionnaire*, según el grado de actividades físicas diarias declaradas durante el año anterior en: inactivos (<4100 METs/min semana), intermedios y activos (>6300 METs/min semana).

De todos los participantes se obtuvieron los datos de ingesta, mediante cuestionarios de recordatorio de 24 horas, medidas antropométricas (peso, altura, IMC, % grasa, presión arterial) y una muestra de sangre venosa en condiciones basales (en reposo y en ayunas). A nivel de laboratorio clínico, glucosa, triglicéridos, colesterol total, HDL, LDL y VLDL, urea, ácido úrico y creatinina fueron determinaron mediante procedimientos estándar utilizando kits clínicos comerciales en un sistema autoanalizador (Technicon DAX System). Los parámetros hematológicos y el hemograma se determinaron mediante citometría de flujo. A nivel de laboratorio de investigación, se realizó la extracción de PBMCs y plasma. En PBMCs se determinaron los niveles de proteínas antioxidantes (CAT, MnSOD, GPx, GRd, TrxR1, UCP3, TLR2, TLR4). También se determinaron niveles de marcadores de daño oxidativo y nitrosativo en proteínas (índice de carbonilos y Nitro-Tyr mediante Dot-Blot,). Se estudió además la expresión relativa de los mRNA de COXIV, PGC1α, MitND5, IL1ra, IL10, IL1β, NFκB, TLR4, TNFα, IL6 mediante Real-Time PCR. Por último se llevó a cabo la determinación de las actividades enzimáticas de CAT, SOD, GPx, GRd. En plasma se evaluaron marcadores de daño oxidativo en lípidos (MDA), las actividades enzimáticas de CAT, SOD y MPO y marcadores de infamación (IL6, TNFα, sCD62L, sICAM3).

Los resultados de este estudio se recogen en los manuscritos III y IV.

Experimento 3. Efectos horméticos del peróxido de hidrógeno

Exposición ex vivo a H_2O_2 sobre el estado redox en inflamatorio en PBMCs de humanos con síndrome metabólico

Para tratar de alcanzar los objetivos 6 y 7 desarrollamos una metodología experimental basada en la emulación de una situación de estrés oxidativo *ex vivo* mediante la exposición continuada durante 2 horas de células inmunitarias a H_2O_2 generada por la enzima glucosa oxidasa. Se seleccionaron un total de 34 muestras provenientes de pacientes incluidos en el estudio PREDIMED-Plus (ISRCT; http://www.isrctn.com/ISRCTN89898870; nº 89898870) para realizar este experimento. El estudio PREDIMED-Plus es un ensayo multicéntrico (23 centros entre Península y Baleares), a 3 años de seguimiento, aleatorizado, con grupos paralelos (control/intervención), de prevención primaria cardiovascular

en hombres (55-75 años) y mujeres (60-75 años) con IMC ≥ 27 y <40 kg/m2 y con síndrome metabólico.

Se obtuvo una muestra de sangre en condiciones de ayuno de cada participante y se realizó la extracción, aislamiento y purificación de PBMCs y neutrófilos, además de la obtención del plasma sanguíneo. Ambos tipos celulares, PBMCs y neutrófilos, fueron incubados durante 2 horas a 37°C en medio RPMI-1640 (2g/L de glucosa) en presencia y ausencia de glucosa oxidasa (GOX) (proveniente de *Aspergillus niger,* ~75% protein, 138,370 U/g solid, Sigma-Aldrich). La GOX se utiliza con el objetivo de inducir la producción de H_2O_2 (una unidad de GOX oxida 1.0 µmol de β-D-glucosa a D-glucono-δ-lactona y H_2O_2 por minuto a pH 5.1 a 35°C). Las concentraciones de sólido (µg GOX/ml medio de cultivo) añadidas al cultivo celular se especifican en el diagrama a continuación:

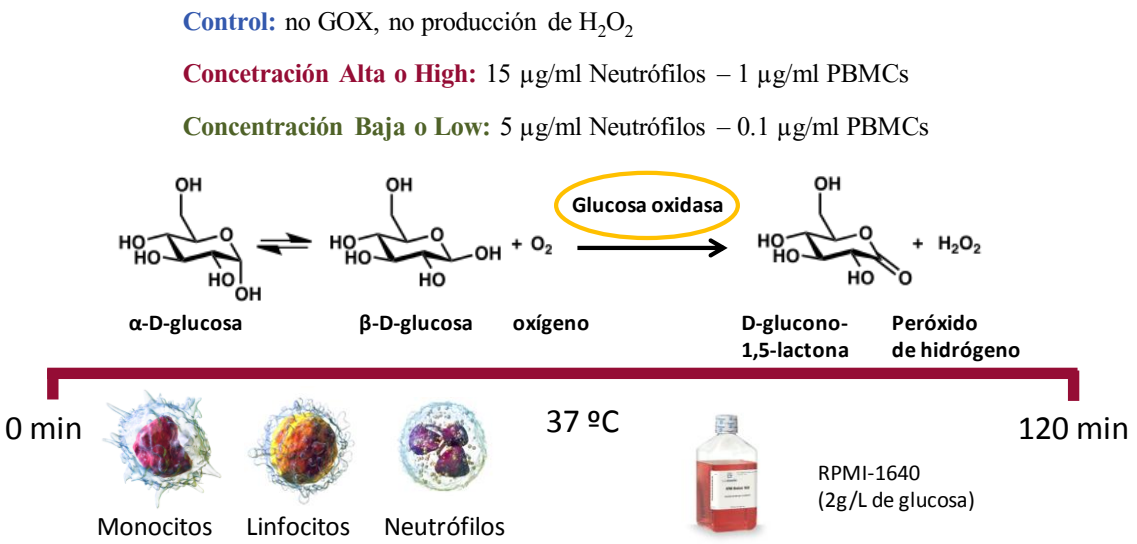


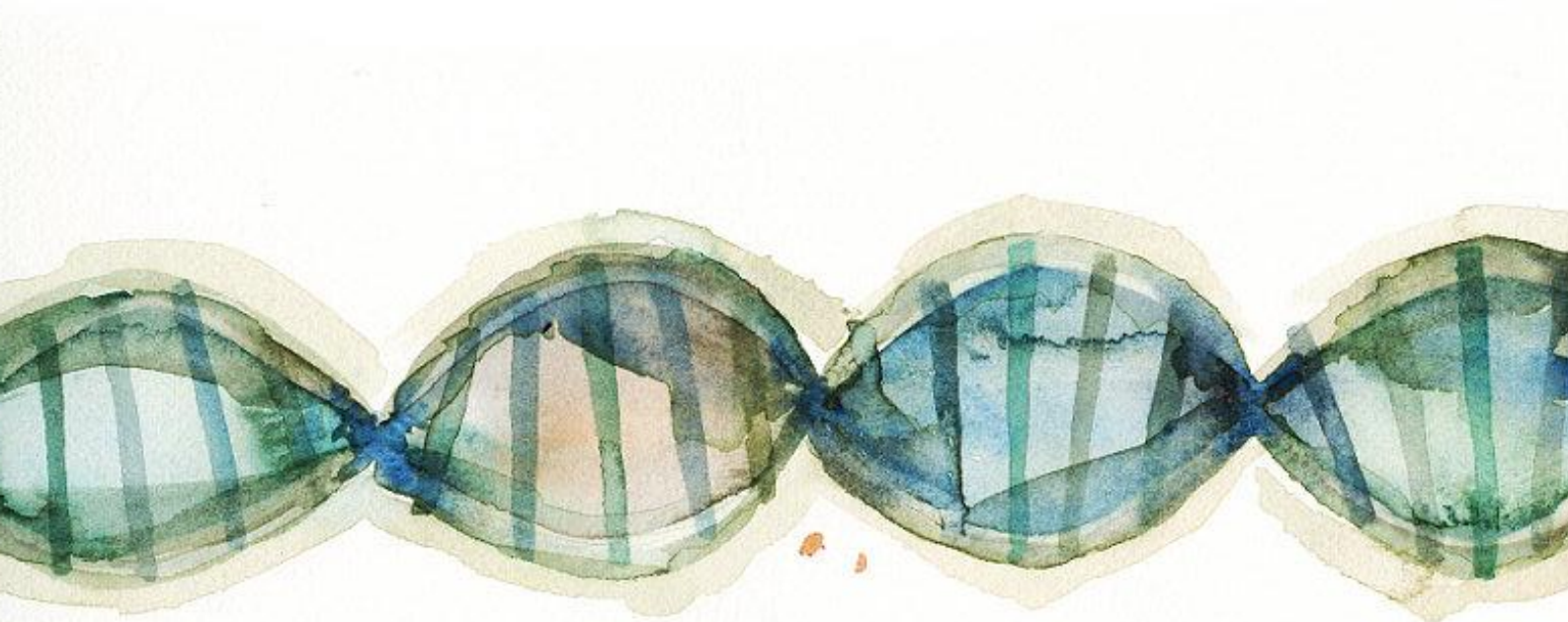
Figura 7. Condiciones de incubación de las PBMCs y los neutrófilos en presencia de glucosa oxidasa. (Recurso: Elaboración propia)

Tras las 2 horas de incubación se centrifugaron las muestras a fin de obtener las células y el sobrenadante del cultivo. Se realizó un ensayo de viabilidad celular mediante el método cristal violeta para comprobar que el tiempo de incubación y la dosis de GOX no resultaban letales para el cultivo celular. Tanto en neutrófilos como en PBMCs se midió la expresión relativa de los mRNA correspondientes a proteínas implicadas en la respuesta inmune (COX2, TLR2, TLR4, NFκB), marcadores proinflamatorios (IL6, Il8, TNFα),

mediadores antiinflamatorios (IL1rα, IL1β), enzimas antioxidantes (CAT, MnSOD, CuZnSOD) y proteínas relacionadas con la dinámica mitocondrial (Mtf1, Mtf2, Tfam y NRF2). En los sobrenadantes de ambos tipos celulares se determinaron los niveles de citoquinas (adiponectina, IL6 y TNFα).

Los resultados de este estudio se recogen en el manuscrito V.

III. Resultados y discusión



Manuscript I

Training and acute exercise modulates mitochondrial dynamics in football player's blood mononuclear cells

Carla-Busquets Cortés, Xavier Capó, Miquel Martorell, Josep A. Tur, Antoni Sureda, Antoni Pons.

2017. European Journal of Applied Physiology, *117*(10), 1977–1987.
doi: 10.1007/s00421-017-3684-z. Epub 2017 Jul 26.

Eur J Appl Physiol (2017) 117:1977–1987
DOI 10.1007/s00421-017-3684-z

ORIGINAL ARTICLE

Training and acute exercise modulates mitochondrial dynamics in football players' blood mononuclear cells

Carla Busquets-Cortés[1] · Xavier Capó[1] · Miquel Martorell[2] · Josep A. Tur[1,3] · Antoni Sureda[1,3] · Antoni Pons[1,3]

Received: 22 December 2016 / Accepted: 10 July 2017 / Published online: 26 July 2017

Abstract

Purpose Regular physical activity induces oxidative stress but also causes adaptations in antioxidant defences including the nuclear factor κB (NF-κB) pathway, which activates target genes related to antioxidant defences such as uncoupling proteins (UCPs), and mitochondrial biogenesis mediated by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). The aim of the study was to determine the effect of long-term training and acute exercise on oxidant/antioxidant status and the expression of mitochondrial biogenesis genes in peripheral blood mononuclear cells (PBMCs).

Methods Twelve professional football players performed an 8-week exercise programme comprising a daily 2-h football training session. Blood samples were taken before and after the training season.

Results The results reported a significant increase in antioxidant protein levels and in mitochondrial proteins in resting conditions after the 8-week training period. PGC1α, UCP-2 and mitofusin 2 protein levels also increased after acute exercise compared to pre-exercise levels. After the training, the expression of PGC1α, cytochrome *c* oxidase subunit IV and mitochondrial NADH dehydrogenase subunit 5 messenger RNA (mRNA) significantly augmented after the acute physical activity compared to pre-exercise levels; while no changes occurred in these mRNA in basal conditions. NF-κB activation and ROS production reported a significant increase after acute exercise.

Conclusions Training increases the levels of proteins related to mitochondrial biogenesis and improves the antioxidant capabilities of mitochondria in PBMCs among well-trained football players. Acute exercise may act as an inducer of mitochondrial biogenesis through NF-κB activation and PGC1α gene expression.

Keywords Biogenesis · Fission · Fusion · Mitochondria · PBMCs · Training

Abbreviations

18S	Ribosomal 18S
ANOVA	Analysis of variance
BMI	Body mass index
CAT	Catalase
cDNA	Complementary DNA
COX-IV	Cytochrome *c* oxidase subunit IV
CuZn-SOD	Cu/Zn superoxide dismutase
DCF	Dichlorofluorescein
DCFH-DA	2,7-Dichlorofluorescin-diacetate
DNA	Desoxy-ribonucleic acid
DRP-1	Dynamin-related protein 1
ELISA	Enzyme-linked immunosorbent assay
FIS-1	Fission Protein 1
HR_{max}	Maximum heart rate
GPx	Glutathione peroxidase
GTPase	Guanosine triphosphate hydrolase

Communicated by Nicolas Place.

✉ Antoni Pons
antonipons@uib.es

[1] Research Group on Community Nutrition and Oxidative Stress, Science Laboratory of Physical Activity, Department of Fundamental Biology and Health Sciences, University of Balearic Islands, 07122 Palma de Mallorca, Spain

[2] Departamento de Nutrición y Dietética, Facultad de Farmacia, Universidad de Concepción, Chile, 4070386 Concepción, Chile

[3] CIBER: CB12/03/30038 Fisiopatología de la Obesidad la Nutrición, CIBEROBN, Instituto de Salud Carlos III (ISCIII), University of Balearic Islands, 07122 Palma De Mallorca, Spain

H_2O_2	Oxygen peroxide
HIIT	High-intensity interval training session
PBMC	Peripheral blood mononuclear cell
MAPK	Mitogen-activated protein kinase
MAS	Maximal aerobic speed (=vVO_{2max})
MitND5	Mitochondrial NADH dehydrogenase subunit 5
Mn-SOD	Mn superoxide dismutase
mRNA	Messenger RNA
Mtf-1	Mitofusin-1
Mtf-2	Mitofusin-2
NRF-1	Nuclear respiratory factor 1
OPA-1	Optic atrophy protein 1
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator
PMA	Phorbol 12-myristate 13-acetate
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
SEM	Standard error of the mean
SDS	Sodium dodecylsulfate
ROS	Reactive oxygen species
Tfam	Mitochondrial transcription factor A
UCP-2	Uncoupling protein 3
UCP-3	Uncoupling protein 3
VO_{2max}	Maximal oxygen consumption
vVO_{2max}	Velocity at maximal oxygen uptake (=MAS)
WHR	Waist–hip ratio

Introduction

Mitochondria are organelles with an essential energetic role characterised by high plasticity that constantly fuse with (fusion) and separate from (fission) the mitochondrial reticulum (Drake et al. 2016; Ni et al. 2015). These phenomena enable the cell to maintain a connected mitochondrial network (structural homeostasis) by joining healthy mitochondria, and preserve quality by dismantling and excluding damaged mitochondria (Van der Bliek et al. 2013). Both events, alongside the assembly of new mitochondria (biogenesis) in response to energy demands, are subject to non-stop, strict control and equilibrium, and are prepared to tip the balance when physiological demands change (Hood et al. 2006; Yun and Finkel 2014; Westrate et al. 2014).

The fusion process is managed by the membrane-bound GTPases mitofusin 1 (Mtf-1), mitofusin 2 (Mtf-2) and optic atrophy protein 1 (OPA-1), which are crucial for the union of the outer and inner mitochondrial membrane, respectively (Romanello and Sandri 2015; Scott and Youle 2010). Through the fusion process, a continuous mitochondrial network is configured, and membranes and matrix lumens can be shared. As a consequence, constituent mitochondria can exchange metabolites and solutes, while metabolic energy can be dissipated by transmission of membrane potential (Kuzmicic et al. 2011; Twig et al. 2008). In turn, fission processes are under the control of dynamin-like GTPases, such as dynamin-related protein 1 (DRP-1) and fission protein 1 (FIS-1). Fission enables the segregation of dysfunctional mitochondria and their elimination through mitophagy processes, while also participating in cell apoptosis by releasing cytochrome *c* (Losón et al. 2013). These fusion and fission events show that mitochondrial dynamics have a fundamental role in regulating metabolic pathways and turn out to be critical in maintaining adequate energy balance and cell viability. Otherwise, any factual error resulting from mitochondrial breakdown can lead to cell problems that could cause serious diseases in mammals (Diaz and Moraes 2008).

Mechanical stress-induced signals, such as p38 mitogen-activated protein kinase (MAPK), produced during muscle contraction have the potential to promote and regulate the activity and expression of exercise-sensitive transcription factors such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). In turn, PGC-1α induces mitochondrial biogenesis by orchestrating the transcription of the nuclear and mitochondrial genome, via nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (Tfam), respectively (Busquets-Cortés et al. 2016; Picca and Lezza 2015). Indeed, mitochondria are responsible for starting off signal transduction cascades to the nucleus that coordinate transcriptional responses after physiological alterations (Merry and Ristow 2016; Yun and Finkel 2014). The positive effects of moderate exercise lie in mitochondrial and non-mitochondrial adaptations that restore and maintain cellular homeostasis. With regard to mitochondria, regular training confers healthy properties owing to coordinated improvements in quality (structure–function) and quantity (amount) through structural merging (fusion) and disjointing (fission), and synthesis-incorporation of mitochondrial DNA and proteins in the existing reticulum (biogenesis) (Drake et al. 2016). Within the adaptive responses associated to exercise, the activation of the NF-κB pathway induces target genes related to antioxidant defences, such as mitochondrial uncoupling proteins (UCP) and antioxidant enzymes (Flandin et al. 2005; Ho et al. 2012). In this way, UCP-2 and UCP-3, both located in the mitochondrial inner membrane, catalyse a proton leak that uncouples oxidative phosphorylation and reduces membrane potential, likely leading to reduced reactive oxygen species (ROS) production (Bézaire et al. 2007). Furthermore, there is an enhancement in cardiac and skeletal muscle mitochondrial respiratory capability and a proper adjustment of mitochondrial life span (Martorell et al. 2014; Yan et al. 2012).

Although this molecular pathway has been thoroughly studied in skeletal muscle, it remains poorly described in other cell types. The practice of regular physical activity

appears to be one of the main contributors to chronic disease prevention and is considered a health promoter. The molecular mechanisms involved have not been fully elucidated, but there are numerous studies that describe functional changes in immune cells such as peripheral blood mononuclear cells (PBMCs) after exercise. Regular physical exercise can potentiate PBMC activation, resulting in an increased cytokine production via TLR signalling pathways; this can lead to improved oxidative stress and inflammatory profiles (Capó et al. 2016a, b; Farinha et al. 2015; Zheng et al. 2015). In addition, blood cells have the advantage of being easily obtained from human tissue in great quantities with minimally invasive techniques. It has been suggested that PBMCs could be used as a surrogate model for skeletal muscle, offering a significant practical advantage for gene expression analysis in nutrigenomic studies (Rudkowska et al. 2011). We hypothesized that physical activity increases the physiological demands and, consequently, oxidative stress would have increased during the acute exercise that could affect cell functionality. Concurrently, mitochondrial biomarkers could undergo changes that have not been characterised in peripheral blood mononuclear cells (PBMCs). Our aim was to investigate the consequences of long-term training and a bout of acute exercise on antioxidant status and the expression of mitochondrial biogenesis proteins in immune cells among professional football players. The procedures for obtaining muscle cells for research purposes involve invasive techniques and involve many ethical difficulties in healthy subjects. Consequently, we selected PBMCs, which are easier and quicker to obtain, as a model for studying the effects of different exercise levels on mitochondria dynamics and for validating their cellular suitability in evidencing changes associated with physical activity.

Materials and methods

Study design and anthropometric characteristics

Twelve male professional football players from the Reial Mallorca B team voluntarily took part in the study at the beginning of their annual sport season. Anthropometric characteristics of the participants were 19.3 ± 0.4 years old, 76.5 ± 1.8 kg of weight and 179.2 ± 2.5 cm of height. The waist circumference was 78.2 ± 0.8 cm, the hip circumference was 97.0 ± 1.0 cm and the waist–hip ratio (WHR) was 0.805 ± 0.012. The value of systolic blood pressure was 117 ± 8 and 56.7 ± 5.9 mmHg for the diastolic blood pressure. The body mass index (BMI) was 24.0 ± 0.6 kg/m^2, the football players had 92.5 ± 0.2% fat-free mass and the calculated maximal oxygen consumption (VO_{2max}) was 60.4 ± 1.8 mL/(kg·min). Participants performed a program of exercise for 8 weeks 5 days/week consisting of 2 h of habitual physical training session and one official match per week. The study was initiated at the beginning of the season. The intense physical activity was 96.4 ± 57.9 min/day and the moderate exercise consisted of 68.6 ± 17.1 min/day. High-intensity training involves repeated bouts of high-intensity exercise at ≥80% maximum heart rate (HR_{max}), interspersed with brief periods of rest or low-intensity exercise, whereas moderate training was comprised between 50–80% HR_{max}. After 8 weeks of regular training the players performed an acute exercise session consisting of 15 min of warm-up, followed by the Léger–Boucher test, which consists of continuous running between two lines 20 m apart in time to recorded beeps that will be progressively closer together (Léger and Boucher 1980) and then, a recovery exercise of control-passing for 15 min. The central part of the training session consisted of small-sided football games. Succinctly, the first exercise was a 5 vs. 5 possession exercise in a 20 × 15 m area (4 repetitions of 5 min with 30 s of recovery between repetitions); the second was a 6 vs. 6 possession exercise in a 30 × 20 m area (3 repetitions of 6 min with 1 min of recovery between repetitions), and finally the athletes played a 5 vs. 5 soccer match in a 30 × 40 m area for 20 min. The exercise was conceived to allow players to work above their 80% VO_{2max} for more than the 50% of the match time, ensuring that under these conditions the exercise caused oxidative stress (Martorell et al. 2015; Sureda et al. 2009). The Léger–Boucher test was performed at the beginning of the study and after 8-weeks training and the maximum oxygen uptake (VO_{2max}) was calculated by the retroextrapolation method (extrapolation to time zero of recovery of the exponential least squares regression of the first four 20-s recovery VO_2 values). Maximal aerobic speed (MAS) is the minimal running velocity at which VO_{2max} occurs, also known as the velocity at VO_{2max} (vVO_{2max}) (Léger and Lambert 1982). All the participants followed a standardized Mediterranean diet with analogous energy, carbohydrate and lipid intake supervised by the medical and nutritionist team. All the participants were informed of the aim and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human participants and was approved by the Ethical Committee of Clinical Investigation of the Comunitat Autónoma de les Illes Balears No. IB 994/08 PI (Palma de Mallorca, Balearic Islands, Spain). The project was registered at http://ClinicalTrial.gov (NCT02177383).

Dietary intake

Dietary habits were assessed using a 7-day dietary record fulfilled at the beginning of the study. A dietician team verified and quantified the food records and every food item

consumed was converted into nutrients using a computerized program according to the European and Spanish food composition tables.

Experimental procedure

For each subject, three different blood samples were obtained from the antecubital vein. One blood sample was taken in basal conditions after overnight fasting before starting the experimental period. Following 8 weeks of training and after a day of rest from the last day of training to ensure the complete recovery of the players two new blood samples were taken; one samples was obtained under fasting conditions (pre-exercise conditions) and the second, 2 h after finishing the physical activity session (post-exercise conditions). Cell counts were determined in an automatic flow cytometer analyzer Technicon H2 (Bayer, Leverkusen, Germany) system. The PBMC fraction was purified following an adaptation of the method described elsewhere (Boyum 1964) using Ficoll-Paque PLUS reagent (GE Healthcare, 190 Chalfont St Giles, UK). Blood was carefully poured on Ficoll in a 1:5:1 proportion and was then centrifuged at 900*g*, at 4 °C for 30 min. The plasma and the Ficoll phases were discarded and PBMCs layer was washed twice with PBS and centrifuged for 10 min at 1000*g*, 4 °C. This process was performed in duplicate; one sample was destined to obtain RNA and the other one was preserved with radioimmunoprecipitation (RIPA) lysis buffer for western blot analysis. Cell lysates were stored at −80 °C until biochemical analyses.

PBMCs RNA extraction and real-time PCR

Cytochrome *c* oxidase subunit IV (COX-IV), PGC-1α and mitochondrial NADH dehydrogenase subunit 5 (MitND5) mRNA expression was determined by multiplex real-time PCR based on incorporation of a fluorescent reporter dye and using human 18S ribosomal as the reference gene. For this purpose, total RNA was isolated from PBMCs by extraction with Tripure (Tripure Isolation Reagent, Roche Diagnostics, Germany) following a procedure previously described (Capó et al. 2014). RNA (1 µg) from each sample was subjected to reverse transcription using 50 units of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol of oligo (dT) for 60 min at 37 °C in a 10 µL final volume, according to the manufacturer's instructions. The resulting cDNA (3 µL) was amplified with the Light Cycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Germany). Target cDNAs were amplified as follows: 10 min, 95 °C followed by 45 cycles of amplification. The specific primers and amplification conditions used for each gene are presented in Table 1. mRNA levels at the beginning of the training season were arbitrarily referred to as 1.

SDS-polyacrylamide gel electrophoresis and western blot analysis

COX-IV, PGC-1α, Mtf-1, Mtf-2, Tfam, UCP-2 and UCP-3 protein levels were determined by western blot. PBMCs were lysed with 250 µL of RIPA buffer [250 mM Tris/HCl, pH 8.0, 4.4% NaCl, 5% IGEPAL®, 2.5% deoxycholic acid, 0.5% sodium dodecylsulfate (SDS)] and heated for 5 min at

Table 1 Primer sequence and conditions used in real-time PCRs

Gene	Primer		Temperature (°C)	Time (s)
UCP-3	Fw	5′-CGT GGT GAT GTT CAT AAC CTA TG-3′	60	10, 10, 15
	Rv	5′-CGG TGA TTC CCG TAA CAT CTG-3′		
PGC-1α	Fw	5′-CACTTACAAGCCAAACCAACAACT-3′	62	10, 10, 15
	Rv	5′-CAATAGTCTTGTTCTCAAATGGGGA-3′		
COXIV	Fw	5′-AGAAGCACTATGTGTACGGCCC-3′	60	10, 10, 15
	Rv	5′-GGTTCACCTTCATGTCCAGCAT-3′		
MitND5	Fw	5′-CGGCTGAGAGGGCGTAGG-3′	60	10, 10, 15
	Rv	5′-GATGAAACCGATATCCGGCCGA-3′		
Mfn1	Fw	5′-TGTTTTGGTCGCAAACTCTG-3′	60	10, 10, 15
	Rv	5′-CTGTCTGCGTACGTCTTCCA-3′		
Mfn2	Fw	5′-ATGCATCCCCACTTAAGCAC-3′	60	10, 10, 15
	Rv	5′-CCAGAGGGCAGAACTTTGTC-3′		
18S	Fw	5′-ATGTGAAGTCACTGTGCCAG-3′	60	10, 10, 15
	Rv	5′-GTGTAATCCGTCTCCACAGA-3′		

100 °C. COX-IV, PGC-1α (30 µg of protein), Mtf-1, Mtf-2, UCP-2, UCP-3 and Tfam (20 µg of protein) aliquots were loaded in each lane and separated by size following SDS-polyacrylamide (12% polyacrylamide) gel electrophoresis. Samples were transferred onto a nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-Rad) and blocked with 5% non-fat dry milk for 3 h. The membrane was incubated overnight at 4 °C with commercially available primary antibodies: anti-COX-IV (1:500), anti-UCP-3 (1:500) (Millipore, Billerica, MA, USA), anti-PGC-1α (1:1000), anti-Tfam (1:500), anti-UCP-2 (1:500), anti-Mtf-1 (1:200) and anti-Mtf-2 (1:1000) antibodies (Santa Cruz Biotechnology, CA, USA). Then, incubation with a secondary peroxidase-conjugated antibody, anti of the primary antibody (1:5000 for PGC1α, Tfam, Mtf-1 and Mtf-2; and 1:10,000 for COX-IV, UCP-2 and UCP-3) was performed. Protein bands were visualized by Immun-Star Western C Kit reagent (Bio-Rad Laboratories) Western blotting detection systems. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad laboratories) and the resulting bands were analyzed using the image analysis program Quantity One-1D Software (Bio-Rad Laboratories). Precision Plus Protein™ Kaleidoscope™ (Bio-Rad) was used as a molecular weight marker and the band density of each protein was quantified according to the loading control (Actin), used as a housekeeping.

Hydrogen peroxide production

H_2O_2 intracellular production, as an indicator of ROS production, by PBMCs was measured after stimulation with phorbol 12-myristate 13-acetate (PMA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator. DCFH-DA is a cell-permeable probe that is hydrolyzed intracellularly to DCFH carboxylate anion, which remains in the cell. Two-electron oxidation of DCFH generates dichlorofluorescein (DCF), a fluorescent product easily monitored by fluorescent-based techniques. A stock solution of DCFH-DA (1 mg/mL) in ethanol and PMA (1 mg/mL) in DMSO was prepared and stored at 20 °C until analysis. DCFH-DA (30 µg/mL) in PBS was added to a 96-well microplate containing 50 µL of fresh PBMCs suspension. PMA (10 ng/mL) was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was measured at 37 °C for 1 h in FL 9800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.). The production of H_2O_2 has been quantified with a standard with known concentrations of H_2O_2.

Determination of NF-κB activation

NF-κB activation was determined before and after the acute exercise after 8 week of training. An isolated suspension of PBMCs was subjected to whole-cell protein extraction for the determination of NF-κB p50 activation, which was performed using an Enzyme-linked Immunosorbent Assay (ELISA) method according to the manufacturer's instructions TransAM NF-κB p50 Chemi (Active Motif®). Briefly, the primary antibody used to detect NF-κB recognizes an epitope on p50 that is accessible only when NF-κB is activated and bound to its target DNA.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.21 for Windows). Results are expressed as mean ± standard error of the mean (SEM), and the level of significance was established at $p < 0.05$ for all statistics. A Kolgomorov–Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by one-way analysis of variance (ANOVA). When significant differences were observed, post hoc analyses were completed using a Student's *t* test paired data analysis.

Results

The participants' nutritional intake before the start of the study is presented in Table 2. The athletes' protein intake was greater than general recommendations, while carbohydrate and fibre intakes were lower. The volunteers' diets showed high levels of cholesterol and lipids with a poor balance of saturated and unsaturated fats according to dietary guidelines, which is a general pattern observed in the Balearic Island population.

The athletes spent 17.42 ± 1.62 min performing the Léger–Boucher test at the beginning of the study and 18.15 ± 1.72 min after 8 weeks of experiment time. The calculated VO_{2max} values of football players recorded after performing the Léger–Boucher test were 60.41 ± 1.8 mL/(kg·min) at baseline and 62.61 ± 3.3 mL/(kg·min) after 8 weeks of intervention. No differences were detected after 8 weeks of training, thus indicating that the players were highly fit.

The changes in PBMCs after training and acute exercise are presented in Table 3. No significant effects from training or acute exercise were observed in total PBMC counts or the percentage of lymphocytes and monocytes.

The effects of training and acute exercise on mRNA expression in football players' PMBCs are shown in Fig. 1. It would appear that 8 weeks of regular training did not modify the gene expression of COX-IV, PGC1α and MitND5 determined in basal conditions, whereas a significant increase was reported in the three genes after the acute physical activity ($p < 0.05$).

Table 2 Daily nutrient intake of the athletes before the start of experiment

Energy (kcal)	2721.08 ± 160.4
Water (mL)	1880 ± 287
Protein energy (%)	18.54 ± 1.1
Carbohydrate energy (%)	51.5 ± 1.8
Lipid energy (%)	39.68 ± 1.9
Saturated fatty acids (%)	13.1 ± 1
Monosaturated fatty acids energy (%)	16.3 ± 1.1
Polyunsaturated fatty acids energy (%)	4.41 ± 0.36
Fiber (g)	18.8 ± 2.3
Cholesterol (mg)	430 ± 21
Alcohol energy (%)	NI ± NI
Sodium (mg)	2931 ± 446
Potassium (mg)	2860 ± 219
Magnesium (mg)	282 ± 15
Phosphorous (mg)	1538 ± 91
Calcium (mg)	821 ± 42
Iron (mg)	22.6 ± 8.6
Copper (mg)	2.05 ± 0.11
Selenium (µg)	94.8 ± 13.1
Iodine (µg)	102 ± 14
Zinc (mg)	13.1 ± 1.3
Retinol (µg)	396 ± 43
Carotenes (µg)	1905 ± 276
Vitamin A (retinol equivalents)	787 ± 56
Thiamine (mg)	1.44 ± 0.08
Riboflavin (mg)	1.88 ± 0.05
Vitamin B_6 (mg)	2.44 ± 0.29
Vitamin B_{12} (µg)	13.7 ± 3.2
Vitamin C (mg)	80.2 ± 12.3
Vitamin D (µg)	2.43 ± 0.4
Vitamin E (mg)	8.06 ± 0.7
Niacin (mg)	27.2 ± 1.3
Pantothenic acid (mg)	5.52 ± 0.18
Folic acid (µg)	262 ± 11

Mean ± SEM, $n = 12$

Table 3 Effects of training and acute exercise supplementation on PBMC counts

	Initial basal	Eight weeks basal	Eight weeks post-exercise
PBMCs (10^3 cells/µL)	2.47 ± 0.6	2.98 ± 0.4	3.19 ± 0.4
Lymphocytes (%)	79.9 ± 6.6	82.9 ± 4.3	69.19 ± 4.6
Monocytes (%)	20.1 ± 5.5	17.0 ± 3.8	24.2 ± 4.3

No significant differences were reported. Mean ± SEM, $n = 12$

Figure 2a, b shows the effects of training and acute exercise on the levels of specific mitochondrial proteins in PBMCs. Exercise training for 8 weeks significantly

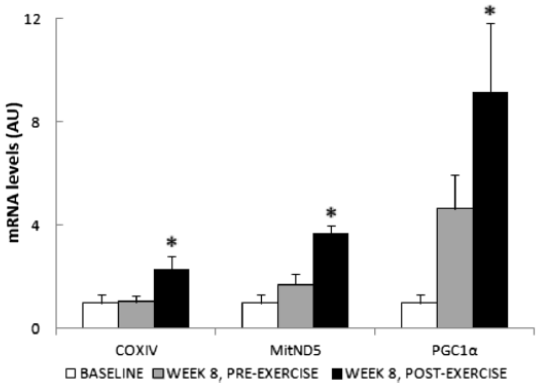


Fig. 1 Changes in mRNAs expression in football players' PMBCs. The *hash symbol* indicates significantly different values (Student's *t* test paired data, $p < 0.05$) between baseline and 8-week pre-exercise values. The *asterisk* indicates significantly different values between 8-week pre-exercise and 8-week post-exercise values. Mean ± SEM, $n = 12$

increased the protein levels of respiratory chain uncoupling proteins (UCP-2 and UCP-3), the inductor of mitochondrial biogenesis Tfam, a marker of mitochondrial respiratory chain function (COX-IV) and a marker of mitochondrial fusion (Mtf-1). In addition, UPC-2 protein levels significantly increased 2 h after acute exercise. The PGC-1α and Mtf-2 protein levels only increased after the acute exercise bout in the 8-week samples compared to pre-exercise levels. Figure 3 also shows ROS production levels in PBMCs stimulated with PMA expressed as a percentage of the baseline hydrogen peroxide production rate. PBMCs from the baseline group and 8-week pre-exercise group produced 72.6 ± 12 and 68.0 ± 19 nmol H_2O_2/min × 10^6 cells, respectively. H_2O_2 production of the post-exercise group's PBMCs significantly increased to 119 ± 33 nmol H_2O_2/min × 10^6 cells.

The nuclear factor NF-κB must be activated before its internalisation into the nucleus and binding to DNA to facilitate the expression of many genes. The levels of activated NF-κB significantly increased 2 h after acute exercise (Fig. 4).

Discussion

The main point of this study was to demonstrate the influence of exercise training and a bout of acute exercise on mitochondrial biogenesis and mitochondrial dynamics for the peripheral blood mononuclear cells. No differences were detected after 8 weeks of training when performing the Leger–Boucher test or in the VO_{2max} and vVO_{2max} values of football players. This indicates their high physical

Eur J Appl Physiol (2017) 117:1977–1987

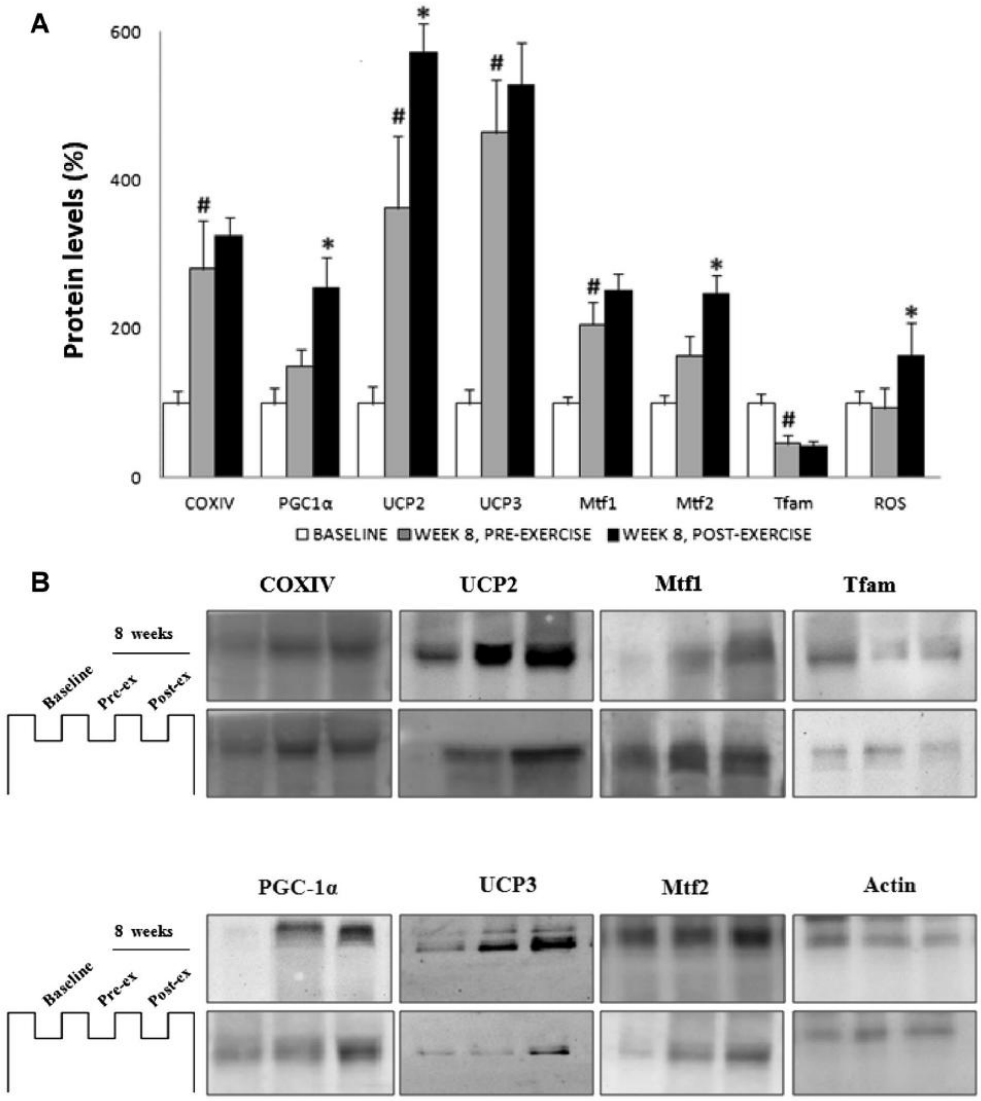


Fig. 2 **a** Changes in the levels of protein in football players' PMBCs. The *hash symbol* indicates significantly different values (Student's *t* test paired data, $p < 0.05$) between baseline and 8-week pre-exercise values. The *asterisk* indicates significantly different values between 8-week pre-exercise and 8-week post-exercise values. Mean ± SEM, $n = 12$. **b** Representative picture of the bands obtained by immunoblotting determined at baseline and at 8 weeks pre-exercise and post-exercise

fitness due to the participants being professional football players that have trained regularly for many years. Our obtained data are comparable to VO_{2max} from professional soccer players of a similar age (Sureda et al. 2007; Wells et al. 2012). Exercise training implies repeated exposure to an acute increase in metabolic, thermoregulatory and mechanical stress that can rapidly trigger cytosolic signalling pathways that ultimately modify nuclear gene expression (Merry and Ristow 2016). Regular physical exercise is characterised by adaptations in antioxidant defences in muscle and immune cells that improve exercise performance (Cases et al. 2005; Gondin et al. 2011). Indeed, the ROS normally produced during exercise cause a transient oxidative stress status that activates the antioxidant regulatory mechanisms in immune system cells (Ferrer et al. 2009). Many sources of ROS production have

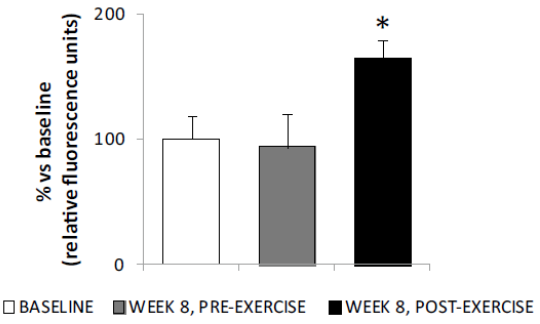


Fig. 3 Changes in ROS production level in PBMCs activated with PMA at the beginning of the study and after 8 weeks of training, under resting and post-exercise conditions. The *asterisk* indicates significantly different values between 8-week pre-exercise and 8-week post-exercise values. Mean ± SEM, $n = 12$

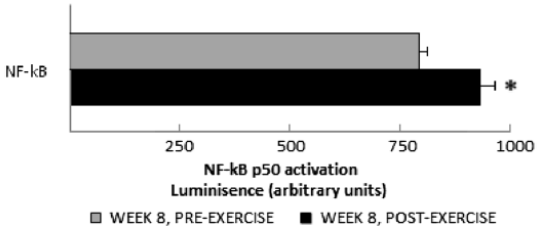


Fig. 4 NF-κB activation assay after 8 weeks of training. Changes in NF-κB activated levels after acute exercise. The *asterisk* indicates significantly different values between 8-week pre-exercise and 8-week post-exercise values. Mean ± SEM, $n = 12$

been reported in muscle during exercise, including the mitochondrial electron transport chain, NAD(P)H oxidase enzymes associated with the sarcoplasmic reticulum and transverse tubules, plasma membrane redox systems, phospholipase A2-dependent processes and xanthine oxidase activity (Ji 1995; Powers and Jackson 2008), which may eventually overwhelm antioxidant defences and result in a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage and cellular degeneration (Radak et al. 2013; Sureda et al. 2012). In the present study, acute exercise stimuli caused an activation of the NF-κB pathway in PBMCs, as has also been observed in these cells after marathon running (Gomez-Cabrera et al. 2007) and after sustained exercise at 80% VO_{2max} (Capó et al. 2016a, b; Vider et al. 2001). Likewise, 8 weeks of regular training enhanced the antioxidant mitochondria capability to counteract mitochondrial ROS production through increasing UCP-2 and UCP-3 protein levels; additionally, UCP-2 protein levels increased significantly after the acute exercise session, contributing to improve the antioxidant response (Pecqueur et al. 2009). The lack of a significant increase in H_2O_2 production after 8 weeks of training may be due to the established good physical fitness of the participants at the start of the study. In this sense, it has been shown that changes in ROS production and in antioxidant response are more evident in untrained people than in trained ones (Bloomer and Fisher-wellman 2008). However, in our study we have not determined the total antioxidant capacity of PBMCs to conclude whether there is an increase in the antioxidant capability or a reduction in the production of ROS. The current study demonstrates that training significantly increased UCP-2 and UCP-3 protein levels in PBMCs, bringing to light a possible augmented number of UCP-2/3 per mitochondria in accordance with studies carried out in skeletal muscle (Jiang et al. 2009) and in PBMCs (Capó et al. 2014). Low/moderate ROS levels are associated with their role as molecular signalling mediators that promote adaptive responses, whereas higher levels are harmful and are typically associated with the processes of senescence and apoptosis (Powers and Jackson 2008; Merry and Ristow 2016). Consequently, to guarantee cell functionality, integrity and to potentiate the adaptive responses to exercise, it is essential to avoid excessive production of ROS induced by each acute bout of exercise. Thus, the evidenced increase in UCP-2/3 protein levels could be a response to maintain ROS production within levels that allow their action as cellular messengers but avoid potential harmful effects. In a previous study, we reported that non-stimulated production of H_2O_2 in lymphocytes only significantly increases when the bout of exercise is high-intensity in well-trained football players (Sureda et al. 2007). Accordingly, the present results show that under maximal stimulation with PMA, PBMCs isolated in post exercise conditions exhibit larger capacity to produce H_2O_2. Concretely, we observed a 64% increase in H_2O_2 production. The higher capability of PBMCs to produce H_2O_2 after exercise supports the function of the reactive molecules as cellular signals via activating redox sensitive pathways including mitochondrial biogenesis and antioxidant defences (Li et al. 2016). In fact, increased production of ROS may trigger the upregulation of UCPs which have been associated to improvement in the mitochondrial efficiency of oxidative phosphorylation due to higher removal of ROS (Bo et al. 2008, 2016).

Our results are in accordance with the effects associated with exercise evidencing an increase in PGC-1α gene expression and protein levels after acute exercise in PBMCs. This suggests the likely involvement of PGC-1α in exercise-induced mitochondrial biogenesis, which is also supported by numerous studies carried out after acute exercise (Cartoni et al. 2005; Pilegaard et al. 2003) and after a training period (Russell et al. 2003; Short et al. 2003) in skeletal muscle. PGC-1α, through interaction with NRF-1 and Tfam, coordinates an upregulation of muscle mitochondrial content, a

capacity for substrate metabolism and oxidative phosphorylation, thus orchestrating compensatory physiological adaptations after repeated stimuli that enhance performance associated with endurance exercise training (Merry and Ristow 2016; Perry et al. 2010). PGC-1α also regulates COX-IV activity, the last oxidase in the mitochondrial electron transport chain and a likely mitochondrial number indicator (Samjoo et al. 2013). Both the increased COX-IV expression gene after acute exercise and higher protein levels after 8 weeks of training in our PBMCs may be due to a larger mitochondria number per cell; in this sense, training and acute exercise could induce new mitochondria biogenesis processes with a reduced ratio of ROS production in PBMCs.

In addition, mitochondrial specific proteins such as Mtf-1 and Mtf-2 experienced augmented protein levels. Both molecules are responsible for maintaining the mitochondrial network and the leading factors for mitochondrial fusion of the outer and inner mitochondrial membrane. Training could enhance mitochondrial remodelling in PBMCs in a similar way as has been pointed out in skeletal muscle (Cartoni et al. 2005). The expression of Mtf-1 and Mtf-2 increased after acute exercise in human skeletal muscle. This finding would underpin the statement that exercise promotes remodelling mitochondrial processes. Accordingly, it seems that the changes in PBMCs are similar to those reported for skeletal muscle after exercise, establishing an initial approach between immune system cell mitochondrial dynamics and the skeletal muscle cell model. However, additional studies looking at the changes that occur in the skeletal muscle and the PBMCs in the same samples are necessary. Since no differences were reported in PBMC counts or in the percentage of lymphocytes and monocytes before and after the training season, and under post-exercise conditions, changes in mitochondria markers may be attributed to training and/or acute exercise and not to changes in the lymphocyte/monocyte proportion.

In conclusion, the present results show that 8 weeks of regular training induces mitochondrial adaptations in terms of fusion processes and antioxidant UCP-2 and UCP-3 protein levels in PBMCs to meet the demands arising from exercise and also induces a larger capacity for H_2O_2 to be produced. Thus, our results could be an opening approximation in defining a link between mitochondrial events in immune cells and skeletal muscle cells, and reveal that regular exercise gradually improves mitochondrial quantity (mitochondrial biogenesis) and quality (balance between biogenesis and dynamics).

Acknowledgements Acción Estratégica en Salud del Ministerio de Ciencia e Innovación DPS2008-07033-C03-03, Programme of Promotion of Biomedical Research and Health Sciences (Projects PI11/01791 and PI14/00636, Red Predimed-RETIC RD06/0045/1004, CIBEROBN CB12/03/30038) and Balearic Island Government and FEDER funds (35/2011 and 23/2012). The authors hereby acknowledge the Ph.D. grant provided by the University of the Balearic Island and the FPI/1648/2014 grant provided by Conselleria d'Educació, Cultura i Universitats, Direcció General d'Universitats i Recerca, Govern de les Illes Balears.

Compliance with ethical standards

Conflict of interest All authors have declared there is not any potential conflict of interests concerning this article.

References

Bézaire V, Seifert EL, Harper M-E (2007) Uncoupling protein-3: clues in an ongoing mitochondrial mystery. FASEB J 21(2):312–324. doi:10.1096/fj.06-6966rev

Bloomer RJ, Fisher-wellman KH (2008) Blood oxidative stress biomarkers: influence of sex, exercise training status, and dietary intake. Gend Med 5:218–228

Bo H, Jiang N, Ma G, Qu J, Zhang G, Cao D, Zhang Y (2008) Regulation of mitochondrial uncoupling respiration during exercise in rat heart: role of reactive oxygen species (ROS) and uncoupling protein 2. Free Radic Biol Med 44:1373–1381. doi:10.1016/j.freeradbiomed.2007.12.033

Bo J, Xie S, Guo Y, Zhang C, Guan Y, Li C, Meng QH (2016) Methylglyoxal impairs insulin secretion of pancreatic? Cells through increased production of ROS and mitochondrial dysfunction mediated by upregulation of UCP2 and MAPKs. J Diabetes Res 9:1–14

Boyum A (1964) Separation of white blood cells. Nature 204:793–794

Busquets-Cortés C, Capó X, Martorell M, Tur JA, Sureda A, Pons A (2016) Training enhances immune cells mitochondrial biosynthesis, fission, fusion, and their antioxidant capabilities synergistically with dietary docosahexaenoic supplementation. Oxid Med Cell Longev 2016:8950384

Capó X, Martorell M, Sureda A, Llompart I, Tur J, Pons A (2014) Diet supplementation with DHA-enriched food in football players during training season enhances the mitochondrial antioxidant capabilities in blood mononuclear cells. Eur J Nutr 54:35–49. doi:10.1007/s00394-014-0683-2

Capó X, Martorell M, Sureda A, Batle JM, Tur JA, Pons A (2016a) Docosahexaenoic diet supplementation, exercise and temperature affect cytokine production by lipopolysaccharide-stimulated mononuclear cells. J Physiol Biochem 72(3):421–434. doi:10.1007/s13105-016-0490-8

Capó X, Martorell M, Sureda A, Tur JA, Pons A (2016b) Effects of dietary Docosahexaenoic, training and acute exercise on lipid mediators. J Int Soc Sports Nutr 13(1):16. doi:10.1186/s12970-016-0126-y

Cartoni R, Leger B, Hock MB, Praz M, Crettenand A, Pich S, Russell AP (2005) Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. J Physiol 567(Pt 1):349–358. doi:10.1113/jphysiol.2005.092031

Cases N, Sureda A, Tauler P, Aguilo A, Tur JA, Pons A, Co A (2005) Relation between oxidative stress markers and antioxidant endogenous defences during exhaustive exercise. Free Radic Res 39(12):1317–1324. doi:10.1080/10715760500177500

Diaz F, Moraes CT (2008) Mitochondrial biogenesis and turnover. Cell Calcium. doi:10.1016/j.ceca.2007.12.004

Drake JC, Wilson RJ, Yan Z (2016) Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle. FASEB J 30(1):13–22. doi:10.1096/fj.15-276337

Farinha JB, Steckling FM, Stefanello ST, Cardoso MS, Nunes LS, Barcelos RP, Soares A (2015) Response of oxidative stress and

inflammatory biomarkers to a 12-week aerobic exercise training in women with metabolic syndrome. Sports Med Open. doi:10.1186/s40798-015-0011-2

Ferrer MD, Tauler P, Sureda A, Tur JA, Pons A (2009) Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise. J Sports Sci 27(1):49–58. doi:10.1080/02640410802409683

Flandin P, Donati Y, Barazzone-argiroffo C, Muzzin P (2005) Hyperoxia-mediated oxidative stress increases expression of UCP3 mRNA and protein in skeletal muscle. FEBS Lett 579:3411–3415. doi:10.1016/j.febslet.2005.04.084

Gomez-Cabrera M-C, Martínez A, Santangelo G, Pallardó FV, Sastre J, Viña J (2007) Oxidative stress in marathon runners: interest of antioxidant supplementation. Br J Nutr 96(S1):S31–S33. doi:10.1079/BJN20061696

Gondin J, Brocca L, Bellinzona E, D'Antona G, Maffiuletti NA, Miotti D, Bottinelli R (2011) Neuromuscular electrical stimulation training induces atypical adaptations of the human skeletal muscle phenotype: a functional and proteomic analysis. J Appl Physiol. doi:10.1152/japplphysiol.00914.2010

Ho PWL, Ho JWM, Liu H, So DHF, Tse ZHM, Chan K, Ho S (2012) Mitochondrial neuronal uncoupling proteins: a target for potential disease-modification in Parkinson' s disease. Transl Neurodegener 1(1):3. doi:10.1186/2047-9158-1-3

Hood DA, Irrcher I, Ljubicic V, Joseph A-M (2006) Coordination of metabolic plasticity in skeletal muscle. J Exp Biol 209:2265–2275. doi:10.1242/jeb.02182

Ji LL (1995) Oxidative stress during exercise: implication of antioxidant nutrients. Free Radic Biol Med 18:1079–1086

Jiang N, Zhang G, Bo H, Qu J, Ma G, Cao D, Zhang Y (2009) Upregulation of uncoupling protein-3 in skeletal muscle during exercise: a potential antioxidant function. Free Radic Biol Med 46(2):138–145. doi:10.1016/j.freeradbiomed.2008.09.026

Kuzmicic J, Del Campo A, López-Crisosto C, Morales PE, Pennanen C, Bravo-Sagua R, Lavandero S (2011) Dinámica mitocondrial: un potencial nuevo blanco terapéutico para la insuficiencia cardiaca. Rev Esp Cardiol 64(10):916–923. doi:10.1016/j.recesp.2011.05.018

Léger L, Boucher R (1980) An indirect continuous running multistage field test: the Université de Montréal track test. Can J Appl Sport Sci 5(2):77–84

Léger LA, Lambert J (1982) A maximal multistage 20-m shuttle run test to predict VO_2 max. Eur J Appl Physiol Occup Physiol 49(1):1–12

Li L, Kang C, Zhang Y (2016) Exercise-induced hormesis and skeletal muscle health. Free Radic Biol Med 98:113–122. doi:10.1016/j.freeradbiomed.2016.02.025

Losón OC, Song Z, Chen H, Chan DC (2013) Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Mol Biol Cell 24(5):659–667. doi:10.1091/mbc.E12-10-0721

Martorell M, Capó X, Sureda A, Batle JM, Llompart I, Argelich E, Pons A (2014) Effect of DHA on plasma fatty acid availability and oxidative stress during training season and football exercise. Food Funct 5(2):1920–1931. doi:10.1039/C4FO00229F

Martorell M, Capó X, Bibiloni MM, Sureda A, Mestre-Alfaro A, Batle JM, Pons A (2015) Docosahexaenoic acid supplementation promotes erythrocyte antioxidant defense and reduces protein nitrosative damage in male athletes. Lipids 50(2):131–148. doi:10.1007/s11745-014-3976-6

Merry TL, Ristow M (2016) Mitohormesis in exercise training. Free Radic Biol Med 98:123–130. doi:10.1016/j.freeradbiomed.2015.11.032

Ni HM, Williams JA, Ding WX (2015) Mitochondrial dynamics and mitochondrial quality control. Redox Biol 4:6–13. doi:10.1016/j.redox.2014.11.006

Pecqueur C, Alves-Guerra C, Ricquier D, Bouillaud F (2009) UCP2, a metabolic sensor coupling glucose oxidation to mitochondrial metabolism? IUBMB Life 61(7):762–767. doi:10.1002/iub.188

Perry CGR, Lally J, Holloway GP, Heigenhauser GJF, Bonen A, Spriet LL (2010) Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. J Physiol 588(Pt 23):4795–4810. doi:10.1113/jphysiol.2010.199448

Picca A, Lezza AMS (2015) Regulation of mitochondrial biogenesis through TFAM-mitochondrial DNA interactions: useful insights from aging and calorie restriction studies. Mitochondrion 25:67–75. doi:10.1016/j.mito.2015.10.001

Pilegaard H, Saltin B, Neufer PD (2003) Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. J Physiol 546(Pt 3):851–858. doi:10.1113/jphysiol.2002.034850

Powers SK, Jackson MJ (2008) Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol Rev 88(4):1243–1276. doi:10.1152/physrev.00031.2007

Radak Z, Zhao Z, Koltai E, Ohno H, Atalay M (2013) Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling. Antioxid Redox Signal 18(10):1208–1246. doi:10.1089/ars.2011.4498

Romanello V, Sandri M (2015) Mitochondrial quality control and muscle mass maintenance. Front Physiol 6:422. doi:10.3389/fphys.2015.00422

Rudkowska I, Raymond C, Lavigne C, Holub BJ (2011) Validation of the use of peripheral blood mononuclear cells as surrogate model for skeletal muscle tissue in nutrigenomic studies. OMICS 15(1–2):1–7. doi:10.1089/omi.2010.0073

Russell AP, Feilchenfeldt J, Schreiber S, Praz M, Crettenand A, Gobelet C, Dériaz O (2003) Endurance training in humans leads to fiber type-specific increases in levels of peroxisome proliferator-activated receptor-gamma coactivator-1 and peroxisome proliferator-activated receptor-alpha in skeletal muscle. Diabetes 52(12):2874–2881. doi:10.2337/diabetes.52.12.2874

Samjoo IA, Safdar A, Hamadeh MJ, Glover AW, Mocellin NJ, Santana J, Tarnopolsky MA (2013) Markers of skeletal muscle mitochondrial function and lipid accumulation are moderately associated with the homeostasis model assessment index of insulin resistance in obese men. PLoS One. doi:10.1371/journal.pone.0066322

Scott I, Youle RJ (2010) Mitochondrial fission and fusion. Essays Biochem 47(11):85–98. doi:10.1042/bse0470085

Short KR, Short KR, Vittone JL, Bigelow ML, Vittone JL, Bigelow ML, Nair KS (2003) Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. Diabetes 52(8):1888–1896. doi:10.2337/diabetes.52.8.1888

Sureda A, Ferrer MD, Tauler P, Romaguera D, Drobnic F, Pujol P, Pons A (2007) Effects of exercise intensity on lymphocyte H2O2 production and antioxidant defences in soccer players. Br J Sports Med 43(3):186–190. doi:10.1136/bjsm.2007.043943

Sureda A, Córdova A, Ferrer MD, Tauler P, Tur JA, Pons A (2009) Effects of L-citrulline oral supplementation on polymorphonuclear neutrophils oxidative burst and nitric oxide production after exercise. Free Radic Res 43(9):828–835. doi:10.1080/10715760903071664

Sureda A, Batle JM, Ferrer MD, Mestre-Alfaro A, Tur JA, Pons A (2012) Scuba diving activates vascular antioxidant system. Int J Sports Med 33(7):531–536. doi:10.1055/s-0031-1297957

Twig G, Elorza A, Molina AJA, Mohamed H, Wikstrom JD, Walzer G, Shirihai OS (2008) Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J 27(2):433–446. doi:10.1038/sj.emboj.7601963

van der Bliek AM, Shen Q, Kawajiri S (2013) Mechanisms of mitochondrial fission and fusion. Cold Spring Harb Perspect Biol 5(6):a011072. doi:10.1101/cshperspect.a011072

Vider J, Laaksonen DE, Kilk ANN, Atalay M, Lehtmaa J, Zilmer M, Sen CK (2001) Physical exercise induces activation of NF-κB in human peripheral blood lymphocytes. Antioxid Redox Signal 3(6):1131–1137

Wells CM, Edwards AM, Winter EM, Fysh ML, Drust B (2012) Sport-specific fitness testing differentiates professional from amateur soccer players where VO_{2max} and VO_2 kinetics do not. J Sports Med Phys Fitness 52(3):245–254

Westrate LM, Drocco JA, Martin KR, Hlavacek WS, MacKeigan JP (2014) Mitochondrial morphological features are associated with fission and fusion events. PLoS One. doi:10.1371/journal.pone.0095265

Yan Z, Lira VA, Greene NP (2012) Exercise training-induced regulation of mitochondrial quality. Exerc Sport Sci Rev 40(3):159–164. doi:10.1097/JES.0b013e3182575599

Yun J, Finkel T (2014) Mitohormesis. Cell Metab 19(5):757–766. doi:10.1016/j.pestbp.2011.02.012.Investigations

Zheng Q, Cui G, Chen J, Gao H, Wei Y, Uede T, Chen Z, Diao H (2015) Regular exercise enhances the immune response against microbial antigens through up-regulation of toll-like receptor signaling pathways. Cell Physiol Biochem 37:735–746. doi:10.1159/000430391

Manuscript II

Training Enhances Immune Cells Mitochondrial Biosynthesis, Fission, Fusion, and Their Antioxidant Capabilities Synergistically with Dietary Docosahexaenoic Supplementation.

Carla-Busquets Cortés, Xavier Capó, Miquel Martorell, Josep A. Tur, Antoni Sureda, Antoni Pons.

2016. *Oxidative Medicine and Cellular Longevity*, *2016*, 2016:8950384.
doi: http://doi.org/10.1155/2016/8950384

Hindawi Publishing Corporation
Oxidative Medicine and Cellular Longevity
Volume 2016, Article ID 8950384, 10 pages
http://dx.doi.org/10.1155/2016/8950384

Clinical Study

Training Enhances Immune Cells Mitochondrial Biosynthesis, Fission, Fusion, and Their Antioxidant Capabilities Synergistically with Dietary Docosahexaenoic Supplementation

Carla Busquets-Cortés,[1] Xavier Capó,[1] Miquel Martorell,[2] Josep A. Tur,[1,3] Antoni Sureda,[1,3] and Antoni Pons[1,3]

[1]*Research Group on Community Nutrition and Oxidative Stress, Science Laboratory of Physical Activity, Department of Fundamental Biology and Health Sciences, University of Balearic Islands, 07122 Palma de Mallorca, Spain*
[2]*Departamento de Nutrición y Dietética, Facultad de Farmacia, Universidad de Concepción, 4070386 Concepción, Chile*
[3]*CIBER: CB12/03/30038 Fisiopatología de la Obesidad la Nutrición, CIBEROBN, Instituto de Salud Carlos III (ISCIII), University of Balearic Islands, 07122 Palma de Mallorca, Spain*

Correspondence should be addressed to Antoni Pons; antonipons@uib.es

Received 27 May 2016; Revised 26 July 2016; Accepted 2 August 2016

Academic Editor: Marcos R. de Oliveira

Exercise training induces adaptations in mitochondrial metabolism, dynamics, and oxidative protection. Omega-3 fatty acids change membrane lipid composition and modulate mitochondrial function. The aim was to investigate the effect of 8-week training and docosahexaenoic acid (DHA) supplementation (1.14 g/day) on the mitochondria dynamics and antioxidant status in peripheral blood mononuclear cells (PBMCs) from sportsmen. Subjects were assigned to an intervention ($N = 9$) or placebo groups ($N = 7$) in a randomized double-blind trial. Nutritional intervention significantly increased the DHA content in erythrocyte membranes from the experimental group. No significant differences were reported in terms of circulating PBMCs, Mn-superoxide dismutase protein levels, and their capability to produce reactive oxygen species. The proteins related to mitochondrial dynamics were, in general, increased after an 8-week training and this increase was enhanced by DHA supplementation. The content in mitofusins Mtf-1 and Mtf-2, optic atrophy protein-1 (Opa-1), and mitochondrial transcription factor A (Tfam) were significantly higher in the DHA-supplemented group after intervention. Cytochrome c oxidase (COX-IV) activity and uncoupling proteins UCP-2 and UCP-3 protein levels were increased after training, with higher UCP-3 levels in the supplemented group. In conclusion, training induced mitochondrial adaptations which may contribute to improved mitochondrial function. This mitochondrial response was modulated by DHA supplementation.

1. Introduction

Assembly of new mitochondria in eukaryotic cells occurs in response to the energy demands of the cell in a process called mitochondrial biogenesis. In addition, this event permits the equal distribution of mitochondria among the two cells formed when cell division occurs. Mitochondria form a dynamic tubular reticulum within eukaryotic cells and the generation of their components requires the lipids and proteins synthesis by nuclear genome and the mitochondrial DNA expansion from the preexisting mitochondrial reticulum [1–3]. Due to its reticular properties and high plasticity, mitochondria can rapidly change its size, shape, and distribution by constantly alternating fusion and fission processes [3]. The merging of discrete mitochondria (fusion) to the reticulum leads to the configuration of a continuous mitochondrial network that allows the preservation of mitochondrial content and structural homeostasis. The fragmentation in the reticulum (fission) permits the separation of impaired mitochondria from the healthy network, improving and maintaining mitochondrial quality [4]. Fusion and fission, strictly regulated and permanently balanced,

allow the cell to reorganize its mitochondrial network and maintain the equilibrium needed to keep mitochondrial morphology and function but can shift the balance in order to cope with the changing physiological demands [5]. In mammals fission, it is controlled by dynamin-like GTPases such as dynamin-related protein 1 (DRP1) and fission protein 1 (FIS-1) [1]. Concretely, fission followed by selective fusion segregates dysfunctional mitochondria and permits their removal by mitophagy and also participates in cell apoptosis by releasing cytochrome c [6]. On the contrary, fusion processes are controlled by the membrane-bound GTPases mitofusin 1 (Mfn-1), mitofusin 2 (Mfn-2), and optic atrophy protein 1 (Opa-1), which are essential for combination of the outer and inner mitochondrial membrane [7]. The process of fusion generates networks with continuous membranes and matrix lumens. As a result, not only metabolites, solutes, and proteins can be exchanged among constituent mitochondria but they also share electrochemical gradient, being capable of dissipate metabolic energy by transmission of membrane potential [8]. These phenomena of fusion and fission show that mitochondrial dynamics plays a central role in controlling cell viability and any error resulting from malfunction can cause cell problems that may lead to serious diseases in mammals [9].

Dietary fatty acids have been described to exert diverse effects on mitochondrial function and dynamic behaviour. While saturated fatty acids are commonly associated to cardiovascular diseases and their excessive consumption is not recommended, omega 3 polyunsaturated fatty acids have been reported to have beneficial effects on mitochondria by improving mitochondrial function and promoting mitochondrial fusion in both *in vivo* and *in vitro* experiments [10]. Docosahexaenoic acid (DHA) is an essential omega-3 polyunsaturated fatty acid (PUFA) mainly found in marine food. Together with eicosapentaenoic acid (EPA), DHA is recognized as a protective molecule against inflammation and oxidative stress and it also promotes the gene expression of key enzymes that introduce fatty acids into the mitochondria and their use as energetic fuel in the respiratory chain [11]. However, the high unsaturation index of long-chain omega-3 PUFA, especially DHA, may make them prone to peroxidation, which may be associated with oxidative processes [12, 13]. Thus, prooxidant and antioxidant properties of omega 3 fatty acids and their physiological effects are still controversial.

Mitochondria are known for initiating signal transduction cascades to the nucleus that coordinate transcriptional responses after physiological perturbations. In skeletal muscle, contraction processes cause an increase in reactive oxygen species (ROS) production and a transient situation of oxidative stress. This transitory oxidative stress induced by exercise has an important role in muscle signalling through release of ROS, Ca^{2+}, metabolites, and myokine levels. Mitochondrial and nonmitochondrial adaptations that restore and maintain the cellular homeostasis are responsible for beneficial outcomes of physical activity, when practised moderately [13]. Skeletal muscle adaptive response to training is translated into increased endurance, enhanced vascular function (angiogenesis), and oxidative myofiber transformation, among others. Concerning mitochondria, training confers healthy benefits due to coordinated improvements in quality (structure and function) and quantity (content) through structural joining (fusion) and separation (fission) and synthesis-incorporation of proteins and mitochondrial DNA in the existing reticulum (biogenesis) [4]. Besides, an improvement in cardiac and skeletal muscle mitochondrial respiratory capability and a proper regulation of mitochondrial life cycle span occur [14, 15]. Physical activity induces molecular adaptive responses mediated by oxidative stress and mitochondrial function including the NF-$\kappa\beta$ pathway, which activates target genes related to antioxidant defenses like uncoupling proteins (UCPs) and the mitochondrial biogenesis mediated by peroxisome proliferator activated receptor gamma (PGC-1α). However, this signalling cascade clearly evidenced in skeletal muscle is poorly studied in other cell types. We hypothesise that the oxidative stress induced by regular training can affect cell functionality and, thereby, can exert some effects on mitochondrial dynamics that have not been described in PBMCs.

Our aim was to investigate the effect of a long-term training and DHA supplementation on the antioxidant status and on the expression of mitochondrial biogenesis proteins in immune cells from professional football players. Obtaining muscle cells to investigate the mitochondrial adaptations to exercise is complex and it implies an invasive procedure with many ethical difficulties in healthy subjects. Accordingly, we selected peripheral blood mononuclear cells (PBMCs) as a cellular model for studying the mitochondria dynamics in a quicker and easier way than muscle biopsies and to validate their usefulness in evidencing changes associated with exercise.

2. Materials and Methods

2.1. Subjects Characteristics and Nutritional Intervention. At the beginning of the competition season, 23 male professional and federated football players (Real Mallorca B) were recruited to take part in the study. Unfortunately, from all subjects selected, only 16 reached the end of study because 6 of them left the football team during the experimental time and joined the professional team, and one player broke the anterior cruciate ligament of the knee. Subjects were randomly classified into two groups: placebo, composed of 7 subjects, and experimental, with 9 subjects. Participants in the study were healthy and nonsmokers. The anthropometric and physical performance characteristics of participating football players were as follows: 18.9 ± 0.5 years old (placebo group) and 20.4 ± 0.5 years old (experimental group), 76.0 ± 1.5 kg of weight (placebo group) and 76.4 ± 3.5 kg (experimental group), and 181 ± 3 cm of height (placebo group) and 180 ± 3 cm (experimental group). The waist circumference was 78.0 ± 0.7 and 78.5 ± 1.1 cm, the hip circumference was 99.0 ± 1.6 and 96.6 ± 1.4 cm, and the waist–hip ratio (WHR) was 0.787 ± 0.018 and 0.814 ± 0.012, in placebo and experimental groups, respectively. The value of systolic blood pressure was 115±6 and 120±4 in placebo group and 56.7±5.9 and 66.7 ± 3.5 mmHg in experimental groups. The body mass index (BMI) was 23.1 ± 0.4 and 23.5 ± 0.5 kg/m^2, and the

football players had 91.5 ± 0.3 and 92.8 ± 0.3% fat-free mass, in placebo and experimental groups, respectively. The VO_{2max} in placebo group was 61.2 ± 1.6 and 62.0 ± 0.9 mL/kg min in experimental group, respectively. Finally, the intense physical activity time was 97.5 ± 58.3 and 52.0 ± 13.7 and 68.6 ± 17.1 and 63.2 ± 14.6 min/day of moderate physical activity time, in placebo and experimental group, respectively. There were no differences in these parameters neither between the placebo and experimental groups of football players nor between initial and final nutritional intervention. Following a randomized double-blind trial, participants in the study ingested daily 1 litre of placebo or experimental drink for 5 days per week (excluding the match day and the day of rest), for a period of 8 weeks of training. The beverages were consumed before starting the physical activity session. The training performed during the experimental time consisted of six physical activity sessions per week and 10 official matches. The exercise consisted of 2 h regular football training [15]. All the subjects were properly informed of the purpose and demands of the study before giving their written consent to participate. The study protocol was in accordance with the Declaration of Helsinki for research on human subjects and was approved by the Ethical Committee of Clinical Investigation of the Autonomic Community of the Balearic Islands No. IB 994/08 PI (Palma de Mallorca, Balearic Islands, Spain). The project was registered at ClinicalTrial.gov (NCT02177383).

2.2. Drink Composition. The beverages were composed of 3.0% almond, 0.8% sucrose, water, lemon and cinnamon and α-tocopherol acetate (vitamin E), and 0.8% of different lipids depending on the kind of beverage (placebo or experimental). The lipid content of the placebo drink was 0.8% refined olive oil, and for the experimental drink was 0.6% olive oil and 0.2% DHA-S Market (Market Biosciences Corporation, Columbia, EEUU). The two almond drinks were manufactured by Liquats Vegetals S.A. (Girona, Spain), and the procedure for obtaining them consisted of bleaching almonds and crushing them in water. Then, the mixture was centrifuged to eliminate insoluble materials and cinnamon, lemon natural flavours, sucrose, and vitamin E were added. Finally, olive oil was added to the placebo drink and olive oil plus DHA-S to the experimental one, but there were no differences in taste. Finally, the beverage was sterilized and packed into apparently identical bottles. The concentration of vitamin E in both placebo and experimental drinks is equivalent to 0.4 mg/mL of α-tocopherol acetate.

2.3. Experimental Procedure. A total of two venous blood samples were obtained from each subject over the experimental time in basal conditions after overnight fasting. One sample was extracted before starting the nutritional intervention and the other one at the end of the nutritional intervention. Both samples were obtained from the antecubital vein of subjects in suitable vacutainers with EDTA as anticoagulant and were immediately used to purify erythrocytes and PBMCs. Cell counts were determined in an automatic flow cytometer analyzer Technicon H2 (Bayer, Leverkusen, Germany) VCS system.

The PBMCs fraction was purified using Ficoll-Paque PLUS reagent (GE Healthcare, Chalfont St Giles, UK) [16, 17] Briefly, blood was carefully introduced on Ficoll in a proportion of 1 : 5 : 1 and was then centrifuged at 900 ×g, at 4°C for 30 min. The PBMCs layer was carefully removed while plasma and Ficoll phases were discarded. The PBMCs slurry was then washed twice with PBS and centrifuged for 10 min at 1,000 ×g, 4°C. This process was performed in duplicate: one of the samples was destined to obtaining RNA and the other one was preserved in RIPA lysis and extraction buffer (250 mM Tris/HCl, pH 8.0, 4.4% NaCl, 5% IGEPAL®, 2.5% deoxycholic acid, 0.5% sodium dodecylsulfate (SDS)) for Western blot analysis. Cell lysates were stored at −80°C until biochemical analyses.

Erythrocytes were purified by centrifugation at 900 ×g at 4°C for 30 min. The erythrocyte phase at the bottom was washed with PBS, centrifuged as above, and finally erythrocytes were reconstituted with distilled water. Fatty acid extraction of erythrocyte samples was performed by a modification of the Folch extraction procedure [17, 18]. The method for individual fatty acid determination in erythrocytes was previously described [19].

2.4. Hydrogen Peroxide (H_2O_2) Production. H_2O_2 production by PBMCs was measured before and after stimulation with phorbol myristate acetate (PMA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator. A stock solution of DCFH-DA (1 mg/mL) in ethanol and PMA (1 mg/mL) in DMSO was prepared and stored at 20°C until analysis. DCFH-DA (30 μg/mL) in PBS was added to a 96-well microplate containing 50 μL PBMCs suspension. PMA (10 ng/mL) prepared in HBSS or HBSS alone was added to the wells, and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37°C for 1 h in FL 9800 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc.).

2.5. SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Antioxidant enzyme and mitochondrial protein levels were determined by Western blot analysis. PBMCs lysed with RIPA buffer were heated for 5 min at 100°C. 30-microgram protein aliquots were loaded in each lane and separated by size using SDS polyacrylamide gel (12% acrylamide) and electrotransferred onto a nitrocellulose membrane. The membrane were blocked (5% non-fat powdered milk in PBS, pH 7,5, containing 0,1% Tween 20) and incubated with the corresponding primary monoclonal antibody. Antibodies for PGC-1α (1 : 1000), Mfn-1 (1 : 500), Mfn-2 (1 : 200), OPA-1 (1 : 500), metalloendopeptidase 1 (OMA1) (1 : 200), FIS1 (1 : 200), actin (1 : 200), and UCP-2 (1 : 500) were supplied by Santa Cruz Biotechnology (Santa Cruz, Ca, USA); anti-nuclear respiratory factor 1 (NRF1) (1 : 1000) and anti-mitochondrial transcription factor A (Tfam) (1 : 1000) antibodies were obtained from Cell Signalling (Danvers, MA, USA); antibodies against UCP-3 (1 : 500), Mn-super oxide dismutase (Mn-SOD) (1 : 1000) and cytochrome c oxidase subunit 4 (COX-IV) (1 : 1000) were from Millipore (Billerica, MA, USA). Blots were then incubated with a secondary peroxidase-conjugated antibody (1 : 5000). Development of

Table 1: Effects of training and DHA supplementation on PBMC counts.

		Initial	Final	ANOVA		
				S	T	$S \times T$
PBMCs (10^3 cells/μL)	Placebo	2.47 ± 0.6	2.98 ± 0.4			
	Experimental	2.92 ± 0.2	3.61 ± 3.6			
Lymphocytes (%)	Placebo	79.9 ± 6.6	82.9 ± 4.3			
	Experimental	85.1 ± 2.9	85.3 ± 3.6			
Monocytes (%)	Placebo	20.1 ± 5.5	17.0 ± 3.8			
	Experimental	14.9 ± 3.9	14.7 ± 3.3			

Statistical analysis: two-way ANOVA, $p < 0.05$. (T) significant effect of time of training, (S) significant effect of supplementation, ($S \times T$) significant interaction between both factors. No significant differences were reported.

immunoblots was performed using and enhanced chemiluminescence kit (Immun-Star® Western C® Kit reagent, Bio-Rad Laboratories). Protein bands were visualized using the image analysis program Quantity One (Bio-Rad). Precision Plus Protein™ Kaleidoscope™ (Bio-Rad) was used as a molecular weight marker. The band density of each protein was quantified in relation to the loading control (actin), used as a housekeeping.

2.6. Malondialdehyde Assay. Malondialdehyde (MDA) in PBMCs was analyzed by colorimetric assay for MDA determination, based on the reaction of MDA with a chromogenic reagent that produces a stable chromophore with maximal absorbance at 586 nm. Succinctly, samples or standards were derivatizated using 1-methyl-2-phenylindole (10.3 mM) in acetonitrile : methanol (3 : 1). Proteins were precipitated with HCl 12 N and the samples were incubated for 1 h at 45°C. Absorbance was then measured at 586 nm.

2.7. Protein Carbonyl Determination. Protein carbonyl derivatives were determined through immunological methods using the OxiSelectTM Protein Carbonyl Immunoblot Kit (Cell Biolabs, INC) by following the manufacturer's instructions. Protein concentrations of an aliquot of cells lysed with distilled water were calculated by the Bradford method [20] using the Bio-Rad protein assay reagent (Bio-Rad, Munich, Germany) and 10 μg of protein was transferred onto a nitrocellulose membrane by the dot blot method. Briefly, samples were placed in a vacuum plate and absorbed. A derivatization protocol was followed, using methanol 50%, HCl 12 N, and 2,4-dinitrophenylhydrazine as reagents. Then, the membrane was incubated with the primary antibody, specific to DNP moiety proteins (1 : 4,000). After this, an incubation with a secondary horseradish peroxidase-conjugated antibody (goat anti-rabbit IgG) (1 : 10,000) was performed. The membrane was finally treated with luminol, which is transformed to a light-emitting form at 428 nm through the antigen/primary antibody/secondary antibody/peroxidase complex. The resulting light was visualized by short exposure to a Chemidoc XRS densitometer imaging system (Bio-Rad Laboratories) and bands quantification was performed by using Quantity One-1D analysis software (Bio-Rad Laboratories).

2.8. Statistical Analysis. Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.19.0 for Windows). Results are expressed as mean ± SEM, and $p < 0.05$ was considered statistically significant. A Kolmogorov–Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analyzed were beverage supplementation (S) and the training period (T). The sets of data in which there was a significant interaction between the factors analyzed were tested by one-way ANOVA. When significant effects of one factor were found, a Student's t test for paired data was used to determine the differences between the groups involved.

3. Results

Diet supplementation with DHA-enriched beverage for 8 weeks changed lipid composition of erythrocytes membranes. No differences were observed between placebo (DHA concentration was 29.0 ± 1.3 nmol/10^9 erythrocytes) and experimental (DHA concentration was 34.0 ± 3.6 nmol/10^9 erythrocytes) groups at the beginning of the study. The erythrocyte from the experimental group increased their content in DHA (43.0 ± 3.7 nmol/10^9 erythrocytes) after 8 weeks of nutritional intervention whereas no effects were observed in placebo group (33.6±3.2 nmol/10^9 erythrocytes). These results imply an increase of 26% in the DHA content with respect to the initial values in erythrocytes from the experimental group.

Total PBMC counts and the % of lymphocytes and monocytes were reported in Table 1. No significant differences between placebo and experimental groups or the training period are observed in any cellular parameter. No effects of 8 weeks training or DHA supplementation were evidenced on the ROS production capabilities of PBMCs in response to PMA stimulation (Figure 1). The MDA levels in PBMCs significantly decreased in placebo and in experimental groups after the 8-week training season, while carbonyl index significantly increased in both groups (Table 3).

Figure 2 shows the effect of 8 weeks of training and DHA supplementation on the mitochondrial antioxidant and cytochrome c oxidase protein levels. No effects of training or DHA diet supplementation were observed on Mn-SOD (Figure 2(c)) protein levels; however, training significantly increases protein levels of UCP-2 (Figure 2(a)) and COX-IV (Figure 2(d)) in both placebo and experimental groups. These results are similar to those obtained for UCP-3 (Figure 2(b))

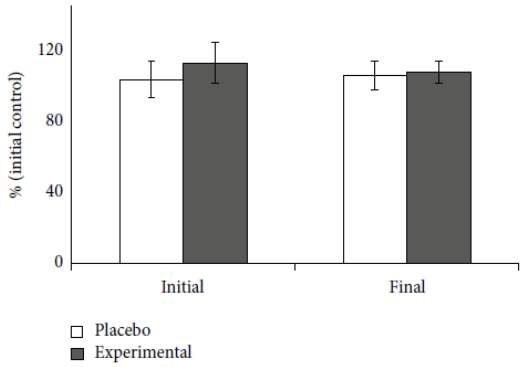


FIGURE 1: Effects of training and DHA supplementation on ROS production by PBMCs after PMA stimuli. Statistical analysis: two-way ANOVA, $p < 0.05$. No significant differences were reported.

protein levels where a significant increase due to training is observed, but in this case the increase was enhanced by DHA diet supplementation.

The effects of training and DHA diet supplementation on mitochondrial dynamics are shown in Table 2 and Figure 3. PGC1α, NRF-1, and OPA-1 protein levels were influenced by the 8-week training period. OPA-1 protein levels significantly increased after training in both placebo and experimental groups, whereas in PGC1α and NRF-1 protein levels the increase was only significant in experimental group. Additionally, Tfam and OMA-1 protein levels were affected by training but also were influenced by DHA diet supplementation. OMA-1 and Tfam protein levels increased after the training in both groups but this increase was significantly higher in experimental group. On the other hand, Mfn-1 and Mfn-2 were affected by training and DHA diet supplementation and by interaction of both factors, reporting a significant increase only in the experimental group after the training period. No effects of training season or DHA supplementation were observed on FIS-1 protein levels.

4. Discussion

The main feature of this study was to evidence the influence of exercise training and dietary supplementation with DHA on the peripheral blood mononuclear cells mitochondrial dynamics and antioxidant function. Regular training induces a greater ROS production in cells, but it also triggers expression of antioxidants enzymes and protection against PBMCs' lipid peroxidation [21]. However, we detected an increase in the levels of protein carbonyl derivates. Oxidative alterations in proteins include the generation of protein carbonyls as a modification of single amino acids [22]. The MDA-amino group reaction can also promote the introduction of carbonyls groups into the protein [23]. We suggest that the lower levels of MDA in PBMCs at the end of the 8-week training period could be a consequence of the MDA reaction with proteins, fact that would increase the carbonyl index.

Eight weeks of regular training enhanced the antioxidant mitochondria capabilities to decrease mitochondrial ROS production through increasing UCP-2 and UCP-3 protein levels; it also improves processes of mitochondrial biosynthesis, fission and fusion that were favoured by the DHA dietary supplementation. Some of these training effects have also been previously described in skeletal muscle as a consequence of the contraction process [24, 25]. Thus, we can translate this exercise-induced muscular response into changes in peripheral blood mononuclear cells, establishing a first approach between immune system cells mitochondrial dynamics and skeletal muscle cells model. Since no differences were reported in PBMC counts and the percentage of lymphocytes and monocytes in both placebo and experimental groups, changes in mitochondria markers may be attributed to training and/or DHA incorporation in PBMC membrane and not to changes in the lymphocyte/monocyte ration.

Both beverages (placebo and experimental) had the same nutritional basis. Concretely, they contain α-tocopherol and phenolic compounds from almond, such as catechin, quercetin, and kaempferol, which have been demonstrated to exert potent free radical scavenging activity and are considered dietary beneficial antioxidants [26–29]. These bioactive ingredients can modulate oxidative stress in cells, but the experiment was designed to study the effect of DHA on PBMCs and not to study the effect of the almond and olive oil based beverage. Consequently, placebo and experimental beverages only differed in the content of DHA, while the rest of the compounds in the beverages were the same. According to our experimental design and taking into account that physical activity parameters were comparable in all football players, observed changes can be attributed to DHA action, but we are not able to discard other bioactive compounds' contributions to counteract oxidative stress when training effect was evidenced in PBMCs. Further studies including a control group supplemented with equally energetic and mineral content beverage but deprived of other bioactive compounds and DHA would be necessary to differentiate the effect of training and the effect of the dietary almond and olive oil based beverage supplementation on PBMCs antioxidant status. There is a lack of studies performed about effects of DHA on mitochondrial dynamics, so any change evidenced with our experimental design could be attributed to DHA action and should be taken into consideration.

Erythrocytes cell membranes are usually used as biomarkers of medium-term fatty acids intake as they reflect the proportion accumulated over the lifespan of red blood cells [30]. In our case, a DHA-enriched diet for 8 weeks induced a change in lipid composition of erythrocytes membranes of football players; those who ingested the experimental drink increased their DHA content compared to those who ingested the placebo drink. This indicates that the participants followed the prescribed beverage intake during the trial and reinforces the idea that DHA can incorporate into immune cell membranes and may exert some effects on peripheral blood mononuclear cells function, even on its mitochondrial dynamics. In fact, 8 weeks of dietary omega-3 supplementation attenuates proinflamatory cytokine production after a bout of acute exercise [18] and enhances antioxidant defenses in professional athletes under resting conditions and after acute exercise [15].

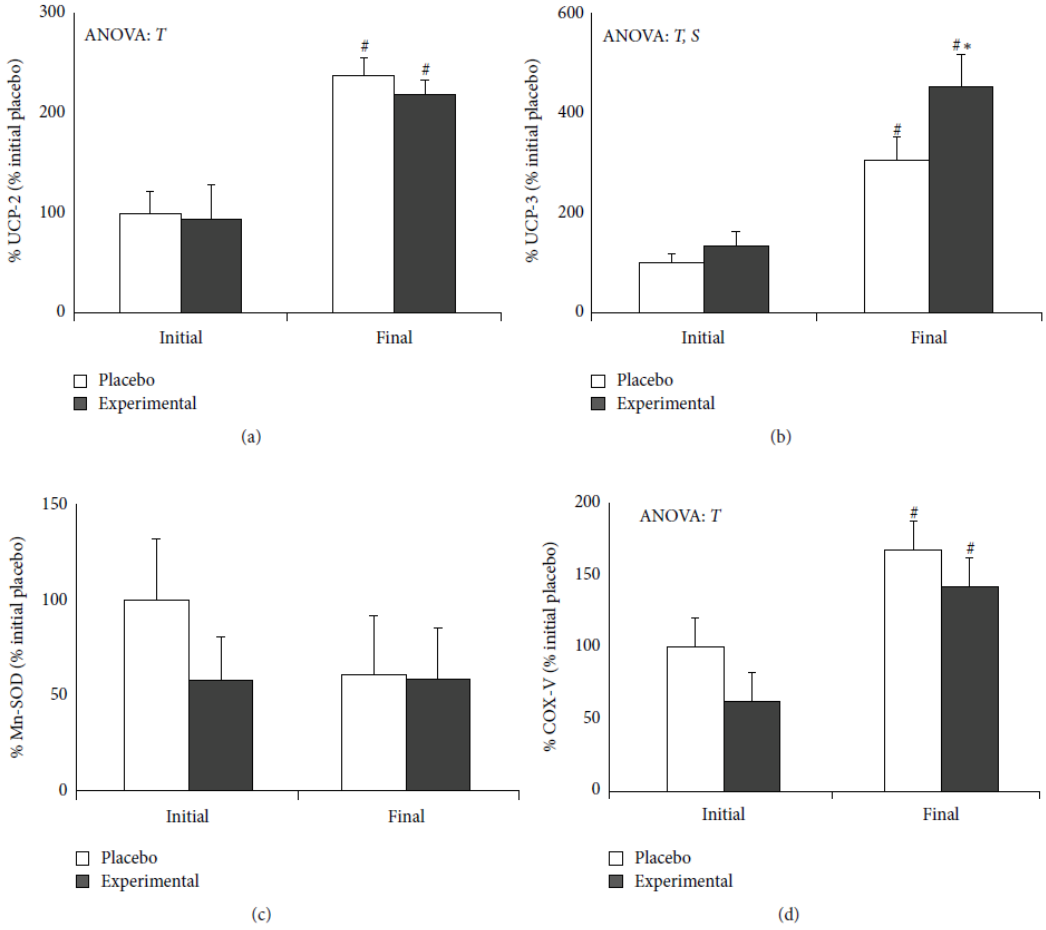


FIGURE 2: Effects of training and DHA supplementation on mitochondrial protein levels of PBMCs. (a) uncoupling protein- (UCP-) 2, (b) UCP-3, (c) Mn-superoxide dismutase (Mn-SOD), and (d) cytochrome c oxidase (COXIV). Statistical analysis: two-way ANOVA, $p < 0.05$. *T*, significant effect of training; *S*, significant effect of DHA supplementation. One-way ANOVA, $p < 0.05$. (∗) Significant differences between placebo and experimental groups; (#) significant differences between initial and final training period.

The capability to modulate mitochondrial function and number is a prominent adaptive response in all eukaryotic cells. In mammals there exist various factors that trigger this mitochondrial reaction, including hormone levels, aging, hypoxia, and local environmental stressors like temperature, physical exercise, and type of food ingested [31]. Concretely, dietary fatty acids have been suggested to exert some effects on mitochondrial dynamics. Beneficial effects of omega 3 polyunsaturated fatty acids have been reported, especially those derived from fish oil rich in EPA and DHA. These PUFAs improve mitochondrial function, promote mitochondrial fusion, and reduce ROS production in rat hepatic mitochondria [32]. In addition, it was described that 1 hour of incubation with omega 3 PUFA upregulates Mfn-2 expression and increases ATP levels in an *in vitro* steatotic hepatocyte model [33].

On the other hand, exercise training implies a reiterated exposure to an acute increase in metabolic, thermoregulatory, hypoxic, oxidative and mechanical stress. Oxygen overconsumption during acute and intense exercise increases ROS production [34] that can overwhelm the antioxidant defenses and lead the cell to an oxidative stress status [27, 28]. However, regular physical exercise originates adaptations in antioxidant defenses in muscle and immune cells and improves exercise performance [29, 30].

Mechanical stress-induced signals, such as p38 MAPK, which gets activated by the elevation of cytosolic Ca^{2+} during muscle contraction, have the potential to stimulate and regulate the activity and expression of exercise-sensitive transcription factors like PGC-1α [35]. Our obtained data are in accordance with these effects associated to exercise evidencing an increase in PGC-1α, which, in turn, induces

Table 2: Effects of training and DHA supplementation on mitochondrial dynamics protein levels of PBMCs.

		Initial	Final	ANOVA		
				S	T	$S \times T$
PGC-1α (%)	Placebo	100 ± 15	152 ± 22		X	
	Experimental	65.3 ± 5.9	152 ± 29$^{\#}$			
NRF-1 (%)	Placebo	100 ± 19	148 ± 9		X	
	Experimental	59.5 ± 14.7	158 ± 23$^{\#}$			
Tfam (%)	Placebo	100 ± 14^{a}	154 ± 24^{b}		X	X
	Experimental	78.8 ± 23.7^{a}	231 ± 15^{c}			
Mfn-1 (%)	Placebo	100 ± 22^{a}	129 ± 15^{a}	X	X	X
	Experimental	92.6 ± 17^{a}	250 ± 26^{b}			
Mfn-2 (%)	Placebo	100 ± 19^{a}	98.9 ± 11.9^{a}	X	X	X
	Experimental	108 ± 33^{a}	203 ± 29^{b}			
OPA-1 (%)	Placebo	100 ± 23	217 ± 84$^{\#}$		X	
	Experimental	134 ± 19	218 ± 25$^{\#}$			
OMA-1 (%)	Placebo	100 ± 11^{a}	163 ± 25^{b}		X	X
	Experimental	64.8 ± 13.7^{a}	219 ± 17^{c}			
FIS-1 (%)	Placebo	100 ± 15	88.0 ± 27.4			
	Experimental	115 ± 16	125 ± 16			

Statistical analysis: two-way ANOVA, $p < 0.05$. (T) significant effect of time of training, (S) significant effect of supplementation, ($S \times T$) significant interaction between both factors. One-way ANOVA, $p < 0.05$. (∗) significant differences between placebo and experimental, (#) significant differences between initial and final training period. When interaction exists between different groups, distinct letters (a, b, and c) reveal significant differences with respect to all other groups.

Table 3: Oxidative damage in PBMCs.

		Initial	Final	ANOVA		
				S	T	$S \times T$
Malondialdehyde (nmol/10^{9} cells)	Placebo	440 ± 70	160 ± 10$^{\#}$		X	
	Experimental	510 ± 70	330 ± 40$^{\#}$			
Protein Carbonyls (%)	Placebo	100 ± 30	838 ± 78$^{\#}$		X	
	Experimental	94 ± 22	792 ± 65$^{\#}$			

Statistical analysis: two-way ANOVA, $p < 0.05$. (T) significant effect of training period, (S) significant effect of supplementation, ($S \times T$) significant interaction between both factors. One-way ANOVA, $p < 0.05$. #: significant differences between initial and final training period.

mitochondrial biogenesis (fusion) by orchestrating transcription of nuclear genome (through interaction with NRF1) and mitochondrial genome (via Tfam gene transcription). These molecules provide a link between physiological stimuli and transcription of nuclear gene that induce compensatory physiological adaptations that enhance tolerance thresholds to subsequent sublethal doses of stressors [13]. Another PGC-1α target is COX-IV, the terminal oxidase in the mitochondrial electron transport chain and a mitochondrial amount indicator [36]. An increased COX-IV protein level expression in our PBMCs could be due to an augmented total mitochondrial number; thus, mitochondrial specific proteins such as mitofusins undergo a raise in their protein expression. This reinforces the idea that exercise promotes biogenesis processes. Studies performed in skeletal muscle associate increases in COX-IV protein levels with training resulting in an improvement in the capacity of mitochondria to produce ATP [37]. Mitofusins and OPA-1 have been characterized as the leading factors for mitochondrial fusion of the outer and inner mitochondrial membrane, respectively [38]. Exercise promotes this process, as it was demonstrated by significant increases in Mtf1/2 and OPA-1 protein levels after training period in both placebo and experimental groups. In addition to training, mitofusins were also influenced by DHA diet supplementation and by interaction of both factors. Fusion allows compensation of damage in mitochondria by sharing components and helps to maintain energy output to face stress situations [2]. Similar results were found in skeletal muscle of rats. Those fed with a high fat diet rich in fish oil diet (HFO) diminished fission process and augmented fusion processes compared to those fed with a high-lard diet (HL). Indeed, skeletal muscle sections from HFO fed rats revealed a greater number of immunoreactive fibers for Mfn2 and Opa1 protein, while sections from HL fed rats showed a weaker immunostaining for Drp1 and Fis1 [11, 24].

Overexpression of FIS-1 has been reported to induce mitochondrial fragmentation and apoptosis in HeLa cells [39] suggesting the involvement of mitochondrial fission in apoptosis. Even so, no effects of training season or DHA supplementation were observed on FIS-1 protein levels in

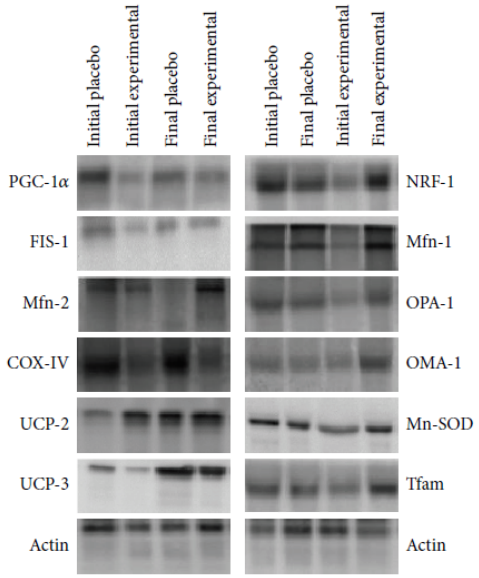


FIGURE 3: Representative picture of the bands obtained by immunoblotting.

our experiment. This may indicate that the addition of new mitochondria to the healthy network (fusion) outweighs the removal of the impaired ones (fission) under exercise conditions. Notwithstanding low levels of damage might be compensated by complementation through mitochondrial fusion; badly injured mitochondria can produce excessive amounts of ROS, by consuming ATP through reversal of ATP synthase. Therefore, rejoining of badly affected mitochondria will infect the healthy mitochondrial reticulum if they are not eliminated. OMA-1 is a metalloprotease involved in the quality system in the inner membrane of mitochondria. Stressing conditions like low levels of ATP or severely impaired mitochondrial activities provoke a loss of mitochondrial membrane potential that causes cleavage and inactivation of OPA-1 mediated by OMA-1, leading to a negative regulation of mitochondrial fusion. Then, the outer membranes of most seriously damaged mitochondria can still fuse but the inner membrane–bound matrix compartments fusion is prevented [1, 3, 40]. OMA-1 protein levels in our PBMCs increased after the training period in placebo and supplemented group, with this increase being significantly higher in the experimental one.

ROS produced during exercise causes a transient oxidative stress status that triggers the antioxidant regulatory mechanisms in immune system cells [41]. These adaptive responses include the NF-$\kappa\beta$ pathway, which activates target genes related to antioxidant defenses such as the mitochondrial uncoupling proteins and antioxidant enzymes like Mn-SOD [42]. UCP-2 and UCP-3 are located in the mitochondrial inner membrane and catalyze a proton leak that uncouples oxidative phosphorylation and dissipates electrochemical gradient across the membrane. This may lead to a reduced ROS production [43]. In the present study regular training significantly increased the UCP-2 and UCP-3 protein levels in PBMCs bringing to light a possible augmented number of UCP-2/3 per mitochondrion in accordance with studies carried out in skeletal muscle [44] and in PBMCs [45]. The increment in UCP-3 was enhanced by DHA diet supplementation which is consistent with other studies carried out with mice cells that show that omega-3 may increase the expression of UCP-3 [46]. This may evidence a synergistic effect of DHA diet supplementation and training on PBMCs antioxidant capabilities. On the contrary, no changes were reported on the antioxidant enzyme Mn-SOD protein levels, suggesting that the increased levels of UCPs could be enough to reduce the exercise induced ROS overproduction. Moreover, subjects from the study were professional athletes and probably their basal antioxidant status allows them to protect against oxidative stress. In addition, the higher UCPs content that is reported to lead to a reduced ROS production could be also responsible of the lack of differences in ROS production between the beginning and the end of the training period. It is important to note that the levels of ROS are key to determine their physiological effect. Low/moderate ROS production rates are associated with their role as molecular signalling inducing adaptive responses, while higher levels are harmful and associated with processes of senescence and apoptosis. Consequently, avoiding excessive production of ROS is essential to ensure cell functionality and to potentiate the adaptive responses to exercise.

In conclusion, the present results evidenced that 8 weeks of regular training induces mitochondrial adaptations in terms of fusion and fission processes and in antioxidant defenses in PBMCs to meet the demands arising from exercise and this response is potentiated by DHA diet supplementation. Thus, our results could be an opening approximation to define a link between mitochondrial events in immune cells and skeletal muscle cells and to reveal that training improves gradually mitochondrial quantity (mitochondria biogenesis) and quality (balance between biogenesis, dynamics and mitophagy). Further work is needed to identify the molecular mechanisms in which omega 3 fatty acids perform their biological activities on mitochondria.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

Thanks are due to Acción Estratégica en Salud del Ministerio de Ciencia e Innovación DPS2008-07033-C03-03, Programme of Promotion of Biomedical Research and Health Sciences, (Projects PI11/01791 and PI14/00636, Red Predimed-RETIC RD06/0045/1004, CIBEROBN CB12/03/30038) and Balearic Island Government and FEDER funds (35/2011 and 23/2012). The authors hereby acknowledge the PhD grant provided by the University of the Balearic Island and the FPI/1648/2014 grant provided by Conselleria d'Educació, Cultura i Universitats, Direcció General d'Universitats i

Recerca, Govern de les Illes Balears, within the program framework cofinanced by Fondo Social Europeo.

References

[1] I. Scott and R. J. Youle, "Mitochondrial fission and fusion," *Essays in Biochemistry*, vol. 47, no. 11, pp. 85–98, 2010.

[2] R. J. Youle and A. M. van der Bliek, "Mitochondrial fission, fusion, and stress," *Science*, vol. 337, no. 6098, pp. 1062–1065, 2012.

[3] H.-M. Ni, J. A. Williams, and W.-X. Ding, "Mitochondrial dynamics and mitochondrial quality control," *Redox Biology*, vol. 4, pp. 6–13, 2015.

[4] J. C. Drake, R. J. Wilson, and Z. Yan, "Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle," *The FASEB Journal*, vol. 30, no. 1, pp. 13–22, 2016.

[5] D. A. Hood, I. Irrcher, V. Ljubicic, and A.-M. Joseph, "Coordination of metabolic plasticity in skeletal muscle," *Journal of Experimental Biology*, vol. 209, no. 12, pp. 2265–2275, 2006.

[6] J. Kuzmicic, A. Del Campo, C. López-Crisosto et al., "Dinámica mitocondrial: un potencial nuevo blanco terapéutico para la insuficiencia cardiaca," *Revista Española de Cardiología*, vol. 64, no. 10, pp. 916–923, 2011.

[7] V. Romanello and M. Sandri, "Mitochondrial quality control and muscle mass maintenance," *Frontiers in Physiology*, vol. 6, article 422, 2016.

[8] G. Twig, A. Elorza, A. J. A. Molina et al., "Fission and selective fusion govern mitochondrial segregation and elimination by autophagy," *The EMBO Journal*, vol. 27, no. 2, pp. 433–446, 2008.

[9] F. Diaz and C. T. Moraes, "Mitochondrial biogenesis and turnover," *Cell Calcium*, vol. 44, no. 1, pp. 24–35, 2008.

[10] R. Putti, R. Sica, V. Migliaccio, and L. Lionetti, "Diet impact on mitochondrial bioenergetics and dynamics," *Frontiers in Physiology*, vol. 6, article 109, 2015.

[11] D. Richard, K. Kefi, U. Barbe, P. Bausero, and F. Visioli, "Polyunsaturated fatty acids as antioxidants," *Pharmacological Research*, vol. 57, no. 6, pp. 451–455, 2008.

[12] E. Filaire, A. Massart, H. Portier et al., "Effect of 6 weeks of $n-3$ fatty-acid supplementation on oxidative stress in judo athletes," *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 20, no. 6, pp. 496–506, 2010.

[13] T. L. Merry and M. Ristow, "Mitohormesis in exercise training," *Free Radical Biology and Medicine*, vol. 98, pp. 123–130, 2016.

[14] Z. Yan, V. A. Lira, and N. P. Greene, "Exercise training-induced regulation of mitochondrial quality," *Exercise and Sport Sciences Reviews*, vol. 40, no. 3, pp. 159–164, 2012.

[15] M. Martorell, X. Capó, M. M. Bibiloni et al., "Docosahexaenoic acid supplementation promotes erythrocyte antioxidant defense and reduces protein nitrosative damage in male athletes," *Lipids*, vol. 50, no. 2, pp. 131–148, 2015.

[16] A. Sureda, P. Tauler, A. Aguiló et al., "Blood cell NO synthesis in response to exercise," *Nitric Oxide—Biology and Chemistry*, vol. 15, no. 1, pp. 5–12, 2006.

[17] Folch,J., M. Lees, and G. H. Sloane Stanley, "A simple method for the isolation and purification of total lipides from animal tissues," *The Journal of Biological Chemistry*, vol. 226, no. 1, pp. 497–509, 1957.

[18] X. Capó, M. Martorell, A. Sureda, J. M. Batle, J. A. Tur, and A. Pons, "Docosahexaenoic diet supplementation, exercise and temperature affect cytokine production by lipopolysaccharide-stimulated mononuclear cells," *Journal of Physiology and Biochemistry*, vol. 72, no. 3, pp. 421–434, 2016.

[19] M. Martorell, X. Capó, A. Sureda, J. A. Tur, and A. Pons, "Effects of docosahexaenoic acid diet supplementation, training, and acute exercise on oxidative balance in neutrophils," *Applied Physiology, Nutrition and Metabolism*, vol. 39, no. 4, pp. 446–457, 2014.

[20] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[21] M. D. Ferrer, P. Tauler, A. Sureda et al., "A soccer match's ability to enhance lymphocyte capability to produce ros and induce oxidative damage," *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 19, no. 3, pp. 243–258, 2009.

[22] M. Kästle and T. Grune, "Protein oxidative modification in the aging organism and the role of the ubiquitin proteasomal system," *Current Pharmaceutical Design*, vol. 17, no. 36, pp. 4007–4022, 2011.

[23] T. Miyata, R. Inagi, K. Asahi et al., "Generation of protein carbonyls by glycoxidation and lipoxidation reactions with autoxidation products of ascorbic acid and polyunsaturated fatty acids," *FEBS Letters*, vol. 437, no. 1-2, pp. 24–28, 1998.

[24] B. Egan, P. L. O'Connor, J. R. Zierath, and D. J. O'Gorman, "Time course analysis reveals gene-specific transcript and protein kinetics of adaptation to short-term aerobic exercise training in human skeletal muscle," *PLoS ONE*, vol. 8, no. 9, Article ID e74098, 2013.

[25] I. Irrcher, P. J. Adhihetty, A.-M. Joseph, V. Ljubicic, and D. A. Hood, "Regulation of mitochondrial biogenesis in muscle by endurance exercise," *Sports Medicine*, vol. 33, no. 11, pp. 783–793, 2003.

[26] C.-Y. Che, P. E. Milbury, K. Lapsley, and J. B. Blumberg, "Flavonoids from almond skins are bioavailable and act synergistically with vitamins C and E to enhance hamster and human LDL resistance to oxidation," *Journal of Nutrition*, vol. 135, no. 6, pp. 1366–1373, 2005.

[27] A. Meshkini, "Acetone extract of almond hulls provides protection against oxidative damage and membrane protein degradation," *Journal of Acupuncture and Meridian Studies*, vol. 9, no. 3, pp. 134–142, 2016.

[28] P. E. Milbury, C.-Y. Chen, G. G. Dolnikowski, and J. B. Blumberg, "Determination of flavonoids and phenolics and their distribution in almonds," *Journal of Agricultural and Food Chemistry*, vol. 54, no. 14, pp. 5027–5033, 2006.

[29] L. Carrera-Quintanar, L. Funes, E. Viudes et al., "Antioxidant effect of lemon verbena extracts in lymphocytes of university students performing aerobic training program," *Scandinavian Journal of Medicine and Science in Sports*, vol. 22, no. 4, pp. 454–461, 2012.

[30] C. Dawczynski, U. Hackermeier, M. Viehweger, R. Stange, M. Springer, and G. Jahreis, "Incorporation of n-3 PUFA and γ-linolenic acid in blood lipids and red blood cell lipids together with their influence on disease activity in patients with chronic inflammatory arthritis—a randomized controlled human intervention trial," *Lipids in Health and Disease*, vol. 10, no. 1, article 130, 2011.

[31] Z. Wu, P. Puigserver, U. Andersson et al., "Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1," *Cell*, vol. 98, no. 1, pp. 115–124, 1999.

[32] L. Lionetti, M. P. Mollica, I. Donizzetti et al., "High-lard and high-fish-oil diets differ in their effects on function and dynamic behaviour of rat hepatic mitochondria," *PLoS ONE*, vol. 9, no. 3, Article ID e92753, 2014.

[33] Y. Zhang, L. Jiang, W. Hu, Q. Zheng, and W. Xiang, "Mitochondrial dysfunction during in vitro hepatocyte steatosis is reversed by omega-3 fatty acid-induced up-regulation of mitofusin 2," *Metabolism: Clinical and Experimental*, vol. 60, no. 6, pp. 767–775, 2011.

[34] L. L. Ji, "Oxidative stress during exercise: implication of antioxidant nutrients," *Free Radical Biology and Medicine*, vol. 18, no. 6, pp. 1079–1086, 1995.

[35] A. Safdar, J. P. Little, A. J. Stokl, B. P. Hettinga, M. Akhtar, and M. A. Tarnopolsky, "Exercise increases mitochondrial PGC-1α content and promotes nuclear-mitochondrial cross-talk to coordinate mitochondrial biogenesis," *The Journal of Biological Chemistry*, vol. 286, no. 12, pp. 10605–10617, 2011.

[36] I. A. Samjoo, A. Safdar, M. J. Hamadeh et al., "Markers of skeletal muscle mitochondrial function and lipid accumulation are moderately associated with the homeostasis model assessment index of insulin resistance in obese men," *PLoS ONE*, vol. 8, no. 6, Article ID e66322, 2013.

[37] N. K. Stepto, B. Benziane, G. D. Wadley et al., "Short-term intensified cycle training alters acute and chronic responses of PGC1α and cytochrome C oxidase IV to exercise in human skeletal muscle," *PLoS ONE*, vol. 7, no. 12, Article ID e53080, 2012.

[38] A. Quintana and M. Hoth, "Mitochondrial dynamics and their impact on T cell function," *Cell Calcium*, vol. 52, no. 1, pp. 57–63, 2012.

[39] D. I. James, P. A. Parone, Y. Mattenberger, and J.-C. Martinou, "hFis1, a novel component of the mammalian mitochondrial fission machinery," *Journal of Biological Chemistry*, vol. 278, no. 38, pp. 36373–36379, 2003.

[40] Z. Radak, Z. Zhao, E. Koltai, H. Ohno, and M. Atalay, "Oxygen consumption and usage during physical exercise: the balance between oxidative stress and ROS-dependent adaptive signaling," *Antioxidants and Redox Signaling*, vol. 18, no. 10, pp. 1208–1246, 2013.

[41] M. D. Ferrer, P. Tauler, A. Sureda, J. A. Tur, and A. Pons, "Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise," *Journal of Sports Sciences*, vol. 27, no. 1, pp. 49–58, 2009.

[42] J. Vina, C. Borras, M.-C. Gomez-Cabrera, and W. C. Orr, "Part of the series: from dietary antioxidants to regulators in cellular signalling and gene expression. Role of reactive oxygen species and (phyto)oestrogens in the modulation of adaptive response to stress," *Free Radical Research*, vol. 40, no. 2, pp. 111–119, 2006.

[43] V. Bézaire, E. L. Seifert, and M.-E. Harper, "Uncoupling protein-3: clues in an ongoing mitochondrial mystery," *The FASEB Journal*, vol. 21, no. 2, pp. 312–324, 2007.

[44] N. Jiang, G. Zhang, H. Bo et al., "Upregulation of uncoupling protein-3 in skeletal muscle during exercise: a potential antioxidant function," *Free Radical Biology and Medicine*, vol. 46, no. 2, pp. 138–145, 2009.

[45] X. Capó, M. Martorell, A. Sureda, I. Llompart, J. A. Tur, and A. Pons, "Diet supplementation with DHA-enriched food in football players during training season enhances the mitochondrial antioxidant capabilities in blood mononuclear cells," *European Journal of Nutrition*, vol. 54, no. 1, pp. 35–49, 2015.

[46] S. H. Cha, A. Fukushima, K. Sakuma, and Y. Kagawa, "Chronic docosahexaenoic acid intake enhances expression of the gene for uncoupling protein 3 and affects pleiotropic mRNA levels in skeletal muscle of aged C57BL/6NJcl mice," *Journal of Nutrition*, vol. 131, no. 10, pp. 2636–2642, 2001.

Manuscript III

Peripheral Blood Mononuclear Cells Antioxidant Adaptations to Regular Physical Activity in Elderly People

Carla Busquets-Cortés, Xavier Capó, Maria del Mar Bibiloni, Miquel Martorell, Miguel D. Ferrer, Emma Argelich, Cristina Bouzas, Sandra Carreres, Josep A. Tur, Antoni Pons and Antoni Sureda

2018. Nutrients, 10, 1555; doi:10.3390/nu10101555

nutrients

MDPI

Article

Peripheral Blood Mononuclear Cells Antioxidant Adaptations to Regular Physical Activity in Elderly People

Carla Busquets-Cortés [1,2], Xavier Capó [1,2], Maria del Mar Bibiloni [1,2], Miquel Martorell [3], Miguel D. Ferrer [1,2], Emma Argelich [1,2], Cristina Bouzas [1,2], Sandra Carreres [1,2], Josep A. Tur [1,2], Antoni Pons [1,2] and Antoni Sureda [1,2,*]

1 Research Group on Community Nutrition and Oxidative Stress & Laboratory of Physical Activity Science, University of Balearic Islands, 07122, Palma de Mallorca, Spain; carla_busquets@hotmail.com (C.B.-C.), xaviercapofiol@hotmail.com (X.C.); mar.bibiloni@uib.es (M.d.M.B.); miguel-david.ferrer@uib.es (M.D.F.); e.argelich@uib.cat (E.A.); cristina.bouzas@uib.es (C.B.); sandra.carreres@uib.cat (S.C.); pep.tur@uib.es (J.A.T.); antonipons@uib.es (A.P.)

2 CIBEROBN, Instituto de Salud Carlos III (ISCIII), 28029 Madrid, Spain

3 Departamento de Nutrición y Dietética, Facultad de Farmacia, Universidad de Concepción, 4070386 Concepción, Chile; martorellpons@gmail.com

* Correspondence: antoni.sureda@uib.es; Tel.: +34-971173142

Received: 18 September 2018; Accepted: 18 October 2018; Published: 20 October 2018

check for updates

Abstract: Regular physical activity prescription is a key point for healthy aging and chronic disease management and prevention. Our aim was to evaluate the antioxidant defense system and the mitochondrial status in peripheral blood mononuclear cells (PBMCs) and the level of oxidative damage in plasma in active, intermediate and inactive elderly. In total, 127 healthy men and women >55 years old participated in the study and were classified according on their level of declared physical activity. A more active lifestyle was accompanied by lower weight, fat mass and body mass index when compared to a more sedentary life-style. Active participants exhibited lower circulating PBMCs than inactive peers. Participants who reported higher levels of exercise had increased antioxidant protein levels when compared to more sedentary partakers. Carbonylated protein levels exhibited similar behavior, accompanied by a significant raise in expression of cytochrome c oxidase subunit IV in PBMCs. No significant changes were found in the activities of antioxidant enzymes and in the expression of structural (MitND5) and mitochondrial dynamic-related (PGC1α and Mitofusins1/2.) proteins. Active lifestyle and daily activities exert beneficial effects on body composition and it enhances the antioxidant defenses and oxidative metabolism capabilities in PBMCs from healthy elderly.

Keywords: active lifestyle; antioxidants; daily habits; elderly; healthy aging; mitochondria; PBMCs; ROS

1. Introduction

Aging is a normal and multi-factorial physiological phenomenon characterized by a progressive generalized deterioration of the different functions in the organism resulting in an increased vulnerability to environmental challenge and a growing risk of disease and death [1]. This decline of homeostatic capacity is also associated with a general decrease in the effectiveness of mechanisms involved in the damage prevention or reparation [2,3]. One of the main problems associated to aging is the appearance of numerous pathological conditions, almost all non-communicable disorders, such as diabetes, cardiovascular diseases, neurodegenerative disorders or various types of cancer. Altogether,

with the increase in life expectancy, the socio-health cost associated to aging increases notably and becomes a problem for the countries. In this sense, it is essential to develop strategies aimed to achieve a healthy aging, improve the quality of life of the elderly and reduce the economic cost associated to aging [4].

It is well reported in the literature that physically active lifestyles promote health and healthy aging [5–8]. The beneficial effects of exercise on various physiological and psychological parameters in the elderly have also been well established [9,10]. The metabolic challenge during the performance of physical activities results in an elevated generation of reactive oxygen species (ROS), produced as normal cellular products of metabolism [11,12]. ROS produced at moderate levels are influential modulators of muscle contraction, generate physiological responses and, although it may seem contradictory, exert antioxidant protection of the organism [13–15]. This fact brings to light that the intensity, duration, and mode of physical activity markedly affect the metabolic and molecular response to a given exercise challenge [6].

Multiple works focus on skeletal muscle biopsies because it is a highly malleable tissue, capable of pronounced metabolic and morphological adaptations in response to contractile activity (i.e., exercise) [16]. However, the assessment of metabolic function in cells isolated from human blood for treatment and diagnosis of disease is a new and important area of translational research. It is now becoming clear that some diseases also modulate mitochondrial energetics in platelets and leukocytes. This opens the possibility that these circulating cells could sense metabolic stress in patients and serve as biomarkers [17,18] of mitochondrial dysfunction in human pathologies such as diabetes, neurodegeneration and cardiovascular disease [19]. The minor invasiveness, reliability and speed in extraction and purification of peripheral blood mononuclear cells (PBMCs) and neutrophils constitute an emerging approach for using blood cells as an in vivo and in vitro tool to assess the impact of diverse stressors on physiological parameters such as antioxidant and mitochondrial status. The potential of isolated PBMCs to determine antioxidant status and mitochondrial status has been previously described. It has been evidenced that acute exercise enhances mitochondrial biosynthesis, fission and fusion processes in PBMCs [20–22] and training stimulates not only the biogenesis of mitochondria but also the removal of old and unhealthy mitochondria through mitochondrial dynamics and autophagy in skeletal muscle [23]. Conversely, other studies have assessed the antioxidant status in lymphocytes, included in PBMCs [24–27]. In addition, dysregulated nuclear factor kappaB (NF-κB) pathway in PBMCs and increased oxidative stress in mitochondria isolated from lymphocytes were found in subjects with mild cognitive impairment and may potentially reflect the brain damage [28,29].

To sum up, the expected increase of the elderly population is an important health challenge in our society, and maintaining an active lifestyle is also a key point for chronic disease management and prevention. The aim of the present study was to evaluate the antioxidant defense system and the mitochondrial status PBMCs of elderly persons, as well as to assess the oxidative damage in plasma, and its association with different degrees and frequency of daily activities.

2. Materials and Methods

2.1. Study Population, Experimental Design and Ethics

This study is encompassed within a cross-sectional study aimed at identifying cardiovascular risk factors in elderly population. The inclusion criteria were men 55–80 and women 60–80 years old with no previously documented cardiovascular disease (stroke, ischemic heart disease, angina, and myocardial infarction) and Diabetes Mellitus type 2 (patients treated with insulin/oral hypoglycemic, basal blood glucose > 126 mg/dL, or casual glycemia > 200 mg/dL with symptoms of diabetes or Glucose Oral Tolerance Test with glycemia > 200 mg/dL in two determinations) or that meet three or more of the following factors: (a) smoking (smokers of more than one cigarette a day or smokers who have stopped smoking in the last year); (b) hypertension (subjects with arterial pressures ≥ 140/90 mm Hg without treatment or those who follow hypotensive treatment regardless of their

tensional numbers); (c) hypercholesterolemia (subjects with LDL-cholesterol > 160 mg/dL without treatment or those who follow a hypolipemiant treatment regardless of your LDL-cholesterol levels); (d) HDL-cholesterol < 40 mg/dL, with or without lipid-lowering treatment; (e) overweight or obesity (body mass index > 25 kg/m^2); and (f) family history of early ischemic heart disease (first-class relatives men <55 years old or women <65 years old).

Exclusion criteria: all those subjects that do not meet the protocol requirements or who have any of the following criteria have been excluded: (a) institutionalized patients, who do not live independently or cannot stand up; (b) patients without fixed residence in the last years or with the impossibility to attend the quarterly controls; (c) patients with acute inflammatory pathology (e.g., pneumonia) may participate in the study after three months of healing; (d) body mass index > 35 kg/m^2; (e) immunosupressed or HIV-infected patients; (f) chronic alcoholics or drug addicts; and (g) patients who have received drugs under investigation during the last year; (h) Illiteracy.

A total sample of 127 participants (61 men and 66 women) met the requirements to be included in the study. The participants were asked to respond questionnaires of nutritional habits, lifestyles and physical activity. The physical activity performed by the subjects was measured using the Minnesota Leisure-Time Physical Activity Questionnaire which was validated for the Spanish old adult population [30,31]. This questionnaire included a list of physical activities and the participants were asked about what type of leisure-time physical activities (LTPA) they had performed during the previous year. To avoid memory bias, as far as possible, the marked activities performed during the last week were collected first, and, in turn, those performed the last month, last quarter, and finally the last year, always including the former periods. For validation purposes, only the information referring to the last year was used. Thus, this questionnaire does not examine the last exercise performed, but rather the frequency and type of activity within the last week/month/year. The metabolic equivalents (METs) corresponding to the activities on the list was defined according to current knowledge [32]. The participants estimated the duration of the activities performed in min/week, and then, the participants were classified according the METsmin/week. Interviewers took basic anthropometric measurements and nurses took blood samples at the health center closest to the address of the respondent.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the Ethics Committee of Research of Balearic Islands (CEIC-IB1295/09 PI). All participants were informed of the purpose and the implications of the study, and informed consent was obtained from all subjects.

2.2. Body Composition and Dietary Intake

Anthropometric measurements were performed by professional observers to minimize the inter-observer coefficients of variation. Height was determined using a mobile anthropometer (Seca 213, SECA Deutchland, Hamburg, Germany) to the nearest millimeter, with the subject's head in the Frankfurt plane. Body weight, body fat and muscle mass were determined using a Segmental Body Composition Analyzer according to manufacturer's protocol (Tanita BC-418, Tanita, Tokyo, Japan). To avoid variability in the values, the measurement was taken after 12 h of rest and subjects refrained from excessive eating and drinking the day before the analysis. The participants were weighed in bare feet and light clothes, and subtracting 0.6 kg for their clothes. Weight and height measures were used to calculate body mass index (BMI, kg/m^2). Dietary habits were assessed using the 24 h dietary recall method, a structured interview intended to track detailed information about all foods and beverages consumed by the respondent in the past 24 h, from midnight to midnight of the previous day [33,34]. A dietician team verified and quantified the food records and every food item consumed was converted into nutrients using a computerized program according to the European and Spanish food composition tables.

2.3. Cell Isolation and Cell Viability Test

Venous blood samples were collected under basal conditions from the antecubital vein of participants in the study in suitable vacutainers with EDTA as anticoagulant in overnight fasting conditions. The PBMC fraction was purified from fresh whole blood and isolated following a protocol described previously [35] using Ficoll–Paque PLUS reagent (GE Healthcare, Chalfont St Giles, UK) [36,37]. Briefly, 6 mL of blood was carefully introduced on 4 mL of Ficoll (proportion of 1.5:1) and was then centrifuged at 900× *g*, at 4 °C for 30 min. The plasma and the Ficoll phases were discarded and PBMCs layer was washed twice with phosphate-buffered saline (PBS), pH 7.4, and centrifuged for 10 min at 1000× *g*, 4 °C. Plasma was obtained by centrifuging whole fresh blood at 1700× *g* for 15 min, 4 °C.

2.4. RNA Extraction and Real-Time PCR

Cytochrome c oxidase subunit IV (COXIV), Peroxisome Proliferator-Activated Receptor Gamma Coactivator (PGC-1α), Mitochondrial NADH Dehydrogenase Subunit 5 (MitND5) and Mitofusins1 and -2 (Mtf1/2) mRNA expression was determined by Real-Time PCR based on incorporation of a fluorescent reporter dye and using human 18S ribosomal as the reference gene. For this purpose, total RNA was isolated from PBMCs by extraction with Tripure® (Tripure Isolation Reagent, Roche Diagnostics, Mannheim, Germany) following a procedure previously described (Capó et al., 2014). RNA (1 µg) from each sample was subjected to reverse transcription using 50 units of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol of oligo (dT) for 60 min at 37 °C in a 10 µL final volume, according to the manufacturer's instructions. The resulting cDNA (3 µL) was amplified with the Light Cycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Germany). Target cDNAs were amplified as follows: 10 min, 95 °C followed by 45 cycles of amplification. The specific primers and amplification conditions used for each gene are presented in Table 1 mRNA levels from inactive women were arbitrarily referred to as 1.

Table 1. Primer sequence and conditions used in Real-Time PCRs.

Gene	Primer		Temp. of Annealing
PGC-1α	Fw:	5′-CACTTACAAGCCAAACCAACAACT-3′	60 °C
	Rv:	5′-CAATAGTCTTGTTCTCAAATGGGGA-3′	
COXIV	Fw:	5′-AGAAGCACTATGTGTACGGCCC-3′	63 °C
	Rv:	5′-GGTTCACCTTCATGTCCAGCAT-3′	
MitND5	Fw:	5′-CGGCTGAGAGGGCGTAGG-3′	63 °C
	Rv:	5′-GATGAAACCGATATCCGGCCGA-3′	
Mtf1	Fw:	5′-TGT TTT GGT CGC AAA CTC TG-3′	60 °C
	Rv:	5′-CTG TCT GCG TAC GTC TTC CA-3′	
Mtf2	Fw:	5′-ATG CAT CCC CAC TTA AGC AC-3′	60 °C
	Rv:	5′-CCA GAG GGC AGA ACT TTG TC-3′	

Fw: Forward; Rv: Reverse; PGC-1α, Peroxisome Proliferator-Activated Receptor Gamma Coactivator; COXIV, cytochrome c oxidase subunit IV; MitND5, Mitochondrial NADH Dehydrogenase Subunit 5; Mtf 1, Mitofusin 1; Mtf2, mitofusin 2.

2.5. Enzymatic Determinations

Catalase (CAT) activity in plasma and PBMCs was measured by the spectrophotometric method of Aebi [38]. Superoxide dismutase (SOD) activity was measured in plasma and PBMCs by an adaptation of the method of McCord and Fridovich [39]. Glutathione reductase (GRd) activity was measured in PBMCs by a modification of the Goldberg and Spooner spectrophotometric method [40]. Glutathione peroxidase (GPx) activity was determined using an adaptation of the spectrophotometric method of Flohé and Gunzler [41]. All activities were estimated in PBMCs and/or plasma samples with a Shimadzu UV-2100 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) at 37 °C.

2.6. SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Antioxidant protein levels in PBMCs were determined by Western blot analysis. Twenty-microgram protein aliquots were loaded in each lane of an sodium dodecyl sulfate (SDS) polyacrylamide gel (15% acrylamide) and electrophoresed by molecular weight at 200 V for 90 min. Bands were electrotransferred onto a nitrocellulose membrane by using Trans-Blot® Turbo™ Transfer System (Bio-Rad, Segrate, Milan, Italy). The membrane was blocked (5% non-fat powdered milk in PBS, pH 7.5, containing 0.1% Tween 20) for 5 h and incubated with the corresponding primary monoclonal antibody. Antibodies anti-catalase (CAT) (1:1000, rabbit), Mn superoxide dismutase (MnSOD) (1:1000, mouse), glutathione reductase (GRd) (1:1000, mouse), glutathione peroxidase (GPx) (1:200, mouse), thioredoxin reductase 1 (TrxR1) (1:200, goat) and uncoupling protein 3 (UCP3) (1:500, mouse) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Blots were then incubated with a secondary peroxidase-conjugated antibody (1:10,000) against specific primary antibody. Development of immunoblots was performed using an enhanced chemiluminescence kit (Immun-Star® Western C® Kit reagent, Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were visualized using the image analysis program Quantity One (Bio-Rad). Precision Plus Protein Kaleidoscope™ (Bio-Rad) was used as a molecular weight marker.

2.7. Malondialdehyde Assay

Malondialdehyde (MDA) concentration (μM), as a marker of lipid peroxidation, was analyzed in plasma by a colorimetric assay kit (Sigma-Aldrich Merck®, St. Louis, MO, USA) following the manufacturer's instructions. The method used is specific for MDA determination and is based on the reaction of MDA with a chromogenic reagent to yield a stable chromophore with maximal absorbance at 586 nm. Briefly, plasma samples or standards were placed in glass tubes containing n-methyl-2-phenylindole (10.3 mM) in acetonitrile: methanol (3:1). HCl 12N was added and the samples were incubated for 1 h at 45 °C. The absorbance was measured at 586 nm. MDA concentration was calculated using a standard curve of known concentration.

2.8. Protein Carbonyls and Nitrotyrosine Determination

Protein carbonyl derivatives and nitrotyrosine (Nitro-Tyr) levels were determined in PBMCs by an immunological method using the OxyBlotTM Protein Oxidation Detection Kit (Chemicon International Inc, Katy, TX, USA) following the manufacturer's instructions. Total protein concentrations were measured by the method of Bradford [42]. Initially, samples (10 μg or 150 μg of protein for carbonyl or nitrotyrosine, respectively) were transferred onto a nitrocellulose membrane by the method of dot-blot using a Bio-Dot® Microfiltration Apparatus (Bio-Rad, Segrate, Milan, Italy). For carbonyl determination, the membrane was incubated in the presence of 2,4-dinitrophenylhydrazine (DNPH) after transference. Once derivatized, the membrane was incubated with the primary antibody, specific to DNP moiety proteins in the case of carbonyl determination or rabbit anti-nitrotyrosine antibody for nitrotyrosine determination. This step was followed by incubation with horseradish peroxidase-antibody (goat anti-rabbit IgG) conjugate directed against the primary antibody. The membrane was then treated with luminol, which is converted into a light-emitting form at wavelength 428 nm by the antigen/primary-antibody/secondary-antibody/peroxidase complex. The light was visualized and detected by short exposure to a Molecular Imager Chemidoc XRS (Bio-Rad, Segrate, Milan, Italy). Image analysis was performed using Quantity One-1D analysis software (Bio-Rad, Segrate, Milan, Italy).

2.9. Statistical Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.21 for Windows, IBM Software Group, Chicago, IL, USA). Results are expressed as mean ± standard error of the mean (SEM), and the level of significance was established at $p < 0.05$ for all statistics. Normality of

data was assessed using Kolmogorov–Smirnov test. The statistical significance of the data was checked by two-way analysis of variance (ANOVA) after adjustment for gender (G) and exercise (E). When significant differences were found between groups, a Bonferroni post hoc test was carried out. Inactive women were taken as a reference group and referred to as 1.

2.10. Limitations of the Study

Firstly, the present cross-sectional design gives limited ability to elucidate causal relationship between exercise and antioxidant adaptations. Secondly, physical activity was not measured objectively such as by using accelerometer. Thirdly, the macronutrient intake was estimated using recall diets instead of food frequency questionnaires that have been questioned in epidemiological studies [43,44]. Two 24-h recall diets tend to underestimate the food intake over a large period compared to food frequency questionnaires, and imply a considerable day-to-day variation in macronutrient intake. Fourthly, underreporting was calculated using energy intake/basal metabolic rate and medications types (e.g., antidepressants, influence weight, etc.) that might influence basal metabolic rate and lifestyle factors as physical activity were not considered in the present study.

3. Results

3.1. Anthropometric Parameters and Dietary Intake

Participants in the study were separated by gender and classified into three groups (active, intermediate and inactive) in accordance to the physical activity performed (Table 2). The variables measured in this study were age, weight, height, body mass index (BMI), body fat percent, and metabolic equivalents (METS, defined as the amount of oxygen consumed while sitting at rest, and is equal to 3.5 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$) [45]. Significant differences in weight and height were found between men and women: men were heavier and taller than women and had higher values of body fat. Women exhibited similar weight, height, without significant differences in any of the anthropometric parameters depending on the degree of physical activity except for BMI: inactive women exhibited a higher percentage of body fat than the intermediate and active groups. Men constituted a more heterogeneous group and some significant differences were found between groups depending on the degree of physical activity. Active men had statistically significant lower weight, body fat and BMI when compared to the inactive peers. The degree of physical activity evidenced a progressive and significant increase in the calculated METs in both women and men.

Table 2. Characteristics of the participants.

	Women (*n* = 66)			**Men (*n* = 61)**			
	Inactive (*n* = 20)	Intermediate (*n* = 26)	Active (*n* = 20)	Inactive (*n* = 20)	Intermediate (*n* = 15)	Active (*n* = 26)	ANOVA
Age (years)	67.2 ± 1.1	67.0 ± 0.8	68.1 ± 0.9	66.0 ± 1.1	66.9 ± 1.3	64.8 ± 1.1 $	G
Weight (kg)	68.6 ± 1.1	64.8 ± 0.8	64.1 ± 0.9	83.4 ± 1.1 $	80.4 ± 1.3 $	77.2 ± 1.1*$	G, E
Height (cm)	157.1 ± 1.1	156.9 ± 0.9	155.3 ± 1.1	170.5 ± 1.3 $	168.6 ± 1.4 $	169.4 ± 1.3 $	G
BMI (kg/m^2)	27.8 ± 0.7	26.4 ± 0.8	26.6 ± 0.9	28.6 ± 0.6	28.2 ± 0.6	26.8 ± 0.6 *	E
Body fat (%)	27.0 ± 1.0	23.9 ± 1.0 *	23.1 ± 1.2 *	22.9 ± 0.9 $	22.7 ± 1.1	19.2 ± 1.3 *$#	G, E
MET min/week	2764 ± 183	5121 ± 125 *	8234 ± 437 *#	2605 ± 236	5064 ± 297 *	9135 ± 567 *#	E

Statistics: Two-way ANOVA. Results are presented as Mean ± SEM, $p < 0.05$. E indicates significant effects of Exercise. G indicates significant effects of Gender. * indicates significant differences between Active/Intermediate and Inactive groups. # indicates significant differences between Intermediate and Active groups. $ indicates significant differences between men and women.

Energy, macronutrient and micronutrient intake of the participants in the study are presented in Table 3. No significant differences were observed in macronutrient, vitamins C and E, selenium and zinc dietary intake between active, intermediate and inactive groups. Daily selenium and zinc intakes were significantly higher in men than women. Energy intakes ranged 983–2996 kcal/day

for women and 823–3348 kcal/day for men. Under-reporters (energy intake/basal metabolic rate < 0.96) were 33.8% (17 women and 26 men) [46]. However, when macronutrient intake was compared between under-reporters and non-under-reporters, no statistical significant differences were found except for daily fiber intake (significances not shown). Thus, under-reporters were not excluded from the present analysis.

Table 3. Energy and nutrient intake of the participants in the study.

	Women (n = 66)			Men (n = 61)			
	Inactive (n = 20)	Intermediate (n = 26)	Active (n = 20)	Inactive (n = 20)	Intermediate (n = 15)	Active (n = 26)	ANOVA
Energy (Kcal)	1810.2 ± 108	1595.7 ± 77	1734.5 ± 107	1754.2 ± 158	1675.9 ± 106	1605.9 ± 68	
Water (mL)	2057.6 ± 123	2023.6 ± 130	1891.8 ± 109	2053.8 ± 157	2025.4 ± 144	2134.6 ± 150	
Proteins (%)	16.9 ± 0.8	16.8 ± 0.6	17.2 ± 1	17.4 ± 1	19.3 ± 0.8	18.2 ± 0.9	
Carbohydrates (%)	43.3 ± 2	45.2 ± 1	44.8 ± 2.1	46.0 ± 2	44.6 ± 2	41.9 ± 2	
Lipids (%)	35.7 ± 2	33.9 ± 1	34.6 ± 2	33.5 ± 2	33.2 ± 2	35.8 ± 1	
Fiber (%)	3.0 ± 1	3.2 ± 0.2	2.9 ± 0.3	3.5 ± 0.4	3.1 ± 0.2	3.2 ± 0.3	
Vitamin E (mg/day)	8.2 ± 0.8	7.5 ± 0.7	8.3 ± 1	8.6 ± 0.9	11.4 ± 1	8.4 ± 1	
Vitamin C (mg/day)	130 ± 17	140 ± 14	171 ± 17	131 ± 17	134 ± 17	137 ± 19	
Selenium (µg/day)	77.5 ± 11	75.7 ± 9	87,9 ± 6	136 ± 6 $	112 ± 11 $	124 ± 11 $	G
Zinc (mg/day)	7.6 ± 0.5	7.5 ± 0.6	8.1 ± 0.5	9.7 ± 0.6 $	9.5 ± 0.8 $	9.2 ± 0.6 $	G

Statistics: Mean ± SEM, $p < 0.05$. Two-way ANOVA. G indicates significant effects of Gender. $ indicates significant differences between men and women.

PBMC blood count and the percent of lymphocytes and monocytes are shown in Table 4. Statistically significant changes in PBMCs absolute counts were found in active groups. Both women and men who reported higher levels of exercises practiced on a regular basis exhibited lower cell count than intermediate and inactive subjects.

Table 4. PBMC blood count.

	Women (n = 66)			Men (n = 61)			
	Inactive (n = 20)	Intermediate (n = 26)	Active (n = 20)	Inactive (n = 20)	Intermediate (n = 15)	Active (n = 26)	ANOVA
PBMCs (10^3 cells/mm^3)	2.13 ± 0.13	1.90 ± 0.19	1.65 ± 0.19 *	1.99 ± 0.19	1.91 ± 0.2	1.33 ± 0.2 *	E
Lymphocytes (%)	33.2 ± 1.5	33.5 ± 1.3	32 ± 1.2	34 ± 1.2	33 ± 1.6	32 ± 1.1	
Monocytes (%)	6.6 ± 0.4	7.3 ± 0.4	6.5 ± 0.3	7.3 ± 0.4	7.6 ± 0.4	6.6 ± 0.3	

Statistics: Two-way ANOVA. Results are presented as Mean ± SEM, $p < 0.05$. E indicates significant effects of Exercise. * indicates significant differences between Active/Intermediate and Inactive groups.

3.2. Antioxidant Protein Levels and Oxidative Stress Markers

The levels of the antioxidant proteins CAT, MnSOD, GRd, GPx, TrxR1 and UCP3 in PBMCs were evaluated in inactive, intermediate and active men and women in basal conditions (Table 5). Except for MnSOD in men and UCP3 in women, the rest of the proteins studied exhibited statistically significant higher values in active participants than inactive subjects, taking inactive women as a reference group. In addition, TrxR1 levels were significantly higher in intermediate men than inactive men. Significant differences between active and intermediate were also found for GRd in women and UCP3 in men. Gender differences were only reported in CAT and GRd in the intermediate group with higher values in men. No significant interactions between gender and exercises were found.

Table 5. Peripheral blood mononuclear cells (PBMCs) protein levels.

		Inactive	Intermediate	Active	ANOVA		
					G	E	SxE
CAT (%)	Women	100 ± 9	114 ± 13	146 ± 17 #	0.001	0.013	0.601
	Men	132 ± 12	170 ± 17 *	175 ± 17 #$			
MnSOD (%)	Women	100 ± 10	116 ± 13	156 ± 21 #	0.420	0.045	0.900
	Men	125 ± 13	127 ± 20	162 ± 24			
GRd (%)	Women	100 ± 10	109 ± 10	165 ± 20 #	0.013	0.000	0.759
	Men	123 ± 20	158 ± 21	209 ± 24 #$			
GPx (%)	Women	100 ± 12	111 ± 14	168 ± 26 #	0.994	0.002	0.720
	Men	108 ± 9	120 ± 17	152 ± 21 #			
TrxR1 (%)	Women	100 ± 13	135 ± 16	172 ± 24 #	0.056	0.001	0.853
	Men	124 ± 24	177 ± 23 *	196 ± 21 #			
UCP3 (%)	Women	100 ± 10	102 ± 9	118 ± 15	0.099	0.039	0.565
	Men	112 ± 10	109 ± 8	152 ± 19 #			

Statistics: Two-way ANOVA. Results are presented as Mean ± SEM, $p < 0.05$. E indicates significant effects of Exercise. G indicates significant effects of Gender. * indicates significant differences between Active/Intermediate and Inactive groups. # indicates significant differences between Intermediate and Active groups. $ indicates significant differences between men and women.

Oxidative stress markers are reported in Figure 1. MDA levels (Figure 1) were measure plasma, whereas Nitro-Tyr (Figure 2) and carbonylated proteins (Figure 3) were measured in PB of inactive, intermediate and active men and women. Although there is a trend in MDA and Nitro to increase with exercise, the differences were not statistically significant. Carbonylated protein PBMCs reported significant higher levels in the intermediate and active groups than the inactive gr but only in women.

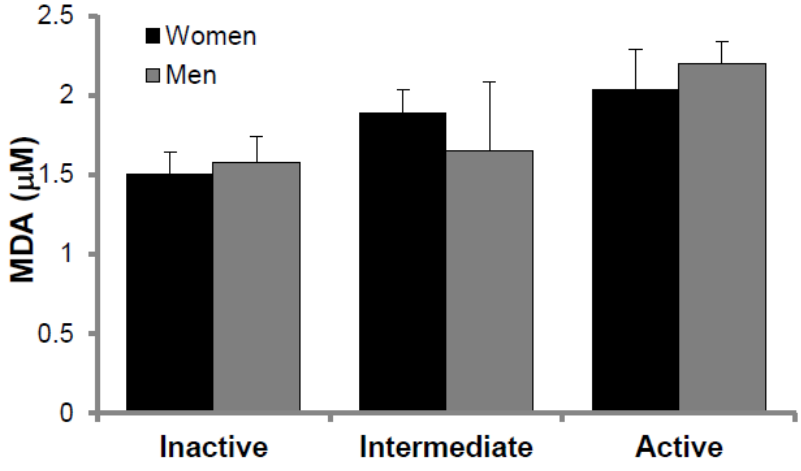


Figure 1. MDA levels in plasma men and women in the study, classified according to physical activity. No significant effects of gender or exercises were found. Statistics: Two-way ANOVA. Results are presented as Mean ± SEM.

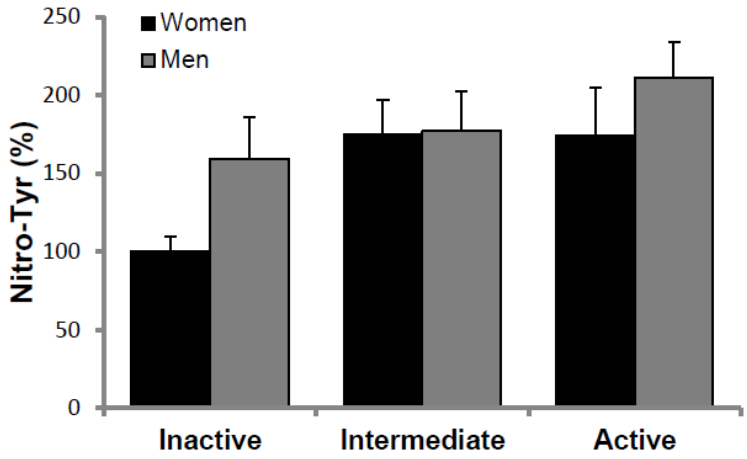


Figure 2. Nitro-Tyr levels in PBMCs men and women in the study, classified according to physical activity. No significant effects of gender or exercises were found. Statistics: Two-way ANOVA. Results are presented as Mean ± SEM.

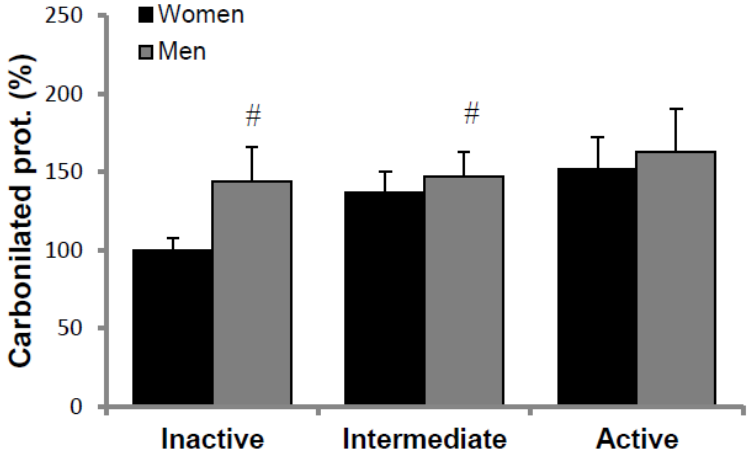


Figure 3. Carbonylated protein levels in PBMCs men and women in the study, classified according to physical activity. Statistics: Two-way ANOVA. Results are presented as Mean ± SEM, $p < 0.05$. E indicates significant effects of exercise. # indicate differences with respect to the inactive group.

3.3. mRNA Relative Expression and Enzymatic Activity

COXIV, PGC1α, MitND5, Mtf1 and Mtf2 relative mRNA expression was assessed in PBMCs. Results are found in Table 6. The mRNA expression of COXIV significantly increased five- and four-fold in active women and men, respectively, compared to the control group (inactive women.). PGC1α, MitND5 and Mtf1/2 exhibited some fluctuations in their mRNA expression depending on the degree of physical activity of the group, but none of these changes were statistically significant.

Table 6. Relative mRNA expression in PBMCs.

	Women (n = 66)			Men (n = 61)			
mRNA levels (%)	Inactive (n = 20)	Intermediate (n = 26)	Active (n = 20)	Inactive (n = 20)	Intermediate (n = 15)	Active (n = 26)	ANOVA
COXIV	1 ± 0.4	1.39 ± 0.5	5.25 ± 1.9 *	1.23 ± 0.4	1.53 ± 0.8	4.37 ± 1.9 *	E
PGC1α	1 ± 0.9	1.11 ± 0.7	1.91 ± 1.0	1.08 ± 0.9	1.12 ± 0.4	1.19 ± 0.6	
MitND5	1 ± 0.4	0.96 ± 0.5	0.72 ± 0.3	1.32 ± 0.5	0.57 ± 0.01	1.05 ± 0.4	
Mtf1	1 ± 0.3	0.80 ± 0.2	1.55 ± 0.8	0.79 ± 0.3	1.48 ± 0.7	1.38 ± 0.5	
Mtf2	1 ± 0.5	1.02 ± 0.7	1.91 ± 0.8	0.83 ± 0.3	1.13 ± 0.7	1.49 ± 0.6	

Statistics: Two-way ANOVA. E indicates significant effects of Exercise. G indicates significant effects of Gender. Results are presented as Mean ± SEM, $p < 0.05$. * indicates significant differences between Active/Intermediate and Inactive groups.

Results of enzymatic activities of CAT and SOD in plasma and CAT, SOD, GPx and GRD in PBMCS are shown in Table 7.

Table 7. Enzymatic activities in PBMCs and plasma.

		Women (n = 66)			Men (n = 61)		
		Inactive (n = 20)	Intermediate (n = 26)	Active (n = 20)	Inactive (n = 20)	Intermediate (n = 15)	Active (n = 26)
PBMCs	CAT (K/10^9 cells)	53 ± 24	44 ± 25	32 ± 13	38 ± 15	36 ± 18	71 ± 30
	SOD (pkat/10^9 cells)	57 ± 16	84 ± 27	110 ± 31	71 ± 39	69 ± 27	64 ± 16
	GPx (nkat/10^9 cells)	102 ± 31	77 ± 14	89 ± 21	80 ± 29	95 ± 38	54 ± 19
	GRd (nkat/10^9 cells)	447 ± 174	406 ± 102	420 ± 166	326 ± 100	324 ± 67	303 ± 84
Plasma	CAT (K/L)	43 ± 20	51 ± 15	54 ± 17	41 ± 17	44 ± 14	64 ± 14
	SOD (pkat/L)	736 ± 117	536 ± 72	526 ± 76	724 ± 108	678 ± 103	649 ± 67

Statistics: Two-way ANOVA. No significant changes between genders were found, and no effects of physical activity were detected.

4. Discussion

The present study attempts to assess the antioxidant system and the mitochondrial status in PBMCs and the level of oxidative damage in plasma, in the elderly according to different degree of physical activity. The changes observed in antioxidant markers in PBMCs cannot be attributed to dissimilar ingests since no significant differences in macro- and micronutrients were found among active, intermediate and inactive groups. The main finding of the present study is the existence of a direct relationship between the self-reported physical activity and protein levels of enzymatic antioxidants and mitochondrial oxidative capacity in both men and women. Men who reported a more active lifestyle exhibited significantly lower weight, BMI and body fat. Women only exhibited significant changes in body fat: it was lower in those who reported more daily activities. The association among body fat, inflammatory markers, and oxidative stress has been previously investigated [47–49]. The adipose tissue is a complex organ with functions other than energy storage, including secretion of several adipokines, such as TNF-α, IL-6, CRP and resistin, among others. Indeed, it has been proposed that adipose tissue may be a significant contributor to increased systemic inflammation in overweight and obese subjects, which can positively correlate to augmented oxidative stress [48]. More active subjects in our study exhibited significant lower BMI and body fat, a fact that would contribute to the attenuated inflammatory status in these elderly participants (observed by the lower circulating PBMCs) and, consequently, enhanced antioxidant machinery (observed by higher antioxidant protein levels). The low number of PBMCs in the active group could derive from the presence of lower amounts of effector memory T cells, exhibiting a reduced proliferative capacity and shorter lifespan, which has been reported to be significantly reduced in a trained group compared with a non-trained group [50].

The significantly higher levels of antioxidant proteins in active participants than inactive partakers match the statement that frequent exposure to moderate exercise (chronic stress) may exert protective effects against oxidative damage. This fact is largely attributed to the upregulation of endogenous antioxidant enzymes [51] such as mitochondrial MnSOD, GPx, and CAT among others [52,53]. These

results would be in accordance with previous investigations reporting that antioxidant defenses are enhanced in trained subjects [54–56]. Thus, the exposure of cells to regular daily activities and an active lifestyle leads to a scenario in which the antioxidant defenses are activated and ready to cope with subsequent bouts of activity and there are no significant raises in oxidative damage markers in PBMCs.

Even though PBMCs from participants who reported regular physical activity exhibited increased antioxidant protein levels, active men and women did not show significant changes in their antioxidant enzymes activities in both plasma and PBMCs. This discrepancy between protein levels and observed activities may be due to the ability of antioxidant enzymes to be induced in the face of a stressful situation, such as exercise, to adapt quickly to the new demanding situation. In this sense, a previous study showed that performing a duathlon in healthy athletes is able to increase enzyme activity, but without altering protein levels suggesting a greater degree of activation of the enzymes present [57]. This fact was later evidenced in in vitro studies, where it was observed how ROS itself at low/moderate levels can activate antioxidant enzymes. The CAT activity of hemolyzed erythrocytes was increased when subjected to xanthine/xanthine-oxidase-generating superoxide anion system [57], while an increase in superoxide anion levels directly activates SOD activity [58]. In another study, the administration of allopurinol to prevent the production of superoxide anion by the action of xanthine oxidase was able to block the stimulating action of ROS on antioxidant defenses after intense exercise, such as a marathon [51]. Thus, the self-reported higher physical activity lead to increased levels of antioxidant enzymes in PBMCs, making it possible for these enzymes to be activated to cope with a subsequent exercise or an increase in ROS production and allowing a better antioxidant response.

MitND5 is a subunit of NADH dehydrogenase (ubiquinone), which is located in the mitochondrial inner membrane, and it can be considered a structural or constitutive mitochondrial protein. MitND5 did not exhibit significant changes in PBMCs between active, intermediate and inactive groups, likely suggesting that mitochondrial reticulum does not need to be more developed in elderly active men and women than in inactive participants. Fusion proteins mitofusins1 and -2 did not exhibit significant changes, while COXIV expression significantly increased five- and four-fold in active women and men, respectively, compared to the control group (inactive women). This might be due to neither a larger mitochondrial reticulum (in accordance with changeless MitND5 mRNA expression) nor enhanced biogenesis (constant Mtf1/2 mRNA expression), but caused by an enhanced respiratory/oxidative capacity of the pre-existent stock of mitochondria. This may suggest improved mitochondrial status in active subjects, but also that an active lifestyle is not enough stimulus to promote mitochondrial biogenesis in elderly active people as well as training does. Similar results were reported in elderly men and women performing resistance exercise training for 14 weeks, with a significant increase in complex IV, but without changes in mtDNA [59]. It is well established that elderly people show reduced mitochondrial content, as well as a lower mitochondrial oxidative capacity [60], but our results may point out that exercise practiced on a regular basis may slow down this decline of mitochondrial function. The present results obtained in PBMC mitochondria might differ in muscle mitochondria. In physiological conditions, peripheral tissues contain resident macrophages and dendritic cells, but other leukocytes (monocytes, neutrophils, and lymphocytes) can transiently infiltrate tissues during pathological situations. Immune cells infiltrate into skeletal muscle during contraction and injury [61]. Muscle cells (myoblasts and myofibers) may produce some of the chemoattracting and activating factors, but other resident cell types such as tissue macrophages are probably more dominant generators of chemokines and inflammatory and growth factors [62], possibly indicating different patterns and pathways modulated in muscle mitochondria.

Widespread physical inactivity is a major public health problem and improving physical activity levels is crucial for healthy aging with positive effects on energy balance and body composition. Physical activity also exerts beneficial effects against a wide range of diseases improving cardiorespiratory and muscular fitness, bone and functional health, and reducing the risk of non-communicable diseases, depression and cognitive decline [63]. Inactive, intermediate and

active participants in our study reported over 2600, 5000 and 8500 METS, respectively, surpassing the general weekly recommendations to get beneficial effects on health. It is well known that regular exercise can ameliorate the oxidant–antioxidant imbalance induced by aging but it remains to be determined what precise dosage (frequency and intensity) of exercise is beneficial [64]. Overall, across all the age ranges, the benefits of implementing the above recommendations, and of being physically active, outweigh the harms.

5. Conclusions

To sum up, elderly people with a more active lifestyle exhibited increased available antioxidant machinery and an attenuated age-associated inflammatory status in PBCMs. Regular daily activities and an active lifestyle leads to a scenario in which the antioxidant defenses are ready to cope with subsequent stressors, allowing a better antioxidant response. No increases in mitochondrial mass or dynamics machinery is observed in subjects with active routine, but an active everyday life in healthy elderly enhances oxidative metabolism capabilities in PBMCs and the antioxidant defenses, but does not necessarily generate significant increases in oxidative stress markers in plasma. Early management of body composition and an active lifestyle could improve and maintain physical function in older adults and promote healthy aging.

Author Contributions: A.S., A.P. and J.A.T. conceived and designed the experiments; C.B., X.C., M.M., M.D.F., C.B., S.C., M.d.M.B. performed the experiments; C.B., A.S. and M.d.M.B. analyzed the data; C.B.-C. and A.S. wrote the paper. All the authors revised and approved the final draft of the manuscript.

Funding: This work was supported by the Institute of Health Carlos III (Projects 11/01791, 14/ 00636, 17/01827, Red Predimed-RETIC RD06/0045/1004, and CIBEROBN CB12/03/30038), grant of support to research groups No. 35/2011 and 01/2017 (Balearic Islands Gov.), EU-COST ACTION CA16112, and Govern de les Illes Balears AAEE26/2017 and EU FEDER funds. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments: The authors hereby acknowledge the PhD FPI/1648/2014 grant provided by Conselleria d'Educació, Cultura i Universitats, Direcció General d'Universitats i Recerca, Govern de les Illes Balears (Fondo Social Europeo) provided to Carla Busquets-Cortés. Xavier Capó acknowledge the PhD grant provided by University of Balearic Isands. Cristina Bouzas acknowledge the "Fernando Tarongí Bauzà" PhD grant.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sureda, A.; Del Mar Bibiloni, M.; Julibert, A.; Aparicio-Ugarriza, R.; Palacios-Le Blé, G.; Pons, A.; Gonzalez-Gross, M. Trace element contents in toenails are related to regular physical activity in older adults. *PLoS ONE* **2017**. [CrossRef] [PubMed]
2. Goloubinoff, P. Mechanisms of protein homeostasis in health, aging and disease. *Swiss Med. Wkly.* **2016**, *146*. [CrossRef] [PubMed]
3. Knight, E.; Stuckey, M.I.; Petrella, R.J. Prescribing physical activity through primary care: Does activity intensity matter? *Phys. Sportsmed.* **2014**, *42*, 78–79. [CrossRef] [PubMed]
4. Jin, K.; Simpkins, J.W.; Ji, X.; Leis, M.; Stambler, I. The Critical Need to Promote Research of Aging and Aging-related Diseases to Improve Health and Longevity of the Elderly Population. *Aging Dis.* **2015**, *6*, 1–5. [CrossRef] [PubMed]
5. Knight, E.; Petrella, R.J. Prescribing physical activity for healthy aging: Longitudinal follow-up and mixed method analysis of a primary care intervention. *Phys. Sportsmed.* **2014**, *42*, 30–38. [CrossRef] [PubMed]
6. Cartee, G.D.; Hepple, R.T.; Bamman, M.M.; Zierath, J.R. Exercise Promotes Healthy Aging of Skeletal Muscle. *Cell Metab.* **2016**, *23*, 1034–1047. [CrossRef] [PubMed]
7. Thompson, P.D.; Buchner, D.; Piña, I.L.; Balady, G.J.; Williams, M.A.; Marcus, B.H.; Berra, K.; Blair, S.N.; Costa, F.; Franklin, B.; et al. Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease. *Circulation* **2003**, *107*, 3109–3116. [CrossRef] [PubMed]
8. Teri, L.; Gibbons, L.E.; Mccurry, S.M.; Logsdon, R.G.; Buchner, D.M.; Barlow, W.E.; Kukull, W.A.; LaCroix, A.Z.; McCormick, W.; Larson, E.B. Exercise plus behavioral management: A randomized controlled trial. *JAMA* **2015**, *290*, 2015–2022. [CrossRef] [PubMed]

9. Daley, M.J.; Spinks, W.L. Exercise, mobility and aging. *Sport Med.* **2000**, *29*, 1–12. [CrossRef]
10. Frankel, J.E.; Bean, J.F.; Frontera, W.R. Exercise in the elderly: Research and clinical practice. *Clin. Geriatr. Med.* **2006**, *22*, 239–256. [CrossRef] [PubMed]
11. Ji, L.L. Antioxidant enzyme response to exercise and aging. *Med. Sci. Sports Exerc.* **1993**, *25*, 225–231. [CrossRef] [PubMed]
12. Birben, E.; Sahiner, U.M.; Sackesen, C.; Erzurum, S.; Kalayci, O. Oxidative stress and antioxidant defense. *World Allergy Organ. J.* **2012**, *5*, 9–19. [CrossRef] [PubMed]
13. Di Meo, S.; Reed, T.T.; Venditti, P.; Victor, V.M. Harmful and beneficial role of ROS. *Oxid. Med. Cell Longev.* **2016**, *2016*, 1–3. [CrossRef] [PubMed]
14. Aldosari, S.; Awad, M.; Harrington, E.; Sellke, F.; Abid, M. Subcellular reactive oxygen species (ROS) in cardiovascular pathophysiology. *Antioxidants* **2018**, *7*, 14. [CrossRef] [PubMed]
15. Ludovico, P.; Burhans, W.C. Reactive oxygen species, ageing and the hormesis police. *FEMS Yeast Res.* **2014**, *14*, 33–39. [CrossRef] [PubMed]
16. Hood, D.A.; Irrcher, I.; Ljubicic, V.; Joseph, A. Coordination of metabolic plasticity in skeletal muscle. *J. Exp. Biol.* **2006**, *209*, 2265–2275. [CrossRef] [PubMed]
17. Reynés, B.; Díaz-Rúa, R.; Cifre, M.; Oliver, P.; Palou, A. Peripheral blood mononuclear cells as a potential source of biomarkers to test the efficacy of weight-loss strategies. *Obesity* **2015**, *23*, 28–31. [CrossRef] [PubMed]
18. Cifre, M.; Díaz-Rúa, R.; Varela-Calviño, R.; Reynés, B.; Pericás-Beltrán, J.; Palou, A.; Oliver, A. Human peripheral blood mononuclear cell in vitro system to test the efficacy of food bioactive compounds: Effects of polyunsaturated fatty acids and their relation with BMI. *Mol. Nutr. Food Res.* **2017**, *61*. [CrossRef] [PubMed]
19. Kramer, P.A.; Ravi, S.; Chacko, B.; Johnson, M.S.; Darley-Usmar, V.M. A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: Implications for their use as bioenergetic biomarkers. *Redox. Biol.* **2014**, *2*, 206–210. [CrossRef] [PubMed]
20. Busquets-Cortés, C.; Capó, X.; Martorell, M.; Tur, J.A.; Sureda, A.; Pons, A. Training and acute exercise modulates mitochondrial dynamics in football players' blood mononuclear cells. *Eur. J. Appl. Physiol.* **2017**, *117*, 1977–1988. [CrossRef] [PubMed]
21. Cartoni, R.; Leger, B.; Hock, M.B.; Praz, M.; Crettenand, A.; Pich, S.; Ziltener, J.-L.; Luthi, F.; Dériaz, O.; Zorzano, A.; et al. Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. *J. Physiol.* **2005**, *567*, 349–358. [CrossRef] [PubMed]
22. Perry, C.G.R.; Lally, J.; Holloway, G.P.; Heigenhauser, G.J.F.; Bonen, A.; Spriet, L.L. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J. Physiol.* **2010**, *588*, 4795–4810. [CrossRef] [PubMed]
23. Yan, Z.; Lira, V.A.; Greene, N.P. Exercise training-induced regulation of mitochondrial quality. *Exerc. Sport Sci. Rev.* **2012**, *40*, 159–164. [CrossRef] [PubMed]
24. Uchiyama, M.; Mihara, M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal. Biochem.* **1978**, *86*, 271–278. [CrossRef]
25. Panayiotidis, M.; Collins, A.R. Ex vivo assessment of lymphocyte antioxidant status using the comet assay. *Free Radic. Res.* **1997**, *27*, 533–537. [CrossRef] [PubMed]
26. Bolin, A.P.; Guerra, B.A.; Nascimento, S.J.S.; Otton, R. Changes in lymphocyte oxidant/antioxidant parameters after carbonyl and antioxidant exposure. *Int. Immunopharmacol.* **2012**, *14*, 690–697. [CrossRef] [PubMed]
27. Campoio, T.R.; Oliveira, F.A.; Otton, R. Oxidative stress in human lymphocytes treated with fatty acid mixture: Role of carotenoid astaxanthin. *Toxicol. Vitr.* **2011**, *25*, 1448–1456. [CrossRef] [PubMed]
28. Ascolani, A.; Balestrieri, E.; Minutolo, A.; Mosti, S.; Spalletta, G.; Bramanti, P.; Mastino, A.; Caltagirone, C.; Macchi, B. Dysregulated NF-κB pathway in peripheral mononuclear cells of Alzheimer's disease patients. *Curr. Alzheimer Res.* **2012**, *9*, 128–137. [CrossRef] [PubMed]
29. Sultana, R.; Baglioni, M.; Cecchetti, R.; Cai, J.; Klein, J.B.; Bastiani, P.; Ruggiero, C.; Mecocci, P.; Butterfield, D.A. Lymphocyte mitochondria: Towards identification of peripheral biomarkers in progression of Alzheimer disease. *Free Radic. Biol. Med.* **2013**, *65*, 595–606. [CrossRef] [PubMed]
30. Elosua, R.; García, M.; Aguilar, A.; Molina, L.; Covas, M.I.; Marrugat, J. Validation of the minnesota leisure time physical activity questionnaire in spanish women. *Med. Sci. Sport Exerc.* **2000**, *32*, 1431–1437. [CrossRef]

31. Pereira, M.A.; FitzGerald, S.J.; Gregg, E.W.; Joswiak, M.L.; Ryan, W.J.; Suminski, R.R.; Utter, A.C.; Zmuda, J.M. A collection of physical activity questionnaires for health-related research. *Med. Sci. Sport Exerc.* **1997**, *29*, S1–S205.
32. Ainsworth, B.; Haskell, W.; Leon, A.; Jacobs, D.; Montoye, H.; Sallis, J.; Paffenbarger, R.S., Jr. Compendium of physical activities: Classification of energy costs of human physical activities. *Med. Sci. Sport Exerc.* **1992**, *25*, 71–80. [CrossRef]
33. Ferrari, M. Intake estimation by means of a 24-hour reminder. *Diaeta* **2013**, *31*, 20–25.
34. Castell, G.S.; Serra-Majem, L.; Ribas-Barba, L. What and how much do we eat? 24-hour dietary recall method. *Nutr. Hosp.* **2015**, *31*, 46–48.
35. Boyum, A. Separation of white blood cells. *Nature* **1964**, *204*, 793–794. [CrossRef] [PubMed]
36. Mestre-Alfaro, A.; Ferrer, M.D.; Sureda, A.; Tauler, P.; Marttínez, E.; Bibiloni, M.M.; Micol, V.; Tur, J.A.; Pons, A. Phytoestrogens enhance antioxidant enzymes after swimming exercise and modulate sex hormone plasma levels in female swimmers. *Eur. J. Appl. Physiol.* **2011**, *111*, 2281–2294. [CrossRef] [PubMed]
37. Busquets-Cortés, C.; Capó, X.; Martorell, M.; Tur, J.A.; Sureda, A.; Pons, A. Training enhances immune cells mitochondrial biosynthesis, fission, fusion, and their antioxidant capabilities synergistically with dietary docosahexaenoic supplementation. *Oxid. Med. Cell Longev.* **2016**. [CrossRef] [PubMed]
38. Aebi, H. Catalase in vitro. *Methods Enzym.* **1984**, *105*, 121–126.
39. McCord, J.M. Superoxide dismutase: An enzymatic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **1969**, *214*, 6049–6055.
40. Bergmeyer, H.U.; Bergmeyer, J.; GraBI, M. Glutathione Reductase. In *Methods in Enzymatic Analysis*, 3rd ed.; Verlag Chemie: Weinheim, Germany, 1984; pp. 258–265.
41. Flohe, L.; Günzler, W.A. Assays of glutathione peroxidase. *Methods Enzym.* **1984**, *105*, 114–120.
42. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *7*, 248–254. [CrossRef]
43. Schatzkin, A.; Kipnis, V.; Carroll, R.J.; Midthune, D.; Subar, A.F.; Bingham, S.; Schoeller, D.A.; Troiano, R.P.; Freedman, L.S. A comparison of a food frequency questionnaire with a 24-hour recall for use in an epidemiological cohort study: Results from the biomarker-based Observing Protein and Energy Nutrition (OPEN) study. *Int. J. Epidemiol.* **2003**, *32*, 1054–1062. [CrossRef] [PubMed]
44. Freedman, L.S.; Potischman, N.; Kipnis, V.; Midthune, D.; Schatzkin, A.; Thompson, F.E.; Troiano, R.P.; Prentice, R.; Patterson, R.; Carroll, R.; et al. A comparison of two dietary instruments for evaluating the fat-breast cancer relationship. *Int. J. Epidemiol.* **2006**, *35*, 1011–1021. [CrossRef] [PubMed]
45. Castro, J.P.; Grune, T.; Speckmann, B. The two faces of reactive oxygen species (ROS) in adipocyte function and dysfunction. *Biol. Chem.* **2016**, *397*, 709–724. [CrossRef] [PubMed]
46. Black, A.E. Critical evaluation of energy intake using the Goldberg cut-off for energy intake: Basal metabolic rate. A practical guide to its calculation, use and limitations. *Int. J. Obes.* **2000**, *24*, 1119–1130. [CrossRef]
47. Axelsson, J.; Heimbürger, O.; Lindholm, B.; Stenvinkel, P. Adipose tissue and its relation to inflammation: The role of adipokines. *J. Ren. Nutr.* **2005**, *15*, 131–136. [CrossRef] [PubMed]
48. Nihi, M.; Manfro, R.; Martins, C. Association between body fat, inflammation and oxidative stress in hemodialysis. *J. Bras.* **2010**, *32*, 9–15.
49. Folsom, A.R.; Pankow, J.S.; Tracy, R.P.; Arnett, D.K.; Peacock, J.M.; Hong, Y.; Djoussé, L.; Eckfeldt, J.H. Association of C-reactive protein with markers of prevalent atherosclerotic disease. *Am. J. Cardiol.* **2001**, *88*, 112–117. [CrossRef]
50. Silva, L.C.R.; de Araújo, A.L.; Fernandes, J.R.; Matias, M.D.S.T.; Silva, P.R.; Duarte, A.J.S. Moderate and intense exercise lifestyles attenuate the effects of aging on telomere length and the survival and composition of T cell subpopulations. *Age* **2016**, *38*, 1–16. [CrossRef] [PubMed]
51. Gomez-Cabrera, M.C.; Domenech, E.; Viña, J. Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. *Free Radic. Biol. Med.* **2008**, *44*, 126–131. [CrossRef] [PubMed]
52. Radak, Z.; Chung, H.Y.; Goto, S. Exercise and hormesis: Oxidative stress-related adaptation for successful aging. *Biogerontology* **2005**, *6*, 71–75. [CrossRef] [PubMed]
53. Ferrer, M.D.; Tauler, P.; Sureda, A.; Tur, J.A.; Pons, A. Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise. *J. Sports Sci.* **2009**, *27*, 49–58. [CrossRef] [PubMed]
54. Gomez-Cabrera, M.C.; Ferrando, B.; Brioche, T.; Sanchis-Gomar, F.; Viña, J. Exercise and antioxidant supplements in the elderly. *J. Sport Heal. Sci.* **2013**, *2*, 94–100. [CrossRef]

55. Radak, Z.; Chung, H.Y.; Goto, S. Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radic. Biol. Med.* **2008**, *44*, 153–159. [CrossRef] [PubMed]
56. Powers, S.K.; Ji, L.L.; Leeuwenburgh, C. Exercise training-induced alterations in skeletal muscle antioxidant capacity: A brief review. *Med. Sci. Sport Exerc.* **1999**, *31*, 987–997. [CrossRef]
57. Tauler, P.; Gimeno, I.; Aguiló, A.; Guix, M.P.; Pons, A. Regulation of erythrocyte antioxidant enzyme activities in athletes during competition and short-term recovery. *Pflugers Arch. Eur. J. Physiol.* **1999**, *438*, 782–787. [CrossRef]
58. Ji, L.L. Modulation of skeletal muscle antioxidant defense by exercise: Role of redox signaling. *Free Radic. Biol. Med.* **2008**, *44*, 142–152. [CrossRef] [PubMed]
59. Parise, G.; Brose, A.N.; Tarnopolsky, M.A. Resistance exercise training decreases oxidative damage to DNA and increases cytochrome oxidase activity in older adults. *Exp. Gerontol.* **2005**, *40*, 173–180. [CrossRef] [PubMed]
60. Conley, K.E.; Jubrias, S.A.; Esselman, P.C. Oxidative capacity and ageing in human muscle. *J. Physiol.* **2000**, *526*, 203–210. [CrossRef] [PubMed]
61. Tidball, J.G.; Villalta, S.A. Regulatory interactions between muscle and the immune system during muscle regeneration. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2010**, *298*, R1173–R1187. [CrossRef] [PubMed]
62. Pillon, N.J.; Bilan, P.J.; Fink, L.N.; Klip, A. Cross-talk between skeletal muscle and immune cells: Muscle-derived mediators and metabolic implications. *Am. J. Physiol. Endocrinol. Metab.* **2013**, *304*, E453–E465. [CrossRef] [PubMed]
63. World Health Organization. *Global Recommendations on Physical Activity for Health*; World Health Organization: Albany, NY, USA, 2010; Volume 60, ISBN 978-92-4-159-997-9.
64. Sallam, N.; Laher, I. Exercise modulates oxidative stress and inflammation in aging and cardiovascular diseases. *Oxid. Med. Cell Longev.* **2016**, *2016*, 46–54. [CrossRef] [PubMed]

Manuscript IV

Regular practice of moderate physical activity by older adults ameliorates their anti-inflammatory status

Miguel D Ferrer; Xavier Capó; Miquel Martorell; Carla Busquets-Cortés; Cristina Bouzas; Sandra Carreres; David Mateos; Antoni Sureda; Josep A. Tur; Antoni Pons.

2018. Nutrients, 10, 1780; doi:10.3390/nu10111780

nutrients

MDPI

Article

Regular Practice of Moderate Physical Activity by Older Adults Ameliorates Their Anti-Inflammatory Status

Miguel D. Ferrer [1,2,3], Xavier Capó [1,2,3], Miquel Martorell [1,3,4], Carla Busquets-Cortés [1,2,3], Cristina Bouzas [1,2], Sandra Carreres [1,2], David Mateos [1,2], Antoni Sureda [1,2,3], Josep A. Tur [1,2] and Antoni Pons [1,2,*]

1 Grup de Nutrició Comunitària i Estrès Oxidatiu, Departament de Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears, 07122 Palma, Spain; miguel-david.ferrer@uib.es (M.D.F.); xavier.capo@uib.es (X.C.); martorellpons@gmail.com (M.M.); carla_busquets@hotmail.com (C.B.-C.); cristina.bouzas@uib.es (C.B.); sandra.carreres@uib.cat (S.C.); david-mateos@hotmail.com (D.M.); antoni.sureda@uib.es (A.S.); pep.tur@uib.es (J.A.T.)

2 CIBER: CB12/03/30038 Fisiopatología de la Obesidad la Nutrición, CIBEROBN, Instituto de Salud Carlos III (ISCIII), University of Balearic Islands, 07122 Palma, Spain

3 Laboratori de Ciències de l'Activitat Física, Departament de Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears, 07122 Palma, Spain

4 Departamento de Nutrición y Dietética, Facultad de Farmacia, Universidad de Concepción, 4070386 Concepción, Chile

* Correspondence: antonipons@uib.es; Tel.: +34-97117-3171

Received: 10 October 2018; Accepted: 14 November 2018; Published: 16 November 2018

Abstract: A chronic inflammatory state is a major characteristic of the aging process, and physical activity is proposed as a key component for healthy aging. Our aim was to evaluate the body composition, hypertension, lipid profile, and inflammatory status of older adults, and these factors' association with physical activity. A total of 116 elderly volunteers were categorized into terciles of quantitative metabolic equivalents of task (MET). Subjects in the first and third terciles were defined as sedentary and active subjects, respectively. Anthropometric and biochemical parameters, hemograms, and inflammatory markers were measured in plasma or peripheral mononuclear blood cells (PBMCs). The active groups exercised more than their sedentary counterparts. The practice of physical activity was accompanied by lower weight, fat mass, body mass index, and diastolic blood pressure when compared to a more sedentary life-style. Physical activity also lowered the haematocrit and total leukocyte, neutrophil, and lymphocyte counts. The practice of exercise induced a decrease in the IL-6 circulating levels and the TLR2 protein levels in PBMCs, while the expression of the anti-inflammatory IL-10 was activated in active subjects. The regular practice of physical activity exerts beneficial effects on body composition and the anti-inflammatory status of old people.

Keywords: immunity; inflammation; metabolism; physical activity

1. Introduction

Ageing is an unavoidable process in all animals and is characterized by progressive accumulation of cell and organ damage, which result in organism malfunction. In the past few centuries, the proportion of elderly people has been continuously increasing worldwide and is projected to reach 19.3% of total population by 2050 [1]. Although ageing has an unavoidable and intrinsic component, it is also importantly modulated by several external factors, such as exposure to chemicals, lifestyle, or nutrition [2]. Therefore, it seems clear that the progression of ageing can be, at least in

part, counteracted by the combination of adequate nutritional intake and a healthy lifestyle, the latter including the regular practice of physical activity [3].

Several studies have evidenced the beneficial effects of physical activity on longevity, showing that regular physical activity is associated with a 30% reduction in the risk of mortality in subjects without CV disease [4], which might correspond to one to two years of additional life [5]. On the other hand, physical inactivity causes 6–10% of the burden of several diseases (including coronary heart disease, diabetes, and cancer) and 9% of premature mortality [6]. In fact, regular physical activity prescription for healthy ageing is a key point for chronic disease management and prevention [4,7]. These positive effects of physical activity might be related to greater conservation of lean tissue [8], lower body mass, and less relative body fat [9] in old adults engaging in high levels of physical activity in comparison to individuals who are more sedentary.

Regular physical activity has also been shown to reduce the risk of several diseases, such as cardiovascular disease, stroke, hypertension, type 2 diabetes, osteoporosis, obesity, colon cancer, breast cancer, anxiety, and depression [10]. Most of these diseases are directly or indirectly related to inflammation processes. In this instance, the benefits of exercise on life-span have been related to different cardioprotective mechanisms, including effects on endothelial function and inflammation [4]. Actually, it has been shown that exercise training exerts anti-inflammatory effects in aged or diseased populations [11], and these effects might be mediated by decreases in TNF-α expression in skeletal muscle, among other effectors [12]. Increased risk of chronic diseases has been associated with elevated inflammation markers [13,14], while the practice of physical activity has shown to reduce pro-inflammatory biomarkers such as C-reactive protein [15], TNF-α [16], or interleukin (IL) 6 [17,18].

Old people usually face a situation of chronic low-grade inflammation. It has been stated that inflammatory cytokines are elevated, and anti-inflammatory cytokine concentrations are lowered, in healthy adults over 50 years of age [19]. This behaviour has been associated with redistribution of body fat and concomitant increases in circulating fatty acids that lead to the activation of proinflammatory macrophages [20]. This chronic low-grade inflammatory status, termed inflamm-aging by some authors [14,21], appears to be a major component of the most common age-related diseases, such as diabetes, osteoporosis, cardiovascular diseases, and cancer.

Therefore, the aim of this study was to evaluate the body composition, hypertension, and lipid metabolic profile, as well as the inflammatory status, of older adults, as well as its association with the regular practice of physical activity.

2. Materials and Methods

2.1. Subjects and Study Design

A total of 116 elderly volunteers (58 men aged between 55 and 80 years and 58 women aged between 60 and 80 years) participated in the study. These volunteers were selected from a larger study population conforming the PHYSMED project (with a total of 380 participants), a multi-centre, cross-sectional study aiming at identifying cardiovascular risk factors in sedentary and active elderly subjects. The 116 volunteers included in this study were recruited in social and municipal clubs, health centres, and sport clubs in different villages and cities of Mallorca, Spain. Exclusion criteria included being institutionalized, suffering from a physical or mental illness that would have limited their participation in physical fitness or their ability to respond to questionnaires, chronic alcoholism or drug addiction, and intake of drugs for clinical research over the past year.

The physical activity performed by the participants was measured using the Minnesota Leisure-time Physical Activity Questionnaire previously validated for the Spanish old adult population [22,23]. This questionnaire included a list of physical activities, and the participants were asked about what type of leisure-time physical activities (LTPA) they had performed during the last year. The participants estimated the duration of the activities performed each hour/week by using metabolic equivalents of task (MET, defined as 1 kcal/kg/hour and equivalent to the energy

cost of sitting quietly) [24]. The resulting quantitative MET for each participant were categorized into terciles [25], and subjects in the first and the third terciles were selected to take part in this study and defined as sedentary and active subjects, respectively.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures were approved by the Ethics Committee of Clinical Research of the Balearic Islands (CEIC-IB, ref. 1295/09 PI). All the subjects were informed of the purpose and demands of the study before giving their written consent to participate.

Venous blood samples were obtained from the antecubital vein of participants in resting conditions after overnight fasting. The peripheral blood mononuclear cell (PBMC) fraction was purified from whole blood following an adaptation of the method described by Boyum [26] using Ficoll-Paque PLUS reagent (GE Healthcare). This procedure ensures a PBMC purity and viability of 95 ± 5%.

2.2. Anthropometric Characteristics

Anthropometric measurements were performed by well-trained dieticians who underwent identical and rigorous training as an effort to minimize the effects of inter-observer variation. Height was determined using a mobile anthropometer (Seca 213, SECA Deutchland, Hamburg, Germany) to the nearest millimetre, with the subject's head in the Frankfurt plane. Body weight, body fat, and muscle mass were determined using a Segmental Body Composition Analyzer (Tanita BC-418, Tanita, Tokyo, Japan). The participants were weighed in bare feet and light clothes, subtracting 0.6 g for their clothes. Body mass index (BMI) was calculated using the following formula: BMI = mass (kg)/squared height (m).

2.3. Biochemical Parameters and Hemogram

Glucose, triglycerides, total, high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) cholesterol, urea, uric acid, and creatinine were determined by standard procedures using commercial clinical kits in an autoanalyzer system (Technicon DAX System).

Haematological parameters and hemogram were determined in an automatic flow cytometer analyser Technicon H2 (Bayer, Leverkusen, Germany) VCS system. Haemoglobin concentration was determined using Drabkin reagent (Sigma Aldrich, St. Louis, MO, USA).

2.4. Circulating Inflammatory Parameters

IL-6, sCD62L, and sICAM3 plasma levels were determined using individual ELISA kits from Diaclone (Besançon, France). TNFα was determined using the RayBiotech (Norcross, GA, USA) ELISA kit. All procedures were performed following the supplier instructions for use.

MPO activity in plasma was measured by guaiacol oxidation, under identical conditions to those previously described [27].

2.5. mRNA Gene Expression

mRNA expressions were determined by real time-polymerase chain reaction (RT-PCR). For this purpose, mRNA was isolated from PBMC by extraction with Tripure Isolation Reagent (Roche, Basel, Switzerland). cDNA was synthesized from 1 µg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics, Basel, Switzerland) with DNA-master SYBR Green I. The primers used are shown in Table 1. For all PCRs, there was one cycle at 95 °C for 10 min, followed by 40 cycles at the conditions shown in Table 1.

Table 1. Primers and conditions used in the PCRs.

Gene		Primer	Conditions	
18S	Fw:	5′-ATG TGA AGT CAC TGT GCC AG-3′	95 °C	10 s
	Rv:	5′-GTG TAA TCC GTC TCC ACA GA-3′	60 °C	10 s
			72 °C	12 s
IL-1ra	Fw:	5′-GAA GAT GTG CCT GTC CTG TGT-3′	95 °C	10 s
	Rv:	5′-CGC TCA GGT CAG TGA TGT TAA-3′	60 °C	10 s
			72 °C	15 s
IL10	Fw:	5′-AGA ACC TGA AGA CCC TCA GGC-3′	95 °C	10 s
	Rv:	5′-CCA CGG CCT TGC TCT TGT T-3′	60 °C	10 s
			72 °C	15 s
IL1β	Fw:	5′-GGA CAG GAT ATG GAG CAA CA-3′	95 °C	10 s
	Rv:	5′-GGC AGA CTC AAA TTC CAG CT-3′	58 °C	10 s
			72 °C	15 s
NFκB	Fw:	5′-AAA CAC TGT GAG GAT GGG ATC TG-3′	95 °C	10 s
	Rv:	5′-CGA AGC CGA CCA CCA TGT-3′	60 °C	10 s
			72 °C	15 s
TLR4	Fw:	5′-GGT CAC CTT TTC TTG ATT CCA-3′	95 °C	10 s
	Rv:	5′-TCA GAG GTC CAT CAA ACA TCA C-3′	60 °C	10 s
			72 °C	15 s
TNFα	Fw:	5′-CCC AGG CAG TCA GAT CAT CTT CTC GGA A-3′	94 °C	10 s
	Rv:	5′-CTG GTT ATC TCT CAG CTC CAC GCC ATT-3′	63 °C	10 s
			72 °C	15 s
IL6	Fw:	5′-ACC TGA ACC TTC CAA AGA TGG C-3′	95 °C	10 s
	Rv:	5′-TCA CCA GGC AAG TCT CCT CAT TG-3′	63 °C	10 s
			72 °C	15 s

The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels of sedentary males were arbitrarily referred to as 1. The expression of the target gene was normalized with respect to ribosomal 18S.

2.6. Western Blot Analysis in PBMCs

Toll-Like Receptor (TLR) 2 and 4 protein levels were determined in PBMCs by Western blot. Protein extracts were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Total protein concentrations were measured by the method of Bradford [28]. 80 µg of total protein was loaded on a 12% agarose gel. Following electrophoresis, samples were transferred onto a nitrocellulose membrane and incubated with a primary monoclonal anti-TLR2 or anti-TLR4 antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and a secondary anti-mouse IgG peroxidase-conjugated antibody. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad Laboratories, Hercules, CA, USA) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and analyzed with Quantity One-1D Software (Bio-Rad Laboratories).

2.7. Statistical Analysis

Statistical analysis was carried out using a statistical package for social sciences (SPSS 22 for Windows, SPSS Inc., Chicago, IL, USA). Results are expressed as mean ± standard error of the mean (SEM) and $p < 0.05$ was considered statistically significant. The statistical significance of the data was assessed by a two-way analysis of variance (ANOVA). The statistical factors analysed were (S) sex and (E) exercise. When significant effects were found, one-way ANOVA was used to determine the differences between the groups involved.

3. Results

The anthropometric characteristics of the participants are shown in Table 2. The active groups (both male and female) exercised more than their sedentary counterparts, as evidenced by the significantly higher degree of physical activity measured in MET-hours/week. No differences in the degree of physical activity performed were evidenced between males and females. However, males

were taller, weighed more, and presented higher fat-free mass and body mass index than females. The practice of regular physical activity was accompanied by significantly lower total weight, fat mass, body mass index, and diastolic blood pressure when compared to a more sedentary life-style.

Table 2. Anthropometric characteristics of the participants.

		Sedentary	Active	ANOVA		
				Sex	Exercise	SxE
Age (years)	Male Female	64.6 ± 1.1 67.3 ± 1.1	62.5 ± 0.9 67.4 ± 1.0 *	0.000	0.339	0.281
Physical activity (MET-hours/week)	Male Female	40.4 ± 4.4 48.4 ± 3.3	141 ± 9 # 126 ± 6 #	0.602	0.000	0.071
Weight (kg)	Male Female	86.1 ± 1.9 69.3 ± 2.2 *	78.2 ± 2.0 # 62.0 ± 1.7 *	0.000	0.000	0.875
Height (cm)	Male Female	170 ± 1 157 ± 1 *	171 ± 1 156 ± 1 *	0.000	0.808	0.624
Fat-free mass (kg)	Male Female	61.1 ± 1.1 41.8 ± 0.9 *	58.8 ± 1.4 40.9 ± 0.6 *	0.000	0.142	0.531
Fat mass (kg)	Male Female	25.0 ± 1.1 27.5 ± 1.6	19.4 ± 0.9 # 21.2 ± 1.2 #	0.090	0.000	0.765
Body Mass Index (kg/m^2)	Male Female	29.6 ± 0.6 28.1 ± 0.8	26.8 ± 0.5 # 25.5 ± 0.7 #	0.038	0.000	0.874
Systolic blood pressure (mm Hg)	Male Female	141 ± 3 138 ± 4	138 ± 4 133 ± 3	0.312	0.312	0.796
Diastolic blood pressure (mm Hg)	Male Female	84.8 ± 1.4 82.0 ± 2.2	81.2 ± 1.8 77.8 ± 1.8	0.099	0.039	0.883

Mean ± SEM. Statistical analysis: two-way ANOVA, $p < 0.05$. (S) effect of sex, (E) effect of exercise, and (SxE) interaction between the two factors. (*) significant differences between sexes; (#) significant differences between sedentary and active groups.

Neither sex or exercise influenced glucose or triglyceride circulating levels (Table 3). Total circulating cholesterol was, however, significantly affected by the sex of the participants, with higher levels observed in women when compared to men. These higher levels of total cholesterol found in females seem attributable to higher HDL-cholesterol levels, which were also higher in women compared to men. HDL-cholesterol circulating levels were also significantly affected by exercise, with those groups of active participants presenting higher levels than their sedentary counterparts. A significant effect of sex was also evidenced in the circulating levels of uric acid and creatinine: females presented significantly lower levels of uric acid and creatinine than their respective male counterparts.

Sex also affected several hemogram parameters, as shown in Table 3. In this instance, females presented significantly lower counts of red blood cells (which resulted in a lower haematocrit and lower haemoglobin content) and eosinophils, as well as a higher platelet count. On the other side, the practice of physical activity lowered the haematocrit, through the significant decrease on total leukocyte count, as well as neutrophil and lymphocyte count.

The circulating levels of key pro-inflammatory proteins are shown in Table 4. No effects of sex were evidenced in any of the circulating pro-inflammatory proteins measured. A significant effect of exercise was observed only in IL-6 levels. The practice of exercise induced a decrease in the circulating levels of IL-6, although this decrease was only significant in the group of females.

Table 3. Biochemical parameters and hemogram of the participants.

		Sedentary	Active	ANOVA		
				Sex	Exercise	SxE
Glucose (mg/dL)	Male Female	100 ± 2 105 ± 12	98.8 ± 2.8 87.8 ± 1.8	0.636	0.153	0.217
Triglycerides (mg/dL)	Male Female	111 ± 7 97.2 ± 6.3	100 ± 6 100 ± 6	0.360	0.564	0.289
Total cholesterol (mg/dL)	Male Female	197 ± 5 214 ± 7	199 + 5 211 ± 6	0.016	0.919	0.732
HDL (mg/dL)	Male Female	44.7 ± 1.6 57.2 ± 2.0 *	51.6 ± 2.2 61.3 ± 2.5 *	0.000	0.011	0.520
LDL (mg/dL)	Male Female	130 ± 5 137 ± 6	127 ± 5 130 ± 5	0.360	0.317	0.676
VLDL (mg/dL)	Male Female	22.3 ± 1.4 19.5 ± 1.3	19.9 ± 1.3 20.1 ± 1.8	0.364	0.532	0.285
Urea (mg/dL)	Male Female	36.3 ± 1.5 36.0 ± 1.5	36.2 ± 1.7 35.6 ± 1.2	0.754	0.883	0.909
Uric acid (mg/dL)	Male Female	6.25 ± 0.21 5.04 ± 0.22 *	6.03 ± 0.18 4.56 ± 0.20 *	0.000	0.087	0.515
Creatinine (mg/dL)	Male Female	0.829 ± 0.018 0.728 ± 0.013 *	0.841 ± 0.016 0.716 ± 0.018 *	0.000	0.978	0.460
Red blood cells (10^6/mm^3)	Male Female	5.03 ± 0.08 4.62 ± 0.06 *	4.90 ± 0.07 4.52 ± 0.08 *	0.000	0.103	0.860
Haemoglobin (g/dL)	Male Female	15.5 ± 0.2 14.2 ± 0.2 *	15.3 ± 0.2 13.8 ± 0.1 *	0.000	0.056	0.798
Haematocrit (%)	Male Female	46.0 ± 0.5 42.1 ± 0.5 *	45.0 ± 0.6 41.1 ± 0.4 *	0.000	0.048	0.971
Mean corpuscular volume (fL)	Male Female	91.7 ± 0.9 91.3 ± 0.7	91.9 ± 0.6 91.2 ± 0.9	0.480	0.916	0.857
Platelets (10^3/mm^3)	Male Female	222 ± 10 246 ± 9	214 ± 8 232 ± 8	0.018	0.195	0.743
Leucocytes (10^3/mm^3)	Male Female	6.39 ± 0.29 6.49 ± 0.32	5.85 ± 0.21 5.33 ± 0.21 #	0.423	0.002	0.244
Neutrophils (10^3/mm^3)	Male Female	3.43 ± 0.21 3.35 ± 0.22	3.15 ± 0.18 2.56 ± 0.13 #	0.074	0.006	0.185
Lymphocytes (10^3/mm^3)	Male Female	2.20 ± 0.13 2.41 ± 0.14	1.94 ± 0.09 2.15 ± 0.12	0.084	0.039	0.997
Monocytes (10^3/mm^3)	Male Female	0.512 ± 0.025 0.511 ± 0.024	0.526 ± 0.027 0.446 ± 0.022	0.114	0.306	0.116
Eosinophils (10^3/mm^3)	Male Female	0.220 ± 0.025 0.187 ± 0.023	0.196 ± 0.020 0.140 ± 0.013	0.037	0.088	0.586
Basophils (10^3/mm^3)	Male Female	0.035 ± 0.004 0.034 ± 0.004	0.037 ± 0.004 0.034 ± 0.003	0.721	0.855	0.775

Mean ± SEM. Statistical analysis: two-way ANOVA, $p < 0.05$. (S) effect of sex, (E) effect of exercise, and (SxE) interaction between the two factors. (*) significant differences between sexes; (#) significant differences between sedentary and active groups.

Table 4. Plasma markers of inflammation.

		Sedentary	Active	ANOVA		
				Sex	Exercise	SxE
IL6 (pg/mL)	Male Female	3.33 ± 0.27 3.39 ± 0.31	2.55 ± 0.18 2.19 ± 0.35 #	0.599	0.001	0.478
TNFα (pg/mL)	Male Female	26.6 ± 3.0 29.1 ± 3.2	21.5 ± 1.8 23.9 ± 5.6	0.480	0.148	0.997
sCD62L (ng/mL)	Male Female	1507 ± 61 1631 ± 239	1298 ± 77 1351 ± 110	0.584	0.132	0.825
sICAM3 (ng/mL)	Male Female	523 ± 24 531 ± 20	496 ± 18 514 ± 25	0.549	0.318	0.832
Myeloperoxidase (μkat/mL)	Male Female	139 ± 43 105 ± 36	179 ± 45 87 ± 29	0.155	0.799	0.514

Mean ± SEM. Statistical analysis: two-way ANOVA, $p < 0.05$. (S) effect of sex, (E) effect of exercise, and (SxE) interaction between the two factors. (#) significant differences between sedentary and active groups.

The inflammatory status of the organism was additionally studied through the gene (Figure and protein (Figure 2) expression of pro- and anti-inflammatory cytokines in PBMC. The regul practice of physical activity influenced the expression of the anti-inflammatory IL-10, with significant higher expression levels in active males compared to sedentary males. A similar pattern of respons although non-significant, was also observed in females. Similarly, exercise also significantly influence the gene expression of NF-κB, tending to higher expressions in active participants when compare to their sedentary counterparts. A significant effect of sex was observed regarding TLR4 ger expression: significantly higher expression of this gene was observed in active females when compare to active males.

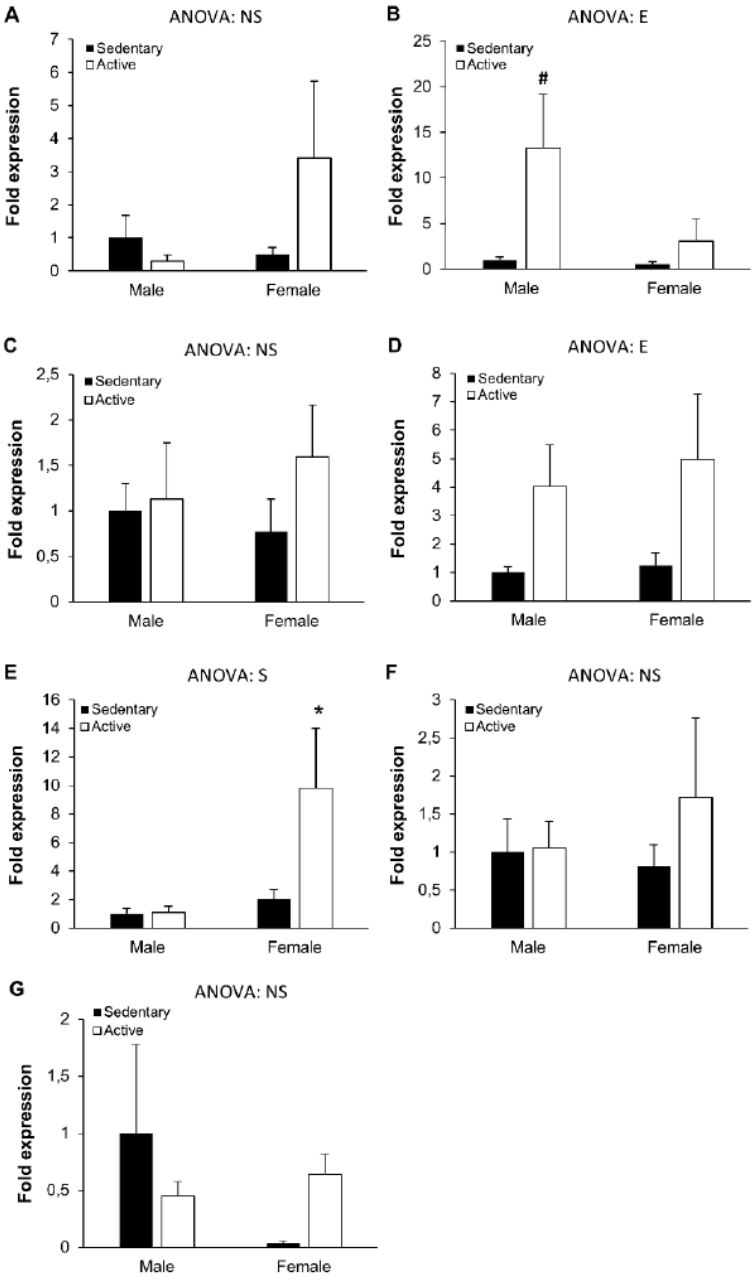


Figure 1. Peripheral blood mononuclear cells gene expression. (**A**) Interleukin(IL)-1 receptor antagonist, (**B**) IL-10, (**C**) IL-1β, (**D**) NF-κB, (**E**) TLR4, (**F**) TNFα, (**G**) IL-6. Results represent mean ± SEM. Statistical analysis: two-way ANOVA, $p < 0.05$. (S) effect of sex, (E) effect of exercise, (NS) non-significant. (*) Significant differences between sexes, (#) significant differences between sedentary and active groups.

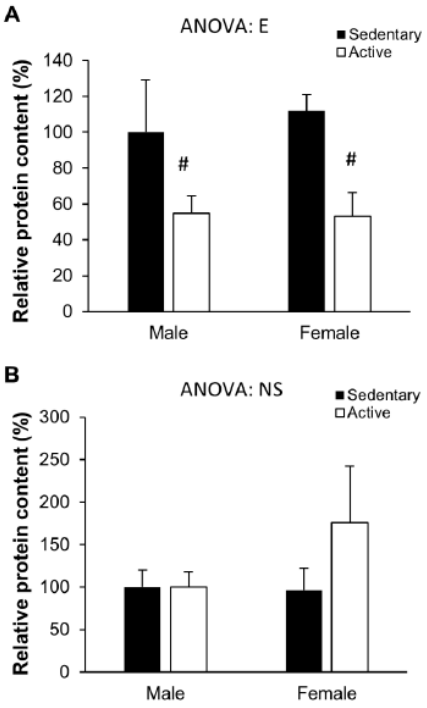


Figure 2. Peripheral blood mononuclear cells protein levels. (**A**) Tol-like receptor (TLR)2 and (**B**) TLR4. Results represent mean ± SEM. Statistical analysis: two-way ANOVA, $p < 0.05$. (E) effect of exercise. (#) Significant differences between sedentary and active groups.

A similar (but non-significant) tendency was also observed in TLR4 protein levels (Figure 2). Finally, TLR2 protein levels were affected by exercise, as evidenced by the significantly lower TLR2 protein levels in the PBMC of active vs sedentary participants (both in males and females).

4. Discussion

Aging has been associated with the functioning of the immune system, and more concretely with inflammatory responses. A chronic, low-grade inflammatory state, called inflamm-aging by some authors [14,21], has been proposed as being responsible for a progressive pro-inflammatory status, which appears to be a major characteristic of the aging process and age-related disease [29]. Therefore, the modulation of the inflammatory status throughout one's life might be an adequate strategy to attain healthy ageing. In this instance, the practice of physical activity has been proposed as a key component of healthy aging [4,30], and the benefits exerted by exercise might be attributable to the acquisition of an anti-inflammatory status. In the present study, we demonstrate that regular practice of physical activity exerts beneficial effects on body composition and the anti-inflammatory status of old people.

The physical activity performed by the participants was measured in the current study using the Minnesota Leisure-time Physical Activity Questionnaire, which had been previously validated for the Spanish old adult population [22,23], and the participants estimated the duration of the activities performed in hour/week by using metabolic equivalents of task (MET, defined as 1 kcal/kg/hour and equivalent to the energy cost of sitting quietly) [24]. The subjects in the first tercile (<82 MET-hours/week) were defined as sedentary, while the subjects in the third tercile (>84 MET-hours/week) were defined as active subjects. As expected by this classification, the active subjects performed around three-fold more physical activity than the sedentary subjects, both in the male and female groups. This regular practice of physical activity translated into a lower weight, a lower fat mass content, and a lower BMI. These results are in accordance with previous reports that

evidenced that the regular practice of physical activity by old people reduces fat mass and BMI and increases fat-free mass [31,32]. These effects on body composition were also accompanied by reduced diastolic blood pressure in the physically active subjects, as has been extensively reported in subjects performing aerobic exercise [33]. The effects of sex on the body composition were also evidenced, as women presented lower fat-free mass and higher fat mass than their male counterparts.

The practice of physical activity also had positive effects on the levels of HDL-cholesterol, which is in accordance with previous reports [34,35]. Lipid parameters were also affected by sex, with women presenting higher total cholesterol circulating levels, which were attributable to higher HDL-cholesterol. These results are in accordance with previous reports on different European populations showing that HDL-cholesterol circulating levels are higher in women than in men [36,37].

The active subjects presented a lower haematocrit than their sedentary counterparts, but similar values of red blood cells counts, haemoglobin levels, and mean corpuscular erythrocyte volume. The lower haematocrit was accompanied by a certain degree of leucopoenia. Although decreases in the number of circulant erythrocytes have been reported in response to acute bouts of physical activity [38], these changes are not always found in well-trained subjects [39], and even increases in the haematocrit and erythrocyte number have been reported in both amateur and professional sportsmen after maximal and submaximal tests and a cycling stage [40]. The leucopoenia found in the active participants of the current study was explained by lower counts of both neutrophils and lymphocytes and is in accordance with previous studies reporting a certain degree of leucopoenia in response to the regular practice of physical activity, which in turn is interpreted as part of an anti-inflammatory response [41].

The systemic inflammatory status of the participants in the study was evaluated through the measurement of circulating pro- and anti-inflammatory proteins and gene and protein expression of different cytokines in PBMCs. A decrease in the circulating levels of IL6 was observed in the active groups. Although IL6 can also exert anti-inflammatory activity (after an acute bout of exercise, IL-6 may induce the anti-inflammatory cytokines IL-10 and IL-1ra [42]), the presence of chronic circulating concentrations of IL6 can induce an acute phase immune response [43], and the regular practice of physical activity induces lower basal concentrations of IL-6 when compared to a sedentary lifestyle [44]. The plasma concentration of this interleukin has been associated with lower muscle mass [45] and higher adiposity [46], although in the present study we have not evidenced differences in fat-free mass. Although a reduction in IL6 levels was observed in the group of active volunteers, no changes in circulating TNF-α were evidenced. Gene expression of pro-inflammatory cytokines such as IL1β or TNF-α or the pro-inflammatory receptor TLR4 in PBMCs were not significantly affected by the practice of regular exercise. These results are in accordance with previous studies reporting that IL1β does not respond to different degrees of exercise, including low intensity aerobic exercise, high intensity aerobic exercise, or a combination of high intensity aerobic and resistance exercise [34]. Although no effects of physical activity were observed either on the gene expression of IL1ra, a significant activation of the anti-inflammatory cytokine IL10 gene expression was observed. Higher levels of IL10 in response to physical activity have been previously reported [34,35], and these increases have been actually related to a decrease in fat mass. In fact, the chronic inflammatory state has been related to the adiposity, and the influence of physical activity in body composition may therefore influence the inflammatory state [31,45,47,48]. The fact that anti-inflammatory cytokine concentrations are lowered in healthy adults over 50 years of age has also been associated with redistribution of body fat [20]. Our current results (lower fat mass and higher IL10 expression in active subjects) are in accordance with previous data and reinforce the anti-inflammatory effect of the regular practice of physical activity in old people.

A significant overexpression of the transcription factor NFκB was also observed in the physically active groups. This nuclear factor can be activated through the action of pro-inflammatory cytokines (such as TNFα), but it can also be activated by ROS and/or RNS [49]. Once activated, the nuclear factor migrates to the nucleus and may induce the expression of a wide variety of genes, including inflammatory cytokines such as TNFα, IL-6, and IL-1β [50,51], but also antioxidant enzymes such as

superoxide dismutase and nitric oxide synthase [52,53]. As the PBMC gene expression profile shows no evidence of a pro-inflammatory phenotype, we might interpret the activation of NFκB through the ROS/RNS route rather than a proinflammatory response. In this instance, the regular practice of physical activity exposes the organism to a sustained and continuous production of low levels of ROS, and these low levels of ROS have been shown to act as second messengers leading an antioxidant and anti-inflammatory response through the activation of NFκB and other genes [52,53].

The protein levels of the inflammation-related receptors TLR2 and TLR4 were also measured. While no effects were observed regarding TLR4 levels, a decrease in both sexes in the protein levels of TLR2 was observed. Activation of the TLR4 signalling pathway stimulates an increase in pro-inflammatory cytokines such as TNFα, IL1β, or IL6, and it has been previously described that physical activity may downregulate TLR4 expression in the immune cells [54,55], together with downstream cytokines such as TNFα [16], IL1β [34], and IL6 [18]. However, a recent study reported that neither TLR4 nor TNFα responded to resistance training with or without weight loss [32], which is in accordance with our own results. Although TLR4 is usually more sensitive TLR in response to physical activity, we observed a down-regulation of TLR2 in active subjects but not of TLR4. TLR2 is another member of the TLRs family that is also involved in the cell response to immune stimuli, and shares with TLR4 its downstream signalling cascade. A recent systematic review showed that chronic exercise has anti-inflammatory effects on the organism through the downregulation of both TLR2 and TLR4 at the protein and gene expression levels [56], which is in accordance with our results.

Taken together, our results show that the regular practice of physical activity by older adults ameliorates their anthropometric characteristics by reducing their weight, fat mass, and body mass index. This effect in their body composition is accompanied by a healthier status, with lower diastolic blood pressure and higher levels of circulating HDL-cholesterol. The changes in the fat body composition and lipid profile might be responsible for the observed attenuation of pro-inflammatory parameters, such as the reduced count of lymphocytes and neutrophils, reduced IL6 circulating levels, and the changes in the expression of pro- and anti-inflammatory proteins in PMBCs. In conclusion, the regular practice of physical activity (> 84 MET-hours/week) by older adults ameliorates their anti-inflammatory status.

Author Contributions: Data curation, C.B., S.C., and D.M.; Formal analysis, C.B., S.C., and D.M.; Investigation, M.D.F., X.C., M.M., C.B.-C., and A.S.; Methodology, A.S. and A.P.; Project administration, J.A.T. and A.P.; Resources, J.A.T. and A.P.; Supervision, J.A.T.; Writing—original draft, M.D.F. and A.P.; Writing—review & editing, X.C., M.M., C.B.-C., C.B., S.C., D.M., A.S., and J.A.T.

Funding: M.D.F. receives funding from Laboratoris Sanifit for an independent project not related to the content of this manuscript. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Acknowledgments: This work was supported by the Spanish Ministry of Economy and Competitiveness, Instituto de Salud Carlos III (Projects 11/01791 and 14/00636, Red Predimed-RETIC RD06/0045/1004, and CIBEROBN CB12/03/30038); Government of the Balearic Islands (grant numbers 35/2011 and AAEE26/2017), and European Union FEDER funds. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest: The authors declare no conflict of interests.

References

1. Tanaka, H.; Seals, D.R. Endurance exercise performance in Masters athletes: Age-associated changes and underlying physiological mechanisms. *J. Physiol.* **2008**, *586*, 55–63. [CrossRef] [PubMed]
2. Tosato, M.; Zamboni, V.; Ferrini, A.; Cesari, M. The aging process and potential interventions to extend life expectancy. *Clin. Interv. Aging* **2007**, *2*, 401–412. [PubMed]
3. Boirie, Y. Physiopathological mechanism of sarcopenia. *J. Nutr. Health Aging* **2009**, *13*, 717–723. [CrossRef] [PubMed]
4. Gremeaux, V.; Gayda, M.; Lepers, R.; Sosner, P.; Juneau, M.; Nigam, A. Exercise and longevity. *Maturitas* **2012**, *73*, 312–317. [CrossRef] [PubMed]

5. Franco, O.H.; de Laet, C.; Peeters, A.; Jonker, J.; Mackenbach, J.; Nusselder, W. Effects of physical activity on life expectancy with cardiovascular disease. *Arch. Intern. Med.* **2005**, *165*, 2355–2360. [CrossRef] [PubMed]
6. Lee, I.M.; Shiroma, E.J.; Lobelo, F.; Puska, P.; Blair, S.N.; Katzmarzyk, P.T. Effect of physical inactivity on major non-communicable diseases worldwide: An analysis of burden of disease and life expectancy. *Lancet* **2012**, *380*, 219–229. [CrossRef]
7. Wen, C.P.; Wai, J.P.; Tsai, M.K.; Yang, Y.C.; Cheng, T.Y.; Lee, M.C.; Chan, H.T.; Tsao, C.K.; Tsai, S.P.; Wu, X. Minimum amount of physical activity for reduced mortality and extended life expectancy: A prospective cohort study. *Lancet* **2011**, *378*, 1244–1253. [CrossRef]
8. Shephard, R.J.; Park, H.; Park, S.; Aoyagi, Y. Objectively measured physical activity and progressive loss of lean tissue in older Japanese adults: Longitudinal data from the Nakanojo study. *J. Am. Geriatr. Soc.* **2013**, *61*, 1887–1893. [CrossRef] [PubMed]
9. Timmerman, K.L.; Flynn, M.G.; Coen, P.M.; Markofski, M.M.; Pence, B.D. Exercise training-induced lowering of inflammatory (CD14+CD16+) monocytes: A role in the anti-inflammatory influence of exercise? *J. Leukoc. Biol.* **2008**, *84*, 1271–1278. [CrossRef] [PubMed]
10. Nelson, M.E.; Rejeski, W.J.; Blair, S.N.; Duncan, P.W.; Judge, J.O.; King, A.C.; Macera, C.A.; Castaneda-Sceppa, C. Physical activity and public health in older adults: Recommendation from the American College of Sports Medicine and the American Heart Association. *Circulation* **2007**, *116*, 1094–1105. [CrossRef] [PubMed]
11. Woods, J.A.; Wilund, K.R.; Martin, S.A.; Kistler, B.M. Exercise, inflammation and aging. *Aging Dis.* **2012**, *3*, 130–140. [PubMed]
12. Olesen, J.; Gliemann, L.; Bienso, R.; Schmidt, J.; Hellsten, Y.; Pilegaard, H. Exercise training, but not resveratrol, improves metabolic and inflammatory status in skeletal muscle of aged men. *J. Physiol.* **2014**, *592*, 1873–1886. [CrossRef] [PubMed]
13. Bastard, J.P.; Maachi, M.; Lagathu, C.; Kim, M.J.; Caron, M.; Vidal, H.; Capeau, J.; Feve, B. Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur. Cytokine Netw.* **2006**, *17*, 4–12. [PubMed]
14. Ferrucci, L.; Fabbri, E. Inflammageing: Chronic inflammation in ageing, cardiovascular disease, and frailty. *Nat. Rev. Cardiol.* **2018**, *15*, 505–522. [CrossRef] [PubMed]
15. Castaneda, C.; Gordon, P.L.; Parker, R.C.; Uhlin, K.L.; Roubenoff, R.; Levey, A.S. Resistance training to reduce the malnutrition-inflammation complex syndrome of chronic kidney disease. *Am. J. Kidney Dis.* **2004**, *43*, 607–616. [CrossRef] [PubMed]
16. Adamopoulos, S.; Parissis, J.; Karatzas, D.; Kroupis, C.; Georgiadis, M.; Karavolias, G.; Paraskevaidis, J.; Koniavitou, K.; Coats, A.J.; Kremastinos, D.T. Physical training modulates proinflammatory cytokines and the soluble Fas/soluble Fas ligand system in patients with chronic heart failure. *J. Am. Coll. Cardiol.* **2002**, *39*, 653–663. [CrossRef]
17. Kohut, M.L.; McCann, D.A.; Russell, D.W.; Konopka, D.N.; Cunnick, J.E.; Franke, W.D.; Castillo, M.C.; Reighard, A.E.; Vanderah, E. Aerobic exercise, but not flexibility/resistance exercise, reduces serum IL-18, CRP, and IL-6 independent of beta-blockers, BMI, and psychosocial factors in older adults. *Brain Behav. Immun.* **2006**, *20*, 201–209. [CrossRef] [PubMed]
18. Phillips, C.M.; Dillon, C.B.; Perry, I.J. Does replacing sedentary behaviour with light or moderate to vigorous physical activity modulate inflammatory status in adults? *Int. J. Behav. Nutr. Phys. Act.* **2017**, *14*, 138. [CrossRef] [PubMed]
19. Pararasa, C.; Ikwuobe, J.; Shigdar, S.; Boukouvalas, A.; Nabney, I.T.; Brown, J.E.; Devitt, A.; Bailey, C.J.; Bennett, S.J.; Griffiths, H.R. Age-associated changes in long-chain fatty acid profile during healthy aging promote pro-inflammatory monocyte polarization via PPARgamma. *Aging Cell* **2016**, *15*, 128–139. [CrossRef] [PubMed]
20. Pararasa, C.; Bailey, C.J.; Griffiths, H.R. Ageing, adipose tissue, fatty acids and inflammation. *Biogerontology* **2015**, *16*, 235–248. [CrossRef] [PubMed]
21. Franceschi, C.; Bonafe, M.; Valensin, S.; Olivieri, F.; De Luca, M.; Ottaviani, E.; De Benedictis, G. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann. N. Y. Acad. Sci.* **2000**, *908*, 244–254. [CrossRef] [PubMed]

22. Elosua, R.; Marrugat, J.; Molina, L.; Pons, S.; Pujol, E. Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. The MARATHOM Investigators. *Am. J. Epidemiol.* **1994**, *139*, 1197–1209. [CrossRef] [PubMed]
23. Elosua, R.; Garcia, M.; Aguilar, A.; Molina, L.; Covas, M.I.; Marrugat, J. Validation of the Minnesota leisure time physical activity questionnaire in Spanish women. Investigators of the MARATDON group. *Med. Sci. Sports. Exerc.* **2000**, *32*, 1431–1437. [CrossRef] [PubMed]
24. Conway, J.M.; Irwin, M.L.; Ainsworth, B.E. Estimating energy expenditure from the Minnesota leisure time physical activity and Tecumseh occupational activity questionnaires—A doubly labeled water validation. *J. Clin. Epidemiol.* **2002**, *55*, 392–399. [CrossRef]
25. Bibiloni, M.D.M.; Julibert, A.; Argelich, E.; Aparicio-Ugarriza, R.; Palacios, G.; Pons, A.; Gonzalez-Gross, M.; Tur, J.A. Western and Mediterranean dietary patterns and physical activity and fitness among Spanish older adults. *Nutrients* **2017**, *9*. [CrossRef] [PubMed]
26. Ferrer, M.D.; Sureda, A.; Batle, J.M.; Tauler, P.; Tur, J.A.; Pons, A. Scuba diving enhances endogenous antioxidant defenses in lymphocytes and neutrophils. *Free Radic. Res.* **2007**, *41*, 274–281. [CrossRef] [PubMed]
27. Sureda, A.; Batle, J.M.; Tauler, P.; Aguilo, A.; Cases, N.; Tur, J.A.; Pons, A. Hypoxia/reoxygenation and vitamin C intake influence NO synthesis and antioxidant defenses of neutrophils. *Free Radic. Biol. Med.* **2004**, *37*, 1744–1755. [CrossRef] [PubMed]
28. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
29. Franceschi, C.; Olivieri, F.; Marchegiani, F.; Cardelli, M.; Cavallone, L.; Capri, M.; Salvioli, S.; Valensin, S.; De Benedictis, G.; Di Iorio, A.; et al. Genes involved in immune response/inflammation, IGF1/insulin pathway and response to oxidative stress play a major role in the genetics of human longevity: The lesson of centenarians. *Mech. Ageing Dev.* **2005**, *126*, 351–361. [CrossRef] [PubMed]
30. Knight, E.; Petrella, R.J. Prescribing physical activity for healthy aging: Longitudinal follow-up and mixed method analysis of a primary care intervention. *Phys. Sportsmed.* **2014**, *42*, 30–38. [CrossRef] [PubMed]
31. Mendham, A.E.; Duffield, R.; Marino, F.; Coutts, A.J. Small-sided games training reduces CRP, IL-6 and leptin in sedentary, middle-aged men. *Eur. J. Appl. Physiol.* **2014**, *114*, 2289–2297. [CrossRef] [PubMed]
32. Markofski, M.M.; Flynn, M.G.; Carrillo, A.E.; Armstrong, C.L.; Campbell, W.W.; Sedlock, D.A. Resistance exercise training-induced decrease in circulating inflammatory CD14+CD16+ monocyte percentage without weight loss in older adults. *Eur. J. Appl. Physiol.* **2014**, *114*, 1737–1748. [CrossRef] [PubMed]
33. Pagonas, N.; Dimeo, F.; Bauer, F.; Seibert, F.; Kiziler, F.; Zidek, W.; Westhoff, T.H. The impact of aerobic exercise on blood pressure variability. *J. Hum. Hypertens.* **2014**, *28*, 367–371. [CrossRef] [PubMed]
34. Balducci, S.; Zanuso, S.; Nicolucci, A.; Fernando, F.; Cavallo, S.; Cardelli, P.; Fallucca, S.; Alessi, E.; Letizia, C.; Jimenez, A.; et al. Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20*, 608–617. [CrossRef] [PubMed]
35. Kadoglou, N.P.; Iliadis, F.; Angelopoulou, N.; Perrea, D.; Ampatzidis, G.; Liapis, C.D.; Alevizos, M. The anti-inflammatory effects of exercise training in patients with type 2 diabetes mellitus. *Eur. J. Cardiovasc. Prev. Rehabil.* **2007**, *14*, 837–843. [CrossRef] [PubMed]
36. Serra-Majem, L.; Pastor-Ferrer, M.C.; Castell, C.; Ribas-Barba, L.; Roman-Vinas, B.; Ribera, L.F.; Plasencia, A.; Salleras, L. Trends in blood lipids and fat soluble vitamins in Catalonia, Spain (1992–2003). *Public Health Nutr.* **2007**, *10*, 1379–1388. [CrossRef] [PubMed]
37. Soriano-Maldonado, A.; Aparicio, V.A.; Felix-Redondo, F.J.; Fernandez-Berges, D. Severity of obesity and cardiometabolic risk factors in adults: Sex differences and role of physical activity. The HERMEX study. *Int. J. Cardiol.* **2016**, *223*, 352–359. [CrossRef] [PubMed]
38. Sureda, A.; Tauler, P.; Aguilo, A.; Cases, N.; Fuentespina, E.; Cordova, A.; Tur, J.A.; Pons, A. Relation between oxidative stress markers and antioxidant endogenous defences during exhaustive exercise. *Free Radic. Res.* **2005**, *39*, 1317–1324. [CrossRef] [PubMed]
39. Tauler, P.; Aguilo, A.; Gimeno, I.; Fuentespina, E.; Tur, J.A.; Pons, A. Response of blood cell antioxidant enzyme defences to antioxidant diet supplementation and to intense exercise. *Eur. J. Nutr.* **2006**, *45*, 187–195. [CrossRef] [PubMed]

40. Tauler, P.; Aguilo, A.; Guix, P.; Jimenez, F.; Villa, G.; Tur, J.A.; Cordova, A.; Pons, A. Pre-exercise antioxidant enzyme activities determine the antioxidant enzyme erythrocyte response to exercise. *J. Sports Sci.* **2005**, *23*, 5–13. [CrossRef] [PubMed]
41. Moro-García, A.; Fernández-García, B.; Alonso-Arias, R.; Rodriguez-Alonso, M.; Suárez, M.; López-Larrea, C. Effects of maintained intense exercise throughout the life on the adaptive immune response in elderly and young athletes. *Br. J. Sports Med.* **2013**, *47*, e3. [CrossRef]
42. Steensberg, A.; Fischer, C.P.; Keller, C.; Moller, K.; Pedersen, B.K. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. *Am. J. Physiol. Endocrinol. Metab.* **2003**, *285*, E433–E437. [CrossRef] [PubMed]
43. Gleeson, M.; Bishop, N.C.; Stensel, D.J.; Lindley, M.R.; Mastana, S.S.; Nimmo, M.A. The anti-inflammatory effects of exercise: Mechanisms and implications for the prevention and treatment of disease. *Nat. Rev. Immunol.* **2011**, *11*, 607–615. [CrossRef] [PubMed]
44. Panagiotakos, D.B.; Pitsavos, C.; Chrysohoou, C.; Kavouras, S.; Stefanadis, C. The associations between leisure-time physical activity and inflammatory and coagulation markers related to cardiovascular disease: The ATTICA Study. *Prev. Med.* **2005**, *40*, 432–437. [CrossRef] [PubMed]
45. Visser, M.; Pahor, M.; Taaffe, D.R.; Goodpaster, B.H.; Simonsick, E.M.; Newman, A.B.; Nevitt, M.; Harris, T.B. Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: The Health ABC Study. *J. Gerontol. A Biol. Sci. Med. Sci.* **2002**, *57*, M326–332. [CrossRef] [PubMed]
46. Nikseresht, M. Comparison of Serum Cytokine Levels in Men Who are Obese or Men Who are Lean: Effects of Nonlinear Periodized Resistance Training and Obesity. *J. Strength Cond. Res.* **2018**, *32*, 1787–1795. [CrossRef] [PubMed]
47. Fried, S.K.; Bunkin, D.A.; Greenberg, A.S. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: Depot difference and regulation by glucocorticoid. *J. Clin. Endocrinol. Metab.* **1998**, *83*, 847–850. [CrossRef] [PubMed]
48. Manna, P.; Jain, S.K. Obesity, Oxidative Stress, Adipose Tissue Dysfunction, and the Associated Health Risks: Causes and Therapeutic Strategies. *Metab. Syndr. Relat. Disord.* **2015**, *13*, 423–444. [CrossRef] [PubMed]
49. Hoesel, B.; Schmid, J.A. The complexity of NF-κB signaling in inflammation and cancer. *Mol. Cancer* **2013**, *12*, 86. [CrossRef] [PubMed]
50. Khansari, N.; Shakiba, Y.; Mahmoudi, M. Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. *Recent Pat. Inflamm. Allergy Drug Discov.* **2009**, *3*, 73–80. [CrossRef] [PubMed]
51. Mendes, K.L.; Lelis, D.F.; Santos, S.H.S. Nuclear sirtuins and inflammatory signaling pathways. *Cytokine Growth Factor Rev.* **2017**, *38*, 98–105. [CrossRef] [PubMed]
52. Gomez-Cabrera, M.C.; Domenech, E.; Vina, J. Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. *Free Radic. Biol. Med.* **2008**, *44*, 126–131. [CrossRef] [PubMed]
53. Ji, L.L.; Gomez-Cabrera, M.C.; Vina, J. Role of nuclear factor κB and mitogen-activated protein kinase signaling in exercise-induced antioxidant enzyme adaptation. *Appl. Physiol. Nutr. Metab.* **2007**, *32*, 930–935. [CrossRef] [PubMed]
54. Flynn, M.G.; McFarlin, B.K.; Phillips, M.D.; Stewart, L.K.; Timmerman, K.L. Toll-like receptor 4 and CD14 mRNA expression are lower in resistive exercise-trained elderly women. *J. Appl. Physiol.* **2003**, *95*, 1833–1842. [CrossRef] [PubMed]
55. Stewart, L.K.; Flynn, M.G.; Campbell, W.W.; Craig, B.A.; Robinson, J.P.; McFarlin, B.K.; Timmerman, K.L.; Coen, P.M.; Felker, J.; Talbert, E. Influence of exercise training and age on CD14+ cell-surface expression of toll-like receptor 2 and 4. *Brain Behav. Immun.* **2005**, *19*, 389–397. [CrossRef] [PubMed]
56. Cavalcante, P.A.M.; Gregnani, M.F.; Henrique, J.S.; Ornellas, F.H.; Araujo, R.C. Aerobic but not Resistance Exercise Can Induce Inflammatory Pathways via Toll-Like 2 and 4: A Systematic Review. *Sports Med. Open* **2017**, *3*, 42. [CrossRef] [PubMed]

Manuscript V

Effects of hydrogen peroxide on inflammatory and redox gene expression in immune cells.
Carla-Busquets Cortés, Xavier Capó, Miquel Martorell, Josep A. Tur, Antoni Sureda, Antoni Pons.
2018. En vías de publicación.

nutrients

MDPI

Article

Effects of Hydrogen Peroxide on Inflammatory and Redox Gene Expression in Immune Cells from Humans with Metabolic Syndrome.

Carla Busquets-Cortés [1], Xavier Capó [1]; Emma Argelich [1], Miguel D. Ferrer [1], David Mateos [1], Cristina Bouzas [1], Manuela Abbate[1], Josep A. Tur [1,2], Antoni, Sureda [1,2] and Antoni Pons [1,2,*].

[1] Research Group on Community Nutrition and Oxidative Stress, Science Laboratory of Physical Activity, Department of Fundamental Biology and Health Sciences, University of Balearic Islands, 07122 Palma de Mallorca, Spain

[2] CIBER: CB12/03/30038 Fisiopatología de la Obesidad la Nutrición, CIBEROBN, Instituto de Salud Carlos III (ISCIII), University of Balearic Islands ,07122 Palma de Mallorca, Spain

* Correspondence: antonipons@uib.es; Tel.: +34 971173171

Received: date; Accepted: date; Published: date

Abstract: Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) can exert opposed effects depending on the dosage: low levels can be involved in signalling and adaptive processes, while higher levels can exert deleterious effects in cells and tissues. Our aim was to emulate a chronic *ex vivo* oxidative stress situation through a 2h exposure of immune cells to sustained H_2O_2 produced by glucose oxidase (GOX), at High or Low production rate, in order to determine dissimilar responses of peripheral blood mononuclear cells (PBMCs) and neutrophils on ROS and cytokine production, and mitochondrial dynamics related proteins, pro/anti-inflammatory and anti-oxidant gene expression. Immune cells were obtained from subjects with metabolic syndrome. H_2O_2 at low concentrations can trigger a transient anti-inflammatory adiponectin secretion and reduced gene expression of Toll-like receptors (TLRs) in PBMCs but may act as stimulator of proinflammatory genes (IL6, IL8) and mitochondrial dynamics related proteins (Mtf2, NRF2, Tfam). H_2O_2 at high concentration enhances the expression of pro-inflammatory genes (TLR2 and IL1β) and diminishes the expression of mitochondrial dynamics related proteins (Mtf1, Tfam) and antioxidant enzymes (Cu/Zn SOD) in PBMCs. The GOX treatments produce dissimilar changes in the immune cells: neutrophils were more resistant to H_2O_2 effects and exhibited a more constant response in terms of gene expression than PBMCs. We observe emerging roles of H_2O_2 on mitochondrial dynamics, redox and inflammation processes in immune cells.

Keywords: gene expression; glucose oxidase; hydrogen peroxide; inflammation; mitochondrial biogenesis; neutrophils; PBMCs; ROS

1. Introduction

Metabolic syndrome is a constellation of metabolic abnormalities including hypertension, hyperglycaemia, abdominal fatness and dyslipidemia. Patients suffering from metabolic syndrome exhibit evident redox imbalance and inflammatory status that affect the cellular responses and subclasses of immune cells [1–4]. Peripheral blood leukocyte subclasses from metabolic syndrome patients have different phenotype compared to non-obese patients, which include clinical biomarkers that largely reflect already manifested comorbidities [5], and they are correlated with subcutaneous adipose tissue macrophages participating in the adipose tissue inflammation [6]. Obesity, a feature of metabolic syndrome, is commonly associated with chronic low grade inflammation with permanently increased levels of reactive oxygen species (ROS) [7–9], such as hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), and hypochlorous acid (HClO) [10]. Concretely H_2O_2, due to its relative stability, mild chemical reactivity, and ability to diffuse [11], is an important metabolite involved in sensing, modulation and signalling of redox metabolism reactions and processes in the cells [12]. Mounting evidence establishes that these H_2O_2–dependent signalling events are triggered by the activation of diverse cell surface receptors [13]. H_2O_2 exhibits the ability to specifically oxidise proteins [14–16] that activate cascades of downstream responses that lead to various metabolic outputs in the cells [17–21]. According to the downstream pathways that are triggered, homeostatic, pathological or protective cascades can be activated [22]. The physiological redox signalling is characterized by reversible oxido-reductive modifications [23] of specific cysteine residues, as thiolate anion (Cys-S-), susceptible of oxidation. The two-electron oxidation of a thiolate by H_2O_2 yields sulfenic acid, which is implicated in a number of important biochemical transformations. Cysteines from several transcription factors (e.g. NF-κB), or proteins involved in cell signalling or metabolism (e.g. GAPDH, glutathione reductase, tyrosine phospatases, kinases, and proteases) can be turned into sulfenic acid [24–26]. The reaction of ROS with protein thiols provides a mechanism by which cells can "sense" changes in the redox balance. Understanding the molecular mechanisms and the dosage by which H_2O_2 exerts the beneficial/adaptive responses more likely than the negative/pathological ones has far-reaching practical significance since several pathological states including as metabolic syndrome are characterized by altered redox biology [16].

To ascertain a role of cellular oxidants in physiological and pathological processes, it is imperative to be able to distinguish and characterize those species involved and monitor its levels in the biological system of interest [27]. Most phenotypic and signalling studies of cellular responses to H_2O_2 are designed using extracellular addition, either with a bolus or with sustained generation using glucose oxidase [19]. Peripheral blood mononuclear cells (PBMCs), consisting in T cells, B cells and monocytes, provide an ideal culture model for the study of inflammation and immunity. Neutrophils also serve as

77 cellular models as well considering their various anti-pathogen functions, among others,
78 in inflammation environment. We aim to emulate a chronic *ex vivo* oxidative stress
79 situation through the continuous exposure of immune cells, from patients with metabolic
80 syndrome, to H_2O_2 produced by glucose oxidase (GOX). The experimental procedure is
81 designed to induce an oxidative stress status in order to distinguish those situations in
82 which H_2O_2 generates oxidative imbalance or promotes beneficial responses. We wanted to
83 bring to light the genes whose expression is upregulated when immune cells are exposed
84 to different H_2O_2 concentrations. Furthermore, we aimed to evidence the effects of H_2O_2 on
85 the expression on the ROS and the cytokine production.

86

87 2. Materials and Methods

88 *Study design*

89 The present study is a cross-sectional analysis on baseline samples within the frame
90 of the PREDIMED-Plus study, a 6-year multicentre, parallel-group, randomised trial
91 conducted in Spain to assess the effect on cardiovascular disease morbimortality of an
92 intensive weight loss intervention programme based on an energy-restricted traditional
93 MedDiet (erMedDiet), physical activity promotion and behavioural support, in
94 comparison with an usual care intervention only with energy-unrestricted MedDiet
95 (control group). Details on the study protocol can be found at http://predimedplus.com/.
96 The trial was registered in 2014 at the International Standard Randomized Controlled Trial
97 (ISRCT; http://www.isrctn.com/ISRCTN89898870) with number 89898870.

98

99 *Participants' characteristics*

100 A total of 34 participants from the randomized, multicenter, clinical trial with
101 parallel groups with metabolic syndrome (PREDIMED-Plus) were enrolled into this study.
102 Inclusion criteria included men aged 55-75 and women between 60-75 years old, with a
103 BMI ≥27 and <40 kg/m^2, that meet at least 3 criteria for the Metabolic Syndrome according
104 to the updated harmonized criteria of the International Diabetes Federation and the
105 American Heart Association and National Heart, Lung and Blood Institute [28]. Criteria
106 exclusion were (a) inability or unwillingness to give informed consent, (b) documented
107 history of previous cardiovascular disease, (c) active cancer or a history of malignant
108 tumours in the last 5 years, (d) Impossibility to follow recommended diet or to carry out
109 physical activity. All participants were informed of the purpose and the implications of the
110 study, and informed consent was obtained from all subjects. All participants were studied
111 at the beginning of the trial; no intervention was yet applied to these subjects. The study

was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the Research Ethics Committee of the Balearic Islands (reference no. 2251/14 PI).

Cell isolation and cell viability test

Venous blood samples were obtained from the antecubital vein of participants in the study in suitable vacutainers with EDTA as anticoagulant in fasting conditions. Cell counts were determined in an automatic flow cytometer analyzer Technicon H2 (Bayer, Leverkusen, Germany) VCS system. The PBMC and neutrophil fractions were purified from fresh whole blood and isolated following the protocol described previously [29] using Ficoll-Paque PLUS reagent (GE Healthcare, Chalfont St Giles, UK) [30,31]. Briefly, 6ml of blood was carefully introduced on 4 ml of Ficoll (proportion of 1.5:1) and was then centrifuged at 900g, at 4 °C for 30 min. The plasma and the Ficoll phases were discarded and PBMCs layer was washed twice with phosphate-buffered saline (PBS), pH 7.4., and centrifuged for 10 min at 1000g, 4°C. The precipitate containing the erythrocytes and neutrophils was incubated at 4°C with 0.15 mol/L of ammonium chloride to haemolyse the erythrocytes. The suspension was centrifuged at 750g, at 4°C for 10 min and the supernatant was then discarded. The neutrophil phase at the bottom was washed first with ammonium chloride and then with PBS. Cell lysates for RNA purification were stored at −80 °C with 1 ml of Tripure Isolation Reagent® (Roche Diagnostics, Mannheim, Germany) until biochemical analysis, while a fresh aliquot was for ROS determination. Cell viability was assessed using crystal violet nuclear staining assay [32]. Violet dye binds to proteins and DNA of living cells. Cells that undergo cell death lose their adherence to culture surface and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture. Briefly, 20 µl of 0.5% crystal violet solution in 30% acetic acid were added to 500 µl of suspension of cells and incubate for 10 minutes at room temperature. Cells were centrifuged (1000g, 10 min) and washed thrice with PBS, until the dye stopped coming off. 100 µl of ethanol were added and all the volume was then transferred onto a 96-well microplate and absorbance at 570 nm was recorded in a microplate reader (Bio-Tek Instruments, Inc.)

Cell treatments and experimental design

All treatments were performed in 2 ml sterilized tubes containing $3 \cdot 10^6$ PBMCs/ml and $3.5 \cdot 10^6$ neutrophils/ml. Cells were resuspended in 2ml of RPMI-1640 medium with glucose (2g/L), L-glutamine and $NaHCO_3$. Cells were incubated at 37ºC in a humidified atmosphere for 2h in absence and in the presence of two different concentrations (High and Low) of glucose glucose oxidase (GOX), following a procedure previously described

[33,34]. Lyophilized glucose oxidase (GOX) type X-S from *Aspergillus niger* (~75% protein, 138,370 U/g solid, Sigma-Aldrich) was used to generate H_2O_2 (one unit of GOX oxidizes 1.0 µmole of β-D-glucose to D-glucono-δ-lactone and H_2O_2 per min at pH 5.1 at 35 °C, equivalent to an O_2 uptake of 22.4 µl per min, according to manufacturer's instructions). GOX was added at concentrations 1 and 0.1 µg solid/ml culture medium (High and Low treatment, respectively) in PBMCs. GOX was added at concentrations 15 and 5 µg solid/ml culture medium (High and Low treatment, respectively) in neutrophils.

Calibration of H_2O_2 to cell exposure

The calibration of H_2O_2 exposition was monitored colorimetrically in PBMCs and neutrophils cultured in presence of High or Low GOX activities. PBMCs were incubated as indicated above in presence of High (1 µg solid/ml culture medium) or Low (0.1 µg solid/ml culture medium) GOX amounts. Neutrophils were incubated as indicated above in presence of High (15 µg solid/ml culture medium) or Low (5 µg solid/ml culture medium) GOX amounts. Aliquots of cell culture were taken at different incubation times, and deproteinized with acetone [35,36] at 4ºC in order to stop the H_2O_2 production by the enzyme. The H_2O_2 level were determined in the deproteinitzation supernatants (centrifugation conditions: 900g at 4º for 5 min) by using horseradish peroxidase and tetramethylbenzidine (TMB) and measuring the absorbance at 450 nm [33,37]. H_2O_2 concentration was calculated with a standard curve of known concentration. Figure 1 summarizes the process to attain steady state of H_2O_2 used in our experimental model. The steady state is a consequence of the extracellular production of hydrogen peroxide by glucose oxidase in the presence of glucose, and of the elimination of H_2O_2 through the enzymatic mechanisms within the neutrophils and PBMCs. When the steady state is attained, the rate of production and elimination is equal.

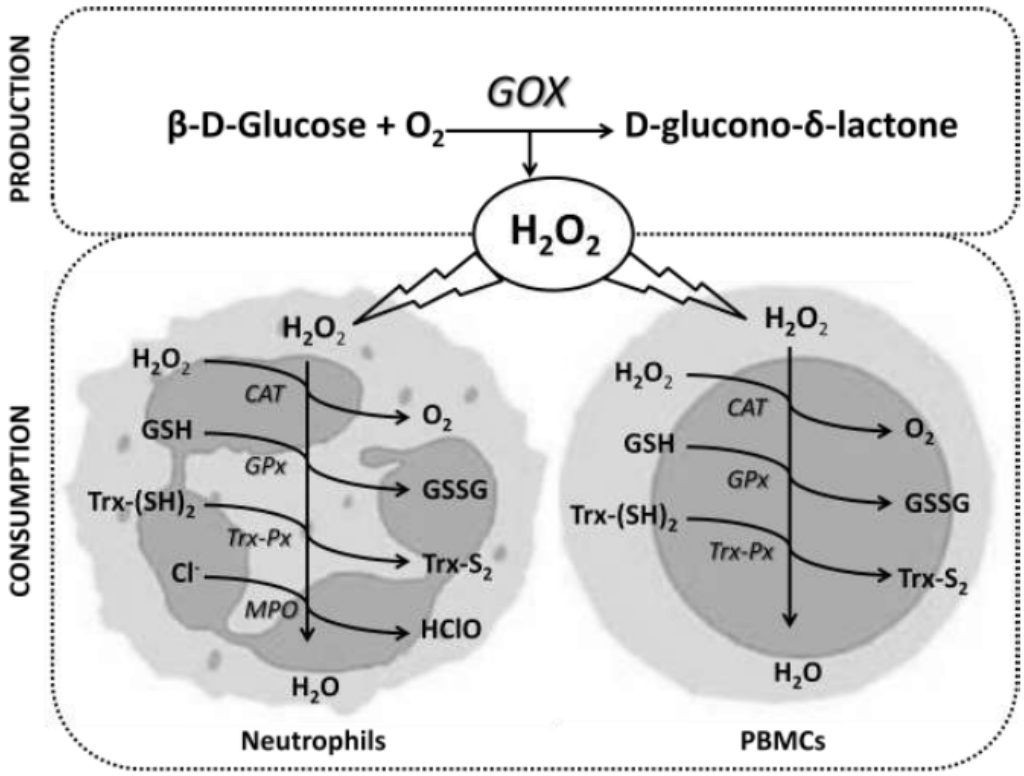


Figure 1. Balance between hydrogen peroxide production and elimination: H_2O_2 extracellularly produced by glucose oxidase in the presence of glucose can be intracellularly decomposed by numerous antioxidant systems within the immune cells. GOX: glucose oxidase; CAT: Catalase; GPx: glutathione peroxidase; TRx-Px: Thioredoxin peroxidase; MPO: myeloperoxidase; GSH: reduced glutathione; GSSG: oxidized glutathione; Trx(SH)2: oxidized thioredoxin; Trx-S2:reduced thioredoxin; Cl-: chloride anion; HClO: hypochlorous acid.

Stimulated PBMCs and neutrophils H_2O_2 *production*

After the 2h incubation at 37ºC, h H_2O_2 production by PBMCs and neutrophils was measured using 2,7- dichlorofluorescin-diacetate (DCFH-DA) as indicator [38,39]. A stock solution of DCFH-DA (2.05 mM) in ethanol was prepared and stored at -20°C until analysis. Hanks' Balanced Salts Medium was prepared in a relation of 9.8 g/L water. H_2O_2 production in PBMCs and neutrophils was measured before and after stimulation with lipopolysaccharide (LPS) (100 µg/mL PBS) from *Escherichia coli* (Sigma-Aldrich) or Zymosan A (ZYM) (1 mg/ml PBS) from *Saccharomyces cerevisiae* (Sigma-Aldrich). A total of 50 µl of LPS or ZYM prepared in PBS was added to the wells. DCFH-DA in ethanol was diluted in Hanks' Balanced Salts Medium (relation 30 µl DCFH-DA/ml Hanks') and added to a 96-well microplate containing 50 µl of cell suspension (containing about 6 x 10^5 cells). The fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37°C for 60 min in FLx800

Microplate Fluorescence Reader (Bio-tek Instruments, Inc.) by punctual ultraviolet light exposures and emission readings were recorded every minute (60 total readings).

RNA isolation and mRNA gene expression

mRNA expressions were determined by Real Time PCR (RT-PCR) based on incorporation of a fluorescent reporter dye and using human 18S ribosomal as the reference gene [40]. For this purpose, total mRNA from PBMCs and neutrophils was isolated by extraction with Tripure Isolation Reagent® (Roche Diagnostics, Germany) following a procedure previously described [41,42]. cDNA was synthesized from 1 µg total RNA using 50 units of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol of oligo (dT) for 60 min at 37°C in a final volume of 10 µL, according to the manufacturer's instructions. Semiquantitative PCR of the resulting cDNA (3 µL) was amplified using the LightCycler® 480 instrument (Roche Diagnostics) with FastStart DNA MasterPLUS SYBR™ Green I kit (Roche Diagnostics, Germany). The specific primers and amplification conditions used for each gene are presented in Table 1. Target cDNAs were amplified as follows: one cycle at 95 °C for 10 min, followed by 45 cycles of amplification (95ºC for 10 s; specific annealing temperature for 10 s; 72ºC for 15 s). mRNA levels in the control situation (no glucose oxidase in the medium) were arbitrarily referred to as 1.

Table 1. Primer sequence and annealing temperatures used for the real-time PCR.

Gene	Primer	Temp
18S	Fw: 5'-GACTCAACACGGGAAACCCTCAC-3' Rv: 5'-GACTCAACACGGGAAACCCTCAC-3'	60°C
COX2	Fw: 5′-TTGCCTGGCAGGGTTGCTGGTGGTA-3′ Rv: 5′-CATCTGCCTGCTCTGGTCAATGGAA-3′	67°C
CAT	Fw: 5′-TTT GGC TAC TTT GAG GTC AC-3′ Rv: 5′-TCC CCA TTT GCA TTA ACC AG-3′	60°C
TNFα	Fw: 5′-CCCAGGCAGTCAGATCATCTTCTCGGAA-3′ Rv: 5′-CTGGTTATCTCTCAGCTCCACGCCATT-3′	63°C
IL6	Fw: 5′-TACATCCTCGACGGCATCTC-3′ Rv: 5′-ACTCATCTGCACAGCTCTGG-3′	63°C
IL1β	Fw: 5′-GGACAGGATATGGAGCAACA-3′ Rv: 5′-GGCAGACTCAAATTCCAGCT-3′	58°C
IL8	Fw: 5′-GCTCTGTGTGAAGGTGCAGTTTTGCCAA-3′ Rv: 5′-TGAACATGGGGAGTGTTTCA-3′	63°C
NFkB	Fw: 5′-AAACACTGTGAGGATGGGATCTG-3′ Rv: 5′-CGAAGCCGACCACCATGT-3′	60°C
IL10	Fw: 5′-AGAACCTGAAGACCCTCAGGC-3′ Rv: 5′-CCACGGCCTTGCTCTTGTT-3′	58°C

Mfn1	Fw: 5'-TGTTTTGGTCGCAAACTCTG-3' Rv: 5'-CTGTCTGCGTACGTCTTCCA-3'	60°C
Mfn2	Fw: 5'-ATGCATCCCCACTTAAGCAC-3' Rv: 5'-CCAGAGGGCAGAACTTTGTC-3'	60°C
Tfam	Fw: 5′-CAAGACAGATGAAACCACCTC-3′ Rv: 5′-AGATTGGGGTCGGGTCACT-3′	60°C
NRF2	Fw: 5′-GCGACGGAAAGAGTATGAGC-3′ Rv: 5′-GTTGGCAGATCCACTGGTTT-3′	60°C
TLR2	Fw: 5′-GGGTTGGAAGCACTGGACAAT-3′ Rv: 5′-TTCTTCCTTGGAGAGGCTGA-3′	55°C
TLR4	Fw: 5′-GGTCACCTTTTCTTGATTCCA-3′ Rv: 5′-TCAGAGGTCCATCAAACATCAC-3′	55°C
SOD Cu/Zn	Fw: 5'-TCA GGA GAC CAT TGC ATC ATT-3' Rv: 5'-CGC TTT CCT GTC TTT GTA CTT TCT TC-3'	63ºC
SOD Mn	Fw: 5′-GAGAAGGTACCAGGAGGCGTTG-3′ Rv: CAAGCCAACCCCAACCTGAGC-3′	64°C

18S, Ribosomal 18S; COX2, Cyclooxigenase 2; CAT, catalase; TNFα, Tumor Necrosis Factor alpha; IL6, Interleukin 6; IL1β, Interleukin 1β; IL8, Interleukin 8; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL10, Interleukin 10; Mfn1, Mitofusin 1; Mfn2, Mitofusin 2; Tfam, transcription factor A, mitocondrial; NRF2, Nuclear Respiratory Factor 2; TLR2, Toll-like Receptor 2; TLR4, Toll-like Receptor 4; SOD Cu/Zn, copper/zinc superoxide dismutase; SOD Mn, manganese superoxide dismutase.

Cytokine determination

Adiponectin, IL6 and and TNFα from cell incubation supernatants were determined using individual Human High Sensitivity ELISA kits (Diaclone, Besaçon, Cedex, France) following the manufacturer's instructions. Intra-assay and inter-assay reproducibility were <5% and <10%, respectively. The rate of cytokine production was calculated by its determination in the cell culture after 2 h of incubation. The amount of cytokine present divided by the counts of PBMCs and by time of incubation was considered the rate of cytokine production (pg/min · 10^6).

Statistical analysis

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS v.21.0 for Windows). Results in figures and tables are expressed as mean ± SEM and *p values* < 0.05 were considered statistically significant. Student's t test for paired data was used to determine the effects of the GOX treatments (High GOX treatments with respect to control, and low GOX treatments with respect to control) in gene expression and cytokine production. One-way ANOVA was performed to determine differences in H_2O_2 production between groups and cell viability between treatments.

3. Results

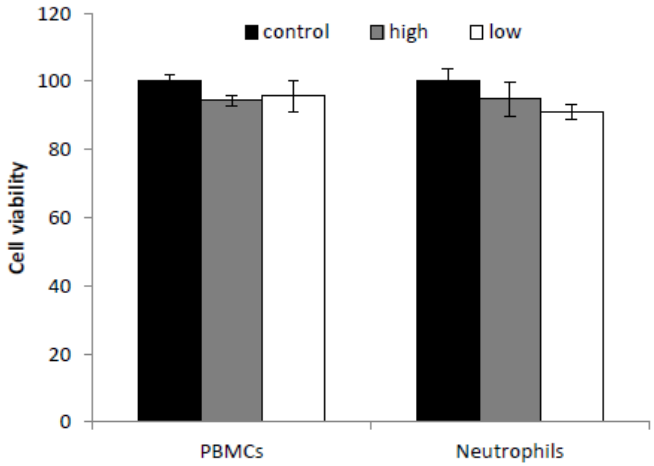
Participants in the study were 34 men and women with documented metabolic syndrome. Anthropometric and haematological characteristics are shown in Table 2. The participants were obese (BMI higher than 30) with values of glucose, cholesterol and triglycerides borderline to pathological cut off values. The blood cell counts were into the normal distribution of healthy subjects.

Table 2. Anthropometric and haematological characteristics of the participants.

	(N = 34)	Reference value
Age (years)	64.2 ± 0.7	-
Weight (kg)	85.5 ± 1.9	-
Height (height)	164.3 ± 1.4	-
Waist circumference (cm)	107.5 ± 1.3	-
BMI (kg/m2)	31.6 ± 0.5	-
Glucose (mg/dL)	112.6 ± 3.1	76- 110
Triglycerydes (mg/dL)	155.9 ± 9.1	10-150
Total cholesterol (mg/dL)	198.8 ± 6.1	<200
PBMCs (10^3cells/mm^3)	3.1 ± 0.2	-
Lymphocytes (10^3cells/mm^3)	2.5 ± 0.1	1-5
Monocytes (10^3cells/mm^3)	0.6 ± 0.01	0-0.8
Neutrophils (10^3cells/mm^3)	3.9 ± 0.3	1.8-7.7

Cell viability after GOX addition, and consequent H_2O_2 generation in the culture medium, was assessed. Results are shown in Figure 2. No significant changes were observed in cell viability when cells were treated with High and Low GOX concentrations with respect to control conditions (no GOX present in the medium).

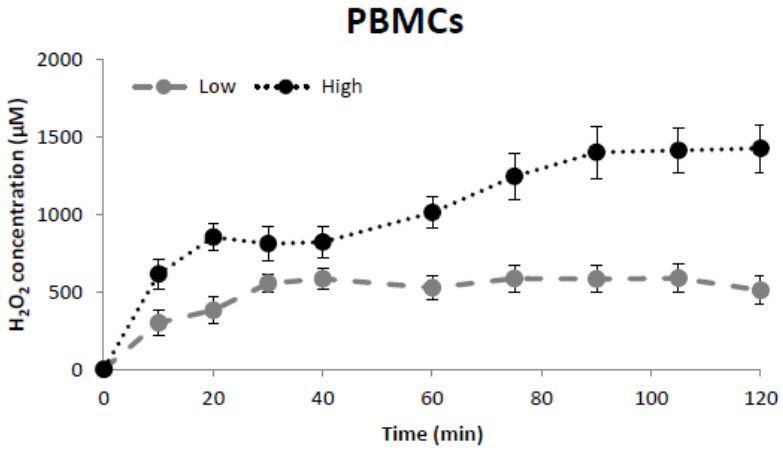
266 **Figure 2. PBMCs and neutrophils viability after 2 hours of exposure to High and**
267 **Low dosage of H_2O_2 at 37ºC.**
268



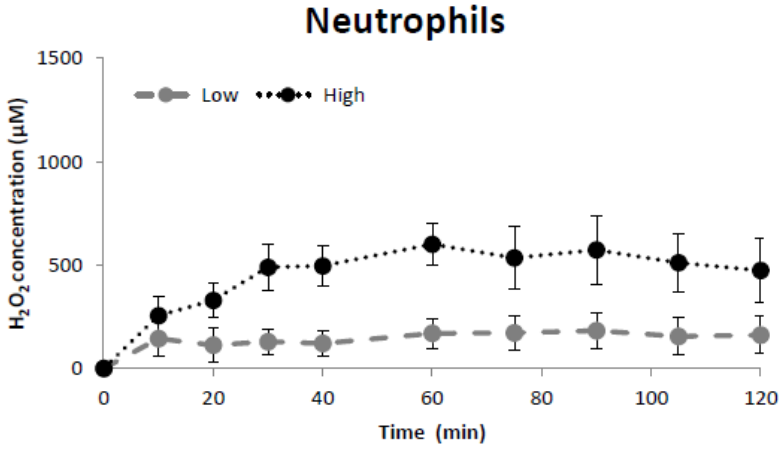
269
270 *Statistical analysis: One way ANOVA, $p < 0.05$. PBMCs: 1 and 0.1 µg solid GOX/ml*
271 *(High and Low, respectively). Neutrophils: 15 and 5 µg solid GOX/ml (High and Low,*
272 *respectively) respectively. Control: no GOX added.*
273

274 Figure 3 shows the progression at different times of H_2O_2 levels in the immune cells
275 culture medium after the addition of High and Low GOX. The cell counts in the cellular
276 culture were the same as in blood (3.1±0.2 10^3PBMCs/mm^3, and 3.9±0.3
277 10^3neutrophils/mm^3). Concentration of H_2O_2 in the culture medium reaches the steady
278 state levels at different times and levels depending on the dosage of GOX applied and on
279 the cell type. Neutrophils reach the steady state after 30 minutes of High GOX addition,
280 while PBMCs it takes almost 70 minutes. The steady state was attained after 10 minutes by
281 neutrophils and after 30 minutes by PBMCs in the case of Low GOX exposure. The H_2O_2
282 steady state levels were different for neutrophils and for PBMCs at Low and High GOX.
283 The stationary state H_2O_2 levels were 1400 ± 154 µM with High GOX concentration (1 µg
284 solid GOX/ml culture media) in PBMCs whereas the steady state levels for neutrophils
285 with High GOX concentration (15 µg solid GOX/ml culture medium) were 500 ± 50 µM.
286 The stationary state H_2O_2 levels were 500 ± 75 µM with Low GOX concentration (0.1 µg
287 solid GOX/ml culture medium) in PBMCs whereas the steady state levels for neutrophils
288 with Low GOX concentration (5 µg solid GOX/ml culture medium) was 180 ± 40 µM.
289
290
291

292 **Figure 3. Levels of H_2O_2 in the culture medium of PBMCs and neutrophils after**
293 **High and Low H_2O_2 exposure by GOX addition for 2 h at 37°C.**



294



295

296 *PBMCs: High indicates H_2O_2 production rate by 1 µg solid GOX/ml culture medium and*
297 *Low indicates H_2O_2 production rate by 0.1 µg solid GOX/ml culture medium.*
298 *Neutrophils: High indicates H_2O_2 production rate by 15 µg solid GOX/ml culture medium*
299 *and Low indicates H_2O_2 production rate by 5 µg solid GOX/ml culture medium.*

300

301 The continuous H_2O_2 production at high or low rate by GOX addition to the culture
302 medium significantly influenced the PBMCs and neutrophils H_2O_2 production following
303 LPS or ZYM stimulation (Table 3). Neutrophils produced H_2O_2 on cellular basis at higher
304 rate than PBMCs, under both LPS and ZYM stimuli and even previously or after the
305 treatment with High or Low H_2O_2 (significances not shown). H_2O_2 production rate was
306 higher under ZYM stimuli than LPS stimuli, in both PBMCs and neutrophils and
307 independently of the H_2O_2 concentration (significances not shown). Previous incubation of

PBMCs with high or low GOX dosages significantly decreased the rate of H_2O_2 production after ZYM but not after LPS stimulation. No significant effects were found when neutrophils previously incubated in the presence of GOX were stimulated with ZYM. However, when LPS was used as stimulus, High GOX concentration exposure significantly augmented H_2O_2 production with respect to control.

Table 3. H_2O_2 production by PBMCs and neutrophils stimulated with LPS or ZYM after High and Low H_2O_2 treatments

			Control	High	Low
RLU/min/ 10^6 cells	PBMCs	ZYM	44.3 ± 6.5	29.7 ± 4.6*	34.7 ± 5.8$
		LPS	12.9 ± 5.4	18.4 ± 4.5	17.7 ± 6.3
	Neutrophils	ZYM	128 ± 19	128 ± 17	129 ± 15
		LPS	35.4 ± 4.8	99.9 ± 14.8*	56.1 ± 11.2

High and Low indicates high and low rate of H_2O_2 production by high and low level of Glucose oxidase in the medium. Statistical analysis: One way ANOVA, $p < 0.05$. (*) Significant effects between High treatment and control; ($) significant effects between Low treatment and control; (#) Significant effects between High treatment and Low treatment. LPS: lipopolysaccharide; ZYM: zymosan.

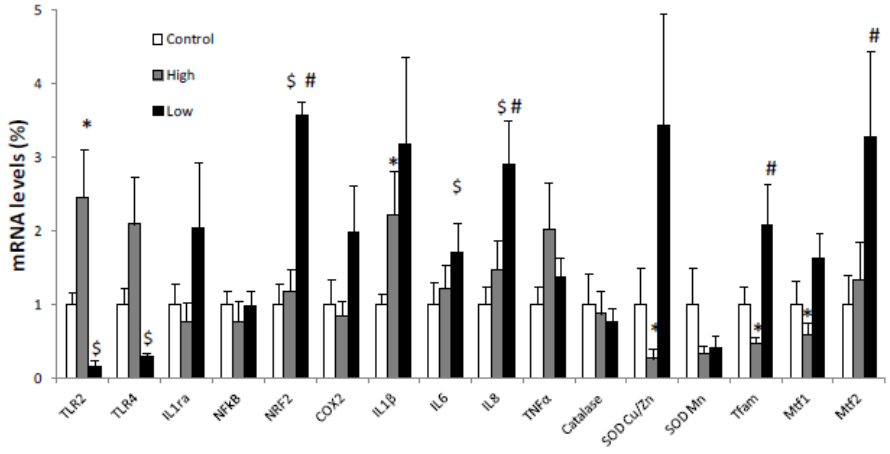
The effects of 2 hours of incubation with GOX on the production of cytokines by PBMCs and neutrophils were analyzed (Table 4). The rate of adiponectin production in PBMCs [43] significantly increased only after High GOX dosage. On the contrary, both GOX treatments provoked a significant decrease in IL-6 production, while TNFα levels remained constant independently of the concentration of GOX applied studied in PBMCs. In neutrophils, no adiponectin production was detected at all, confirming the inability of this cellular type to synthesize this adipokine. IL-6 and TNFα production remained changeless under H_2O_2 exposure in neutrophils.

Table. 4 Cytokine levels in supernatants of PBMCs and neutrophils samples after 2-h continuous exposure to High and Low GOX treatments.

	(pg/min · 10^6)	Control	High	Low
PBMCs	Adiponectin	61.1 ± 11.9	92.1 ± 14.7*	83.6 ± 15.7
	IL-6	116 ± 51	12.7 ± 4.7*	38.8 ± 24.6$
	TNFα	1587 ± 220	1643 ± 264	1506 ± 172
Neutrophils	Adiponectin	ND	ND	ND
	IL-6	38.3 ± 11.41	31.1 ± 8.6	46.8 ± 15.7
	TNFα	374 ± 90	382 ± 112	406 ± 64.6

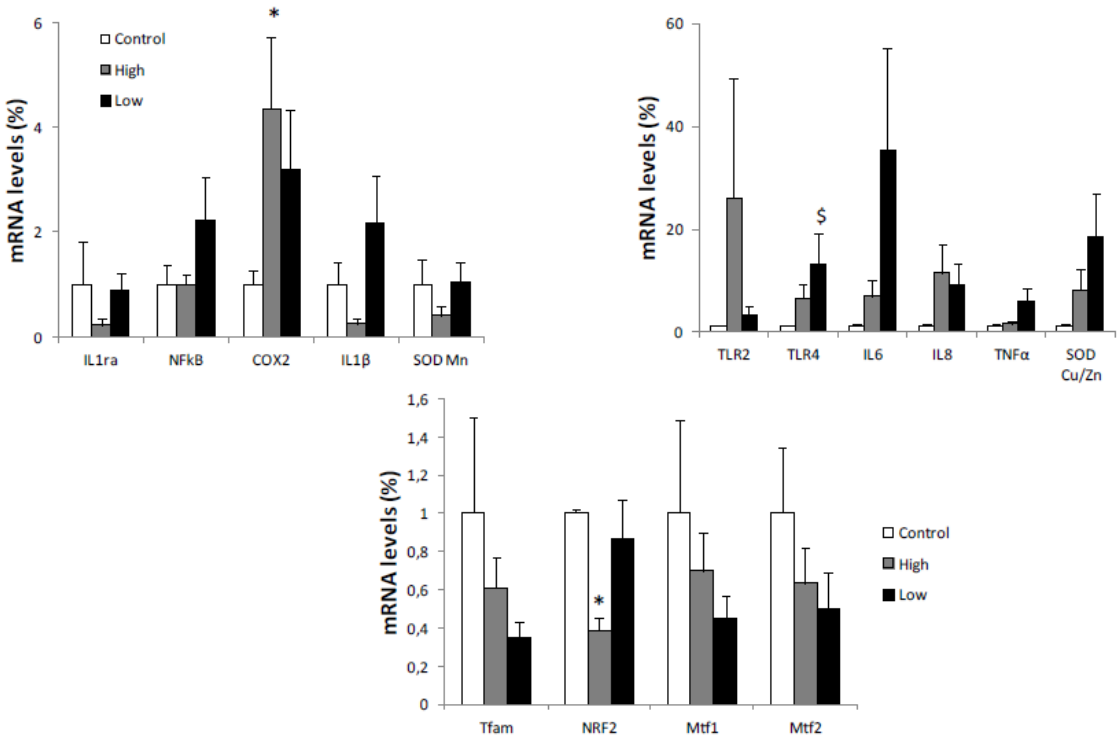
335 *High and Low indicates high and low rate of H_2O_2 production by high and low level of Glucose*
336 *oxidase in the medium. Statistical analysis: Student's t test for paired data, $p<0.05$. (*) Significant*
337 *effects between High treatment and control; ($) significant effects between Low treatment and*
338 *control. ND: non-detected.*
339
340 Changes in gene expression in PBMCs and neutrophils were studied after
341 continuous exposure to two different concentrations of H_2O_2 produced by two different
342 dosages of GOX (Figures 3 and 4). The expression of genes encoding immune and
343 inflammatory-related proteins (COX2, NFkB, TNFα, IL6, IL1β, IL1α, IL8, TLR2, TLR4),
344 antioxidant enzymes (CAT, SOD Cu/Zn, SOD Mn) and mitochondrial dynamics related
345 proteins (Mtf1, Mtf2, Tfam and NRF2) were analysed.
346 In PBMCs (Figure 4) High H_2O_2 exposure significantly increased the expression of
347 immune and inflammatory-related proteins as IL1β and TLR2, while it provoked a
348 decrease in the expression of SOD Cu/Zn, Mtf1 and Tfam. Low H_2O_2 exposure promoted a
349 raise in the expression of mitochondrial proteins Tfam, NRF2 and Mtf2 and
350 proinflammatory mediators IL6 and IL8, while it diminished the expression of receptors
351 TLR2 and TLR4. Significant differences between the two high and low GOX treatments
352 were also detected in IL8, NRF2 and Tfam that increased their mRNA expression in Low
353 with respect to High GOX treatments.
354 In neutrophils (Figure 5), the continuous production of high or low extracellular
355 H_2O_2 exerted lower influence than in PBMCs on the expression of inflammatory,
356 mitochondrial dynamics and antioxidant genes. In fact, Low and High rate of H_2O_2
357 production maintained the control expression of inflammatory genes as NFkB, TNFα,
358 IL1rα, IL1β, IL6, IL8, TLR2, and the control expression of antioxidant and mitochondrial
359 dynamics genes as SOD Cu/Zn, SODMn, Mtf1, Mtf2 and Tfam. Only the expression of
360 COX2, TLR4 and NRF2 evidenced significant influence of the exposition to High or Low
361 H_2O_2 production in neutrophils. High H_2O_2 production significantly increased the COX2
362 expression but decreased the expression of NRF2, whereas the Low H_2O_2 production
363 significantly increased the expression of TLR4 in neutrophils.
364
365
366
367

370 **Figure 4. Relative gene expression of pro/anti-inflammatory proteins, antioxidant**
371 **enzymes and mitochondrial dynamics related proteins in PBMCs.**
372



373
374 *Ribosomal 18S as a reference. Control was arbitrarily referred to as 1. (control: only cells present in*
375 *the medium; High: 1 µg solid GOX/ml; Low: 0,1 µg solid GOX/ml). Statistical analysis: Student's*
376 ***t test** for paired data, $p < 0.05$. (*) Significant effects between High treatment and control; ($)*
377 *significant effects between Low treatment and control; (#) Significant effects between High*
378 *treatment and Low treatment.*
379
380
381
382
383
384
385
386
387
388
389
390
391

Figure 5. Relative expression of pro/anti-inflammatory proteins, antioxidant enzymes and mitochondrial dynamics related proteins in neutrophils.



Ribosomal 18S as a reference (control: only cells present in the medium; High: 15 µg solid GOX/ml; Low: 5 µg solid GOX/ml). Statistical analysis: Student's t test for paired data, $p < 0.05$. (*)Significant effects between High treatment and control; ($) significant effects between Low treatment and control; (#) Significant effects between High treatment and Low treatment.

4. Discussion

A relevant feature of this study is that neutrophils, in spite of being stimulated with higher GOX concentrations than PBMCs, apparently exhibit a steady state of H_2O_2 levels three-time lower than PBMCs during exposure to both GOX concentrations. Furthermore, neutrophils reach the steady state three times faster than PBMCs. These facts enlighten a higher capacity of neutrophils to eliminate H_2O_2 than PBMCs [44]. The method developed is useful to maintain cells to an extracellular sustained production of H_2O_2 and to reach a steady state of H_2O_2 levels in the culture media in less than 2 hours.

Optimal level of inflammation is required for immunity enhancement while chronic inflammation is associated with several metabolic disorders like type 2 diabetes, overweigh and obesity. Indeed, the latter is characterized by chronic low grade inflammation with permanently increased oxidative stress [7,8,45,46]. This pathological condition normally leads to low levels of plasmatic adiponectin (with anti-inflammatory properties) and high levels of TNFα and IL6 (pro-inflammatory) [47]. Adiponectin is one of the most abundantly secreted adipose tissue proteins that is negatively correlated with obesity; the expression of the anti-inflammatory adiponectin is reduced in adipose tissue of obese individuals compared to lean individuals [48,49]. Previous research showed that adiponectin can reduce the secretion of markers involved in the activation of NFκB including TNF-α, IL-6 by adipocytes and macrophages through TLRs [50]. We observed that IL6 production decreases while the adiponectin secretion concurrently increases as a result of exposure to H_2O_2 in PBMCs. This fact could be related to direct or indirect anti-inflammatory proprieties of H_2O_2 [51–53]. We also evidence that PBMCs secrete adiponectin, as documented before, [43] and it is increased by the different GOX treatments.

The observed increase in H_2O_2 production by immune cells after immunostimulatory exposure could be a multifactorial response derived from the increased cellular metabolism induced by the immune activation, and it is probably cell type-dependent. The higher rate of production of H_2O_2 observed in neutrophils than in PBMCs after LPS or ZYM stimulation, could be consequence of the two mitochondrial and lysosomal pathways of oxidant species generation in neutrophils [54–56] versus the only mitochondrial pathway in PBMCs [57,58]. LPS exerts its action mainly through interaction with TLR4 [59] whereas ZYM directly binds TLR2 [60]. The H_2O_2 production is higher under ZYM stimuli than LPS stimuli, in both PBMCs and neutrophils, likely indicating a higher reception by TLR2 than the TLR4 way. However, the effects of the GOX treatments on the expression of TLR2 and TLR4 in PBMC and neutrophils are not coincident with the effects of GOX treatments on H_2O_2 production after immune stimulation. It has been indicated the possibility to internalize the receptor TLR4 into the membrane in a physiological situation with high H_2O_2 production rate, such as acute physical activity [61], thus being the TLRs less available for immunostimulators recognition. The observed effects after the LPS and ZYM immune stimulation in terms of ROS production could also be attributable to an action subsequent to the interaction of immunostimulators with TLRs receptors such as the activation of NFκβ pathway or even at mitochondrial or lisosomal levels [62].

The results obtained reveal that the 2h exposure of PBMCs and neutrophils to different levels H_2O_2 produced by High and Low dosage of GOX from patients with metabolic syndrome induce dissimilar changes in these cellular types, with a more

resistance of neutrophils to H_2O_2 effects in terms of gene expression than PBMCs. In PBMCs, expression of proinflammatory proteins IL6, IL8 and IL1β augment after the 2h incubation with GOX; this pattern in PBMCs suggests that chronic exposure to H_2O_2, either high or low production rate, promotes a transient inflammatory response in immune cells. Mtf2, a mediator of mitochondrial fusion, also exhibits increased expression after H_2O_2 incubation, pointing out an emerging role of mitochondrial dynamics related proteins in inflammation processes. Indeed, it has been reported that Mtf2 interacts with NLRP3 and enhances inflammasome activation, a multiproteic complex responsible for activation for inflammatory processes [63]. Mounting evidence involve Mtf2 in multiple signalling pathways not only restricted to the regulation of mitochondrial shape [64].

The H_2O_2 exposure at Low GOX dosage activates the expression of Mtf2 and Tfam in PBMCs and puts in evidence the induction of mitochondrial remodelling and biogenesis by H_2O_2. Conversely, high H_2O_2 exposure reduces the gene expression of Mtf1 and Tfam in PBMCs. The mitochondrial biogenesis might be considered as an antioxidant mechanism to avoid oxidative stress and also a protective quality-control process for mitochondria [65–67], being mitochondria one of the main sites of ROS production. Mitochondrial fusion and fission in conjunction with mitochondrial autophagy preserve and control organelle quality [68].

The antioxidant enzymatic mechanisms are apparently unaffected in neutrophils and lightly inhibited after H_2O_2 exposure in PBMCs. The effects of H_2O_2 on the antioxidant enzymes expression are dependent of the dosage of H_2O_2 [33]. PBMCs exhibit a decrease in the expression of Cu/Zn SOD at high H_2O_2 exposure while the other antioxidant enzymes are not affected. Neutrophils seem to be more resistant to the effects of H_2O_2 on the gene expression of antioxidant enzyme and mitochondrial proteins than PBMCs. Neutrophils are more resistant cells towards oxidative damage than PBMCs [69–71]; in fact, we had to apply higher glucose oxidase concentrations to these cell type to induce oxidative stress. The treatment with High GOX reduces the expression of NRF2 in neutrophils. NRF2, also called GABPA [72], functions as a transcription factor that activates the expression of some key metabolic genes regulating cellular growth and nuclear genes required for mitochondrial respiration as well as mitochondrial biogenesis, DNA transcription and replication [73–75]. The decreased NRF2 expression induced by high H_2O_2 exposure could influence mitochondrial respiration in neutrophils. In addition, the treatment with high H_2O_2 increases the expression of COX2, an inducible enzyme responsible for synthesizing pro-inflammatory prostaglandins from araquidonic acid and resolving-inflammation molecules as resolvins from eicosapentanoic and docosahexaenoic acid [76,77]. This fact put in evidence a possible pro-inflammatory effect of exposure to high levels of H_2O_2 in neutrophils.

To sum up, the dosage of exposure to of H_2O_2 seems to represent a key variable that influences the side of the double-edge role of this oxidant specie at any given moment: different H_2O_2 extracellular levels influence the pro/anti-inflammatory, pro/antioxidant and mitochondrial status in the cells. Therefore, a close control of these levels may have medically relevance in terms of diagnosis/prognosis of diseases with altered inflammatory and oxidative status as metabolic syndrome.

5. Conclusions

Neutrophils exhibit higher capacity than PBMCs to eliminate H_2O_2 extracellularly produced by glucose oxidase in a medium with glucose. The exogenous H_2O_2 exposure of immune cells from patients with metabolic syndrome induce dissimilar changes in these cellular types, with a more resistance of neutrophils to H_2O_2 effects in terms of gene expression than PBMCs. Indeed, H_2O_2, constantly produced (e.g. for 2 h) and *ex vivo* controlled, triggers a transient anti-inflammatory adipokine secretion in PBMCs but acts as a genetic stimulator of proinflammatory genes in both PBMCs and neutrophils. Antioxidant defences are downregulated by high H_2O_2 levels in PBMCs but cushioned in neutrophils. H_2O_2 influences on mitochondrial dynamics related protein gene expression. At low production rate, H_2O_2 promotes biogenesis and remodelling mitochondria which might be considered as a hormetic protective quality-control process towards oxidative stress, meanwhile at high H_2O_2 production rate induces the downregulation of mitochondrial biogenesis and structural remodelling. A close control of H_2O_2 levels may have medically relevance in pathological processes with altered inflammatory and oxidative status as metabolic syndrome.

Author Contributions: "A.S., A.P. and JA.T. conceived and designed the experiments; C.B., X.C., M.M., MD.F., D.M., E.A. and E.A. performed the experiments; C.B., A.S. and A.P. analyzed the data; C.B., A.S. and A.P. wrote the paper. All the authors revised and approved the final draft of the manuscript".

Funding and acknowledgements:
The PREDIMED-Plus trial was supported by the official funding agency for biomedical research of the Spanish government, Institute of Health Carlos III (ISCIII) through the Fondo de Investigación para la Salud (FIS), which is co-funded by the European Regional Development Fund (Projects 14/00636 and 17/01827, Red Predimed-RETIC RD06/0045/1004, and CIBEROBN CB12/03/30038), Grant of support to research groups no. 35/2011, AAEE01/2017 and AAEE26/2017 (Balearic

519 Islands Gov.), and EU Cost ACTION CA16112. The authors hereby acknowledge
520 the PhD grant provided by the University of the Balearic Island to Xavier Capó and
521 the FPI/1648/2014 grant provided by Conselleria d'Educació, Cultura i
522 Universitats, Direcció General d'Universitats i Recerca, Govern de les Illes Balears
523 (Fondo Social Europeo) to Carla Busquets-Cortés. The funders had no role in study
524 design, data collection and analysis, decision to publish, or preparation of the
525 manuscript.

526

527 **Conflicts of Interest**: The authors declare no conflict of interest.

528

529 References

530 [1] Khaybullina Z. Inflammation and Oxidative Stress: Critical Role for Metabolic
531 Syndrome. J Vasc Med Surg 2017;05:1–3. doi:10.4172/2329-6925.1000302.

532 [2] Holvoet P, P H. Relations between metabolic syndrome, oxidative stress and
533 inflammation and cardiovascular disease. Verh K Acad Geneeskd Belg 2008;70:193–
534 219.

535 [3] Devaraj S, Goyal R, Jialal I. Inflammation, Oxidative Stress, and the Metabolic
536 Syndrome. US Endocrinol 2008;04:32. doi:10.17925/USE.2008.04.2.32.

537 [4] Carrier A. Metabolic Syndrome and Oxidative Stress: A Complex Relationship.
538 Antioxid Redox Signal 2017;26:429–31. doi:10.1089/ars.2016.6929.

539 [5] Pecht T, Gutman-Tirosh A, Bashan N, Rudich A. Peripheral blood leucocyte
540 subclasses as potential biomarkers of adipose tissue inflammation and obesity
541 subphenotypes in humans. Obes Rev 2014;15:322–37. doi:10.1111/obr.12133.

542 [6] Powell LA, Crowe P, Kankara C, McPeake J, McCance DR, Young IS, et al.
543 Restoration of adipose function in obese glucose-tolerant men following
544 pioglitazone treatment is associated with CCAAT enhancer-binding protein β up-
545 regulation. Clin Sci 2012;123:135–46. doi:10.1042/CS20110662.

546 [7] Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity & inflammation: The
547 linking mechanism & the complications. Arch Med Sci 2017;13:851–63.
548 doi:10.5114/aoms.2016.58928.

549 [8] Marseglia L, Manti S, D'Angelo G, Nicotera A, Parisi E, Di Rosa G, et al. Oxidative
550 stress in obesity: A critical component in human diseases. Int J Mol Sci 2015;16:378–
551 400. doi:10.3390/ijms16010378.

552 [9] McMurray F, Patten DA, Harper ME. Reactive Oxygen Species and Oxidative Stress
553 in Obesity—Recent Findings and Empirical Approaches. Obesity 2016;24:2301–10.
554 doi:10.1002/oby.21654.

555 [10] Mittal M, Siddiqui M, Tran K, Reddy S, Malik A. Reactive Oxygen Species in
556 Inflammation and Tissue Injury. Antioxid Redox Signal 2014;20:1126–67.
557 doi:10.1089/ars.2012.5149.

558 [11] Li L, Kang C, Zhang Y. Exercise-induced hormesis and skeletal muscle health. Free
559 Radic Biol Med 2016;98:113–22. doi:10.1016/j.freeradbiomed.2016.02.025.

560 [12] Di Marzo N, Chisci E, Giovannoni R, Di Marzo N, Chisci E, Giovannoni R. The Role
561 of Hydrogen Peroxide in Redox-Dependent Signaling: Homeostatic and
562 Pathological Responses in Mammalian Cells. Cells 2018;7:156.
563 doi:10.3390/cells7100156.

564 [13] Rhee SG. Cellular Regulation by Hydrogen Peroxide. J Am Soc Nephrol
565 2003;14:211S – 215. doi:10.1097/01.ASN.0000077404.45564.7E.

566 [14] Y BH, Veronique M, Hua L. Hydrogen peroxide in the human body 2000;486:14–7.

567 [15] Burdon RH, Rice-Evans C. Free radicals and the regulation of mammalian cell
568 proliferation. Free Radic Res Commun 1989;6:345–58.
569 doi:10.3109/10715768909087918.

570 [16] Huang BK, Sikes HD. Quantifying intracellular hydrogen peroxide perturbations in
571 terms of concentration. Redox Biol 2014;2:955–62. doi:10.1016/j.redox.2014.08.001.

572 [17] Sies H. Hydrogen peroxide as a central redox signaling molecule in physiological
573 oxidative stress: Oxidative eustress. Redox Biol 2017;11:613–9.
574 doi:10.1016/j.redox.2016.12.035.

575 [18] Ludovico P, Burhans WC. Reactive oxygen species, ageing and the hormesis police.
576 FEMS Yeast Res 2014;14:33–9. doi:10.1111/1567-1364.12070.

577 [19] Di Meo S, Reed TT, Venditti P, Victor VM. Harmful and Beneficial Role of ROS.
578 Oxid Med Cell Longev 2016;2016:1–3. doi:10.1155/2016/7909186.

579 [20] Aldosari S, Awad M, Harrington E, Sellke F, Abid M. Subcellular Reactive Oxygen
580 Species (ROS) in Cardiovascular Pathophysiology. Antioxidants 2018;7:14.
581 doi:10.3390/antiox7010014.

582 [21] Castro JP, Grune T, Speckmann B. The two faces of reactive oxygen species (ROS) in
583 adipocyte function and dysfunction. Biol Chem 2016;397:709–24. doi:10.1515/hsz-
584 2015-0305.

585 [22] Marinho, Susana H, Real C, Cyrne L, Soares H, Antunes F. Redox Biology
586 Hydrogen peroxide sensing , signaling and regulation of transcription factors
587 2014;2:535–62. doi:10.1016/j.redox.2014.02.006.

588 [23] Antunes F, Brito PM. Quantitative biology of hydrogen peroxide signaling. Redox
589 Biol 2017;13:1–7. doi:10.1016/j.redox.2017.04.039.

590 [24] Paulsen C. Peroxide-dependent sulfenylation of the EGFR catalytic site enhances
591 kinase activity. Nat Chem Biol 2012;8:57–64. doi:10.1038/nchembio.736.Peroxide-
592 dependent.

593 [25] Pan J, Carroll KS. Chemical Biology Approaches to Study Protein Cysteine
594 Sulfenylation. Biopolymers 2014;101:165–72. doi:10.1016/j.pmrj.2014.02.014.Lumbar.

595 [26] Paulsen CE, Carroll KS. Cysteine-Mediated Redox Signaling: Chemistry, Biology,
596 and Tools for Discovery. Am Chem Soc 2012.

597 [27] Zielonka J, Kalyanaraman B. Small-molecule luminescent probes for the detection of
598 cellular oxidizing and nitrating species. Free Radic Biol Med 2018;128:3–22.
599 doi:10.1016/j.freeradbiomed.2018.03.032.

600 [28] Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al.
601 Harmonizing the metabolic syndrome: A joint interim statement of the international
602 diabetes federation task force on epidemiology and prevention; National heart,
603 lung, and blood institute; American heart association; World heart federation;
604 International . Circulation 2009;120:1640–5.
605 doi:10.1161/CIRCULATIONAHA.109.192644.

606 [29] Boyum A. Separation of white blood cells. Nature 1964;204:793–4.

607 [30] Mestre-Alfaro A, Ferrer MD, Sureda A, Tauler P, Marttínez E, Bibiloni MM, et al.
608 Phytoestrogens enhance antioxidant enzymes after swimming exercise and
609 modulate sex hormone plasma levels in female swimmers. Eur J Appl Physiol
610 2011;111:2281–94. doi:10.1007/s00421-011-1862-y.

611 [31] Busquets-Cortés C, Capó X, Martorell M, Tur JA, Sureda A, Pons A. Training
612 Enhances Immune Cells Mitochondrial Biosynthesis, Fission, Fusion, and Their
613 Antioxidant Capabilities Synergistically with Dietary Docosahexaenoic
614 Supplementation. Oxid Med Cell Longev 2016;2016:2016:8950384.
615 doi:10.1155/2016/8950384.

616 [32] Feoktistova M, Geserick P, Leverkus M. Crystal Violet Assay for Determining
617 Viability of Cultured Cells. Cold Spring Harb Protoc 2016;2016:pdb.prot087379.
618 doi:10.1101/pdb.prot087379.

619 [33] Ferrer MD, Sureda A, Mestre A, Tur J a, Pons A. The Double Edge of Reactive
620 Oxygen Species as Damaging and Signaling Molecules in HL60 Cell Culture. Cell
621 Physiol Biochem 2010;25:241–52.

622 [34] Sureda A, Hebling U, Pons A, Mueller S, Sureda A, Hebling U, et al. Extracellular H
623 2 O 2 and not superoxide determines the compartment-specific activation of
624 transferrin receptor by iron regulatory protein 1 2005;5762.
625 doi:10.1080/10715760500164045.

626 [35] Sedgwick GW, Fenton TW, Thompson JR. Effect of Protein Precipitating Agents on

627 the Recovery of Plasma-Free Amino-Acids. Can Jounal Anim Sci 1991;71:953–7.
628 doi:10.4141/cjas91-116.

629 [36] Arola L, Herrera E, Alemany M. A new method for deproteinization of small
630 samples of blood plasma for amino acid determination. Anal Biochem 1977;82:236–
631 9. doi:10.1016/0003-2697(77)90153-1.

632 [37] McArdle F, Spiers S, Aldemir H, Vasilaki A, Beaver A, Iwanejko L, et al.
633 Preconditioning of skeletal muscle against contraction-induced damage: The role of
634 adaptations to oxidants in mice. J Physiol 2004;561:233–44.
635 doi:10.1113/jphysiol.2004.069914.

636 [38] Capó X, Martorell M, Sureda A, Llompart I, Tur J a., Pons A. Diet supplementation
637 with DHA-enriched food in football players during training season enhances the
638 mitochondrial antioxidant capabilities in blood mononuclear cells. Eur J Nutr
639 2014;54:35–49. doi:10.1007/s00394-014-0683-2.

640 [39] Ferrer MD, Sureda A, Batle JM, Tauler P, Tur JA, Pons A. Scuba diving enhances
641 endogenous antioxidant defenses in lymphocytes and neutrophils. Free Radic Res
642 2007;41:274–81. doi:10.1080/10715760601080371.

643 [40] Rapacz M. Reference genes in real-time PCR 2013:391–406. doi:10.1007/s13353-013-
644 0173-x.

645 [41] Capó X, Martorell M, Sureda A, Batle JM, Tur JA, Pons A. Docosahexaenoic diet
646 supplementation, exercise and temperature affect cytokine production by
647 lipopolysaccharide-stimulated mononuclear cells. J Physiol Biochem 2016;72(3):421–
648 34. doi:10.1007/s13105-016-0490-8.

649 [42] Capó X, Martorell M, Sureda A, Tur JA, Pons A. Effects of dietary Docosahexaenoic,
650 training and acute exercise on lipid mediators. J Int Soc Sports Nutr 2016;13:16.
651 doi:10.1186/s12970-016-0126-y.

652 [43] Crawford LJ a, Peake R, Price S, Morris TCM, Irvine AE. Adiponectin is produced
653 by lymphocytes and is a negative regulator of granulopoiesis. J Leukoc Biol
654 2010;88:807–11. doi:10.1189/jlb.1109723.

655 [44] Pantopoulos K, Schipper H. Principles of Free Radical Biomedicine. Vol I. 2014.

656 [45] Saltiel AR, Olefsky JM. Inflammatory mechanisms linking obesity and metabolic
657 disease. J Clin Investig 2017;127:4–7. doi:10.1172/JCI92035.systems.

658 [46] Monteiro R, Azevedo I. Chronic inflammation in obesity and the metabolic
659 syndrome. Mediators Inflamm 2010;2010. doi:10.1155/2010/289645.

660 [47] Upadhyaya S, Kadamkode V, Mahammed R, Doraiswami C, Banerjee G.
661 Adiponectin and IL-6: Mediators of inflammation in progression of healthy to type
662 2 diabetes in Indian population. Adipocyte 2014;3:39–45. doi:10.4161/adip.26553.

663 [48] Yukio Arita, Shinji Kihara, Noriyuki Ouchi et all. Paradoxical Decrease of an
664 Adipose-Specific Protein,.pdf 1999;83:5.

665 [49] Kang YE, Kim JM, Joung KH, Lee JH, You BR, Choi MJ, et al. The roles of
666 adipokines, proinflammatory cytokines, and adipose tissue macrophages in obesity-
667 associated insulin resistance in modest obesity and early metabolic dysfunction.
668 PLoS One 2016;11:1–14. doi:10.1371/journal.pone.0154003.

669 [50] Sargolzaei J, Chamani E, Kazemi T, Fallah S, Soori H. The role of adiponectin and
670 adipolin as anti-inflammatory adipokines in the formation of macrophage foam
671 cells and their association with cardiovascular diseases. Clin Biochem 2018;54:1–10.
672 doi:10.1016/j.clinbiochem.2018.02.008.

673 [51] Banerjee S, Zmijewski JW, Lorne E, Liu G, Sha Y, Abraham E. Modulation of SCFβ-
674 TrCP-dependent IκBα ubiquitination by hydrogen peroxide. J Biol Chem
675 2010;285:2665–75. doi:10.1074/jbc.M109.060822.

676 [52] Zmijewski JW, Lorne E, Zhao X, Tsuruta Y, Sha Y, Liu G, et al. Antiinflammatory
677 effects of hydrogen peroxide in neutrophil activation and acute lung injury. Am J
678 Respir Crit Care Med 2009;179:694–704. doi:10.1164/rccm.200806-851OC.

679 [53] Essick EE, Wilson RM, Pimentel DR, Shimano M, Baid S, Ouchi N, et al.
680 Adiponectin Modulates Oxidative Stress-Induced Autophagy in Cardiomyocytes.
681 PLoS One 2013;8:1–13. doi:10.1371/journal.pone.0068697.

682 [54] Gomez-Cabrera MC, Salvador-Pascual A, Cabo H, Ferrando B, Vina J. Redox
683 modulation of mitochondriogenesis in exercise. Does antioxidant supplementation
684 blunt the benefits of exercise training? Free Radic Biol Med 2015;86:37–46.
685 doi:10.1016/j.freeradbiomed.2015.04.006.

686 [55] Robinson JM. Phagocytic leukocytes and reactive oxygen species. Histochem Cell
687 Biol 2009;131:465–9. doi:10.1007/s00418-009-0565-5.

688 [56] Segal AW. Europe PMC Funders Group How Neutrophils Kill Microbes. Annu Rev
689 Immunol 2007;2. doi:10.1146/annurev.immunol.23.021704.115653.How.

690 [57] Dan Dunn J, Alvarez LAJ, Zhang X, Soldati T. Reactive oxygen species and
691 mitochondria: A nexus of cellular homeostasis. Redox Biol 2015;6:472–85.
692 doi:10.1016/j.redox.2015.09.005.

693 [58] Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial Reactive Oxygen Species (ROS)
694 and ROS-Induced ROS Release. Physiol Rev 2014;94:909–50.
695 doi:10.1152/physrev.00026.2013.

696 [59] Pålsson-McDermott EM, O'Neill LAJ. Signal transduction by the lipopolysaccharide
697 receptor, Toll-like receptor-4. Immunology 2004;113:153–62. doi:10.1111/j.1365-
698 2567.2004.01976.x.

699 [60] Sato M, Sano H, Iwaki D, Kudo K, Konishi M, Takahashi H, et al. Direct Binding of
700 Toll-Like Receptor 2 to Zymosan, and Zymosan-Induced NF- B Activation and
701 TNF- Secretion Are Down-Regulated by Lung Collectin Surfactant Protein A. J
702 Immunol 2003;171:417–25. doi:10.4049/jimmunol.171.1.417.

703 [61] Gleeson M, McFarlin B, Flynn M. Exercise and Toll-like receptors. Exerc Immunol
704 Rev 2006;12:34–53.

705 [62] Tanaka SI, Isoda F, Ishihara Y, Kimura M, Yamakawa T. T lymphopaenia in relation
706 to body mass index and TNF-α in human obesity: Adequate weight reduction can
707 be corrective. Clin Endocrinol (Oxf) 2001;54:347–54. doi:10.1046/j.1365-
708 2265.2001.1139/cn2155.x.

709 [63] Ichinohe T, Yamazaki T, Koshiba T, Yanagi Y. Mitochondrial protein mitofusin 2 is
710 required for NLRP3 inflammasome activation after RNA virus infection. Proc Natl
711 Acad Sci 2013;110:17963–8. doi:10.1073/pnas.1312571110.

712 [64] de Brito OM, Scorrano L. Mitofusin 2: A Mitochondria-Shaping Protein with
713 Signaling Roles Beyond Fusion. Antioxid Redox Signal 2008;10:621–34.
714 doi:10.1089/ars.2007.1934.

715 [65] Cartoni R, Leger B, Hock MB, Praz M, Crettenand A, Pich S, et al. Mitofusins 1/2
716 and ERRalpha expression are increased in human skeletal muscle after physical
717 exercise. J Physiol 2005;567:349–58. doi:10.1113/jphysiol.2005.092031.

718 [66] Ishihara N. Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions
719 via GTPase activity. J Cell Sci 2004;117:6535–46. doi:10.1242/jcs.01565.

720 [67] Yoboue ED, Mougeolle A, Kaiser L, Averet N, Rigoulet M, Devin A. The role of
721 mitochondrial biogenesis and ROS in the control of energy supply in proliferating
722 cells. Biochim Biophys Acta 2014;1837:1093–8. doi:10.1016/j.bbabio.2014.02.023.

723 [68] Romanello V, Sandri M. Mitochondrial Quality Control and Muscle Mass
724 Maintenance. Front Physiol 2015;6:422. doi:10.3389/fphys.2015.00422.

725 [69] Tauler P, Sureda A, Cases N, Aguiló A, Rodríguez-Marroyo JA, Villa G, et al.
726 Increased lymphocyte antioxidant defences in response to exhaustive exercise do
727 not prevent oxidative damage. J Nutr Biochem 2006;17:665–71.
728 doi:10.1016/j.jnutbio.2005.10.013.

729 [70] Ferrer MD, Tauler P, Sureda A, Pujol P, Drobnic F, Tur JA, et al. A soccer match's
730 ability to enhance lymphocyte capability to produce ROS and induce oxidative
731 damage. Int J Sport Nutr Exerc Metab 2009;19:243–58. doi:10.1123/ijsnem.19.3.243.

732 [71] Sureda A, Ferrer MD, Tauler P, Romaguera D, Drobnic F, Pujol P, et al. Effects of
733 exercise intensity on lymphocyte H2O2 production and antioxidant defences in
734 soccer players. Br J Sports Med 2007;43:186–90. doi:10.1136/bjsm.2007.043943.

735 [72] Baldelli S, Aquilano K, Ciriolo MR. Punctum on two different transcription factors
736 regulated by PGC-1α: Nuclear factor erythroid-derived 2-like 2 and nuclear
737 respiratory factor 2. Biochim Biophys Acta - Gen Subj 2013;1830:4137–46.
738 doi:10.1016/j.bbagen.2013.04.006.

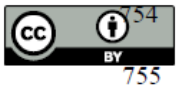
739 [73] Bruni F, Polosa PL, Gadaleta MN, Cantatore P, Roberti M. Nuclear respiratory
740 factor 2 induces the expression of many but not all human proteins acting in
741 mitochondrial DNA transcription and replication. J Biol Chem 2010;285:3939–48.
742 doi:10.1074/jbc.M109.044305.

743 [74] Yang Z-F, Drumea K, Mott S, Wang J, Rosmarin AG. GABP Transcription Factor
744 (Nuclear Respiratory Factor 2) Is Required for Mitochondrial Biogenesis. Mol Cell
745 Biol 2014;34:3194–201. doi:10.1128/MCB.00492-12.

746 [75] Putti R, Sica R, Migliaccio V, Lionetti L. Diet impact on Mitochondrial Bioenergetics
747 and Dynamics. Front Physiol 2015;6:1–7. doi:10.3389/fphys.2015.00109.

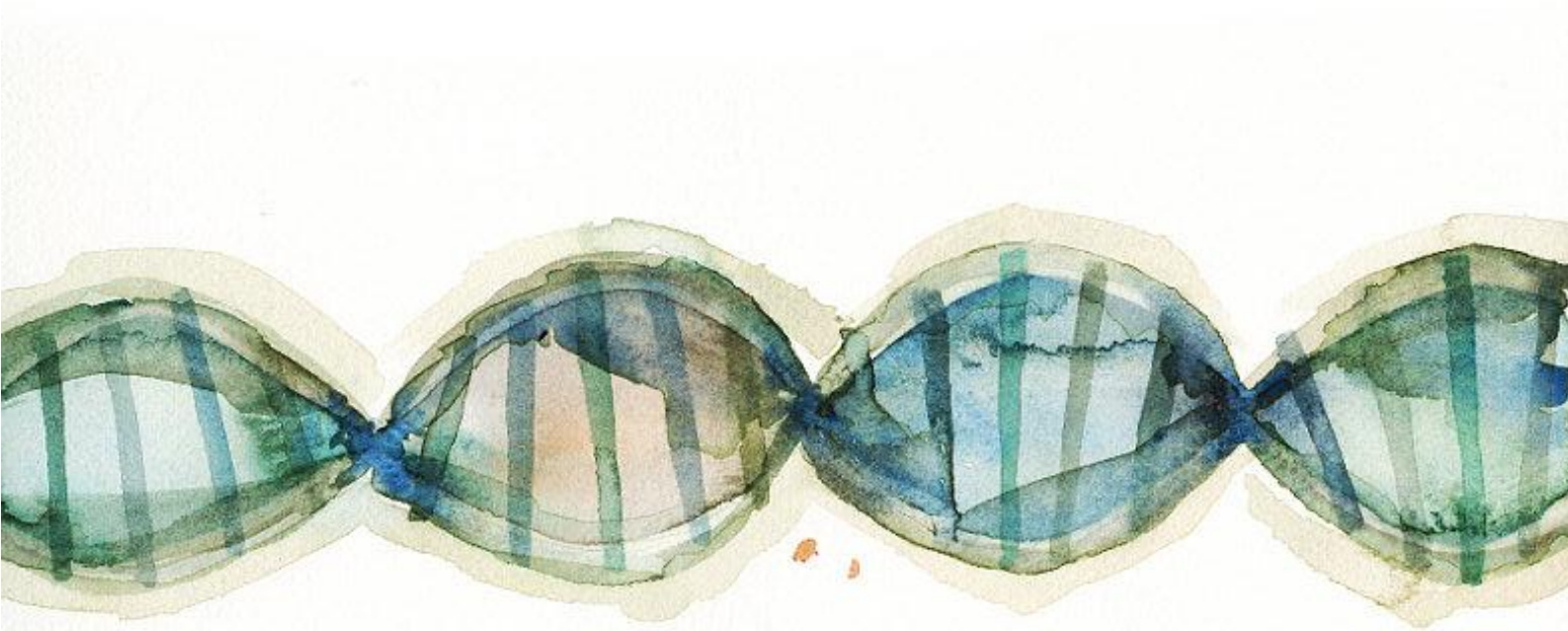
748 [76] Ferrer MD, Busquests-Cortes C, Capo X, Tejada S, Tur JA, Pons A, et al.
749 Cyclooxygenase-2 inhibitors as a therapeutic target in inflammatory diseases. Curr
750 Med Chem 2018;25:1–15. doi:10.2174/0929867325666180514112124.

751 [77] Capó X, Martorell M, Busquets-Cortés C, Tejada S, Tur JA, Pons A, et al. Resolvins
752 as proresolving inflammatory mediators in cardiovascular disease. Eur J Med Chem
753 2018;153. doi:10.1016/j.ejmech.2017.07.018.



754
755

IV. Recapitulación



La presente tesis postula que la práctica de diferentes modalidades de ejercicio físico (agudo, entrenamiento, estilo de vida activo), analizadas tanto *in vivo* como *ex vivo*, opera como estímulo prooxidante suficiente para desencadenar una respuesta en el organismo con capacidad para modular los parámetros antioxidantes, inflamatorios y mitocondriales en PBMCs y neutrófilos, con efectos beneficiosos sobre la generación de daño oxidativo y las adaptaciones al ejercicio en este contexto. Además, también postula el hecho que las respuestas antioxidante y mitocondrial tras la realización de los distintos modelos de actividad física anteriormente comentados se ven afectadas de manera favorable con la suplementación de la dieta ácidos grasos poliinsaturados omega 3, concretamente el DHA. La facilidad en la extracción y purificación de las células inmunitarias constituye un enfoque emergente para el uso de las células sanguíneas como una herramienta *in vivo* e *in vitro* para evaluar el impacto de diversos factores de estrés sobre parámetros fisiológicos. A continuación, se exponen los efectos y respuestas celulares más relevantes que ejerce el estrés oxidativo -derivado de la práctica de actividad física aguda y regular, de un estilo de vida activo y de la exposición exógena a agentes oxidantes- sobre marcadores de estrés y daño oxidativo, dinámica mitocondrial e y marcadores de inflamación.

1. Estrés oxidativo

La práctica de actividad física ha ido adquiriendo con el paso de los años una nueva dimensión y, a día de hoy, es considerada como una herramienta de salud pública, preventiva y terapéutica. Las adaptaciones inducidas por el ejercicio y un estilo de vida activo no son sólo físicas sino también fisiológicas y bioquímicas, y contribuyen de manera positiva sobre el estado de salud y la calidad de vida de la población [153,179,295–297]. En este sentido, el ejercicio es un modelo adecuado para el estudio de la respuesta adaptativa y los mecanismos de regulación que se activan frente a estímulos que alteran el estado redox del organismo. El conocimiento sobre la bioquímica de los radicales libres y el estrés oxidativo ha avanzado enormemente en las últimas décadas. De hecho, con el paso de los años se ha evidenciado un fundamental papel regulador y señalizador de las especies reactivas de oxígeno (ROS) cuando son generadas en cantidades moderadas [65,67,288]. Este rol hormético de las ROS como mensajeros celulares implicados en la inducción de respuestas

adaptativas favorables al estado de salud es materia de estudio constante.

1.1 Estrés oxidativo: efectos del ejercicio agudo y regular y la suplementación con DHA

El ejercicio físico cumple con las características que definen de la teoría de la hormesis [66,298]. Durante la práctica de actividad regular de intensidad leve a moderada se generan cantidades tolerables de ROS que promueven mejoras sobre la salud al potenciar las defensas antioxidantes en el organismo. Al ejercicio agudo de alta intensidad, en cambio, se le asocia la generación de cantidades más elevadas ROS con capacidad para dañar células y tejidos [299–301]. Por otra parte, a pesar de que existen toda una serie de moléculas derivadas de los ácidos grasos omega 3 que tienen un potente efecto resolutivo de la inflamación [302,303], el consumo de ácidos grasos omega 3 como el DHA y el EPA puede contribuir a incrementar el daño oxidativo debido la presencia en su estructura de dobles enlaces altamente susceptibles a la peroxidación [304]. Tanto la suplementación de la dieta con DHA como el ejercicio, por separado o en conjunto, pueden alterar la situación de equilibrio oxidativo produciendo adaptaciones celulares que afectan al daño oxidativo, a las defensas antioxidantes y a la producción de ROS [305–307].

Con el objetivo de evaluar y diferenciar los efectos del estrés oxidativo derivado de la práctica de actividad física aguda y regular junto con la suplementación de la dieta con ácidos grasos omega 3 sobre las defensas antioxidantes y el daño oxidativo en PBMCs, se lleva a cabo una intervención deportivo-nutricional con suplementación de la dieta con ácidos grasos omega 3 en futbolistas jóvenes federados. Específicamente, la intervención consiste en un período de 8 semanas de entrenamiento, con seis sesiones de entrenamiento semanales más los partidos oficiales, junto con la ingesta de un litro diario de una bebida funcional enriquecida con DHA (1.14 g/día) o una bebida placebo (sin aporte extra de DHA). Al final de dicho período se realiza una sesión de entrenamiento intenso y controlado, a modo de ejercicio agudo. Observamos un aumento significativo en el contenido de DHA en las membranas eritrocitarias tras ocho semanas de suplementación con la bebida enriquecida con este omega 3 en comparación con el grupo placebo. Este hecho hace pensar que otros tipos celulares, como las PBMCs, también puedan haber

incorporado e incrementado su disponibilidad de DHA [308]. Así, cambios observados en las adaptaciones a la situación de estrés oxidativo derivado del ejercicio en PBMCs podrían ser atribuidos en parte a la modificación en la composición de sus membranas con un enriquecimiento en DHA.

En cuanto al daño oxidativo tras ocho semanas de entrenamiento y suplementación con DHA, observamos un descenso significativo en los niveles de MDA en PBMCs, tanto en el grupo que consume la bebida enriquecida con DHA como en el grupo placebo. Los productos de peroxidación de lípidos como el MDA, entre otros, se consideran no sólo biomarcadores de estrés oxidativo sino también compuestos biológicamente activos con elevada relevancia fisiológica y patológica [43], ya que su acumulación en tejidos humanos es una causa importante de disfunción tisular y celular [46,309]. Sin embargo, detectamos un aumento significativo en los niveles de carbonilos en ambos grupos, placebo y sumplementado, tras dicho período de entrenamiento. Los productos de la peroxidación lipídica, como el MDA, son fuertes electrófilos y presentan la capacidad de reaccionar con grupos amino, nucleófilos, de residuos de lisina, arginina, histidina y cisteína de las proteínas [55,310,311]. Esta reacción del grupo MDA-amino puede promover la introducción de grupos carbonilos en las proteínas y, a su vez, reducir los niveles detectados de MDA [312,313]. Por ello, sugerimos que los niveles inferiores de MDA en PBMCs al final del período de entrenamiento de ocho semanas son consecuencia de la reacción del MDA con residuos amina de proteínas, hecho que aumentaría el índice de carbonilos. La carbonilación es una modificación post-traduccional irreversible. Si el grado de carbonilación es leve, marca a las proteínas para su degradación por el sistema proteasomal de la célula. Si el grado de carbonilación es elevado, conduce a la acumulación de agregados proteicos de elevado peso molecular resistentes a la degradación. Por tanto, resulta evidente que las reacciones subyacentes a estos procesos tienen un impacto severo en la maquinaria y homeostasis celular. Las evidencias que apoyan el papel de la carbonilación de proteínas en la patogénesis de los trastornos humanos proporcionan un estrecho vínculo entre la aparición/progresión de diversas enfermedades y el estrés oxidativo [52–56]. Sin embargo, la carbonilación puede también activar varios sistemas de señalización cuando las modificaciones tienen lugar en proteínas de señalización. Algunos datos revelan efectos celulares positivos y su papel en las cascadas de señalización específicas (por ejemplo, la activación de la defensa antioxidante mediada por Nrf2 a través

de la carbonilación de Keap1) [314]. Por tanto, ocho semanas de entrenamiento intensivo en futbolistas semi-profesionales inducen una mejora en el perfil del daño oxidativo lipídico pero ello deriva en la generación de daño oxidativo proteico, estando ambos eventos estrechamente relacionados en el contexto del estrés oxidativo inherente a la práctica de ejercicio.

Las ROS producidas durante el ejercicio provocan un estado de desequilibrio oxidativo transitorio que desencadena diversos mecanismos antioxidantes en las células del sistema inmunitario [315]. Estas respuestas adaptativas incluyen la vía de NF-κβ, que induce la expresión de genes diana relacionados con las defensas antioxidantes como las proteínas desacoplantes mitocondriales, anteriormente comentadas, y enzimas antioxidantes como Mn-SOD. Sin embargo, no observamos cambios en los niveles proteicos de Mn-SOD tras ocho semanas de entrenamiento y suplementación con DHA. Cabe recordar que los sujetos del estudio son atletas profesionales entrenados y, probablemente, su forma física al inicio del periodo de entrenamiento conlleve asociado un buen nivel enzimático antioxidante basal para contrarrestar la producción de ROS asociada al entrenamiento. Todo ello hace que estos atletas sean probablemente menos sensibles al estímulo derivado del ejercicio regular, al estar ya bien preparados desde el punto de vista antioxidante, y por tanto que no tenga lugar una mayor adaptación a nivel de MnSOD.

Sin embargo, sí observamos un incremento significativo en los niveles proteicos de UCP-2 y -3 en PBMCs de los futbolistas tras ocho semanas de entrenamiento. Aunque siguen siendo objeto de estudio constante, los efectos antioxidantes de algunas proteínas desacoplantes y su participación en la señalización redox están bien documentados [112,240,316,317]. Adicionalmente, detectamos en PBMCs de los futbolistas niveles de UCP3 significativamente más elevados en el grupo que había ingerido la bebida funcional enriquecida con omega 3 respecto al grupo placebo. Por tanto, este incremento en los niveles de esta proteína desacoplante puede ser atribuible a la suplementación de la dieta de los futbolistas con DHA. Las UCP-2 y UCP-3 están ubicadas en la membrana interna mitocondrial y catalizan una fuga de protones que desacopla la fosforilación oxidativa y disipa el gradiente electroquímico a través de la membrana. Esto puede conducir a una disminución en la generación de ROS mitocondriales [112]. Por tanto, el entrenamiento parece ser un factor que contribuye a la potenciación de las

defensas antioxidantes a nivel de proteínas desacoplantes, y este efecto se ve incrementado sinérgicamente por el aporte extra diario de DHA.

Se ha comprobado que la expresión de UCP-3 en el músculo esquelético se regula rápidamente durante el ejercicio prolongado, posiblemente asociado a la mayor generación de ROS asociada a la actividad física. Mayores niveles de UCP-3 suponen una mayor protección antioxidante que protege a las células musculares de un mayor flujo electrónico en la cadena respiratoria mitocondrial inducido por el ejercicio [113,318]. Este evento podría estar relacionado con el hecho que no evidenciamos aumentos significativos en la capacidad de producción de ROS por parte de las PBMCs en respuesta a la estimulación con PMA [319] tras las ocho semanas de entrenamiento, tanto en el grupo suplementado con DHA como el placebo. Sí observamos que, tras una prueba de esfuerzo extenuante, la producción de ROS por parte de las PBMCs estimuladas con PMA es mayor con respecto a la producción de ROS en condiciones de reposo (pre-ejercicio agudo). El PMA es un diéster de forbol empleado a menudo en la investigación biomédica para activar la vía de transducción de señales de la proteína quinasa C (PKC) [320,321]. La activación de la PKC tras exposición a PMA desencadena señales celulares que conducen a la activación del factor de transcripción NF-κβ en varios tipos celulares [322]. El PMA causa una amplia gama de efectos en células y tejidos, destacando su capacidad para activar la expresión de algunos genes antioxidantes en neutrófilos y células HL60 [24,319,323], pero a su vez induce incrementos en la producción de ROS dado que induce la activación de la NADPH oxidasa [324]. También observamos un aumento significativo en la expresión de UCP-2 tras la sesión de ejercicio agudo en PBMCs, lo que también contribuye a la mejora de la respuesta antioxidante [325], pero en este caso no resulta suficiente como para contrarrestar la producción de ROS inducida por PMA. Así, el papel antioxidante de las UCP-2 y UCP-3 en PBMCs tras un ejercicio agudo se encuentra limitado.

En conclusión, los resultados actuales evidencian que un período de ocho semanas de entrenamiento regular induce adaptaciones antioxidantes en PBMCs. Estas adaptaciones contribuyen a reducir la producción de ROS derivadas del ejercicio ya que, a pesar de no observar cambios en los niveles basales de Mn-SOD, sí aumenta la expresión de las proteínas UCP2 y UCP3, con capacidad antioxidante, y no se potencia la producción de ROS tras estimulación

de PBMCs con PMA en condiciones de reposo. Esta respuesta, además, es potenciada por la suplementación de DHA. Sin embargo, el incremento de UCP2 observado tras la sesión de ejercicio agudo no resulta suficiente como para contrarrestar la aumentada producción de ROS por parte de PBMCs activados con PMA tras dicha prueba de esfuerzo ejercicio, lo que indica una limitada capacidad antioxidante de las UCPs. Tanto en ejercicio físico agudo como regular son buenos modelos fisiológicos para el estudio y caracterización de los mecanismos adaptativos y de respuesta mitocondrial y pro-antioxidante, y su modulación por ácidos grasos poliinsaturados omega 3, en células inmunitarias.

1.2 Estrés oxidativo: efectos de un estilo de vida activo

Los beneficios físicos y fisiológicos del ejercicio son numerosos [326], destacando entre ellos la mejora sobre distintos parámetros como la forma física, la resistencia, la flexibilidad y la movilidad de las articulaciones, entre otros. Adicionalmente la actividad física también promueve el aumento del tono y la fuerza muscular, la regulación de la presión arterial, una mejora en la resistencia a la insulina y el mantenimiento del peso corporal. Además, la liberación de endorfinas y otras hormonas durante el ejercicio comportan también beneficios a nivel psicológico, como mejoras en la autoestima, reducción del estrés y la depresión, entre otros muchos. [153,239,327,328]. Por ello, la práctica regular de actividad física moderada y el llevar un estilo de vida activo, alejado del sedentarismo, constituyen puntos clave para el envejecimiento saludable y el control y prevención de enfermedades crónicas asociadas con el envejecimiento [153,243,329].

Con el objetivo de evaluar y asociar mejoras en la composición corporal, el estado antioxidante y la generación de daño oxidativo a un estilo de vida activo en adultos mayores, se lleva a cabo el siguiente diseño experimental: se clasifican como “activos”, “intermedios” o “inactivos” a 61 hombres y 66 mujeres mayores de 55 años según el grado de actividad física declarada practicada durante el año anterior. La clasificación se realiza de acuerdo con los equivalentes metabólicos (METs min/semana) obtenidos mediante cuestionarios de actividad física. Los sujetos clasificados como activos realizan casi tres veces más actividades diarias que los consideras sujetos inactivos, también llamados sedentarios. Este estilo de vida más activo se traduce en

menor peso, masa grasa, IMC y en una presión arterial diastólica reducida en aquellos sujetos físicamente más activos con respecto a los sedentarios.

En cuanto a marcadores de daño oxidativo, aunque se observa una tendencia en los niveles de MDA -en plasma- y Nitro-Tyr y carbonilos -en PBMCs- a aumentar conforme aumenta el grado de actividades físicas diarias reportada (es decir, más METs min/semana), únicamente el aumento en los niveles de carbonilos en PBMCs resulta significativo en mujeres activas e intermedias respecto a sedentarias. Por tanto, el hecho de haber llevado un estilo de vida y una rutina diaria no contribuye de manera significativa al aumento de marcadores de daño oxidativo en plasma y PBMCs en hombres sanos mayores de 55 años. En mujeres sólo el aumento de las proteínas carboniladas parece estar correlacionado con la mayor actividad diaria reportada. Esta ausencia de daño oxidativo derivado de un estilo de vida más activo podría ser consecuencia de la modulación de las defensas antioxidantes por la práctica regular de actividades físicas. De hecho, observamos en condiciones basales mayores niveles de proteínas antioxidantes (CAT, MnSOD, GPx, GRd, TrxR1) en las PBMCs de sujetos que habían llevado una vida activa con respecto a los sujetos clasificados como sedentarios. De esta manera, se evidencia la potenciación de la capacidad antioxidante endógena inducida por la práctica regular y frecuente de actividades físicas moderadas. Sin embargo, no observamos cambios significativos en las actividades de estos mismos enzimas. La actividad de los enzimas antioxidantes es modulable por moléculas como el $O_2^{\cdot-}$ o el H_2O_2 [132,291,330], observándose que enzimas como la CAT o la GPx en eritrocitos, que no disponen de mecanismos de síntesis de nuevas proteínas, incrementan su actividad tras un ejercicio intenso de larga duración como es una competición de duatlón [132,158,291,330]. Los sujetos mayores de 55 años que llevaron una vida activa presentan mayores niveles, aunque no mayores actividades, de enzimas antioxidantes en condiciones basales que sujetos sedentarios, aunque en caso de necesidad les facultaría para ser activados e incrementar su actividad antioxidante frente a la aparición de futuras y mayores producciones de $O_2^{\cdot-}$. Por tanto, las personas activas exhiben una capacidad más elevada y rápida para activar su respuesta antioxidante tras un estímulo oxidante de mayor intensidad como una actividad física.

En conclusión, un estilo de vida activo conlleva asociadas mejoras en los parámetros antropométricos en mujeres y hombres sanos mayores de 55 años,

mientras que no promueve aumentos significativos en los niveles de marcadores de daño oxidativo, pero sí mayores niveles de proteínas antioxidantes. Así, se evidencia la potenciación de la capacidad antioxidante endógena inducida por la práctica regular de actividades físicas moderadas y un estilo de vida activo.

1.3 Estrés oxidativo: efectos de la exposición continua a H_2O_2

El síndrome metabólico agrupa un conjunto de alteraciones metabólicas comúnmente asociadas a un estado inflamatorio leve crónico y a niveles permanentemente elevados ROS [331–333] como el H_2O_2, el $O2^{\bullet -}$, el $OH^{\bullet}$ y ácido hipocloroso (HClO) [334]. Concretamente, el H_2O_2, debido a su relativa estabilidad, moderada reactividad química y capacidad de difusión en condiciones fisiológicas [298], constituye un metabolito importante involucrado en la modulación y señalización de reacciones del metabolismo redox [335]. El H_2O_2 posee la capacidad de oxidar específicamente a proteínas [99] implicadas tanto en la activación de cascadas homeostáticas, patológicas o de protección [289], de acuerdo con la teoría hormética de las ROS [65,93,96,288,336]. La comprensión de los mecanismos moleculares y las dosis de H_2O_2 que promueven en el organismo las respuestas beneficiosas/adaptativas o, en cambio, favorecen respuestas negativas/patológicas, tiene un significado práctico de gran alcance, ya que multitud de estados patológicos se caracterizan por estados Redox alterados [81]. Para determinar el papel de los oxidantes celulares en los procesos fisiológicos y patológicos, es esencial poder distinguir y caracterizar aquellas especies involucradas y monitorizar sus niveles en los sistemas biológico de interés [337].

Con el objetivo de determinar el efecto diferencial de dosis distintas de estímulo oxidante sobre la producción de ROS y la expresión génica de proteínas antioxidantes, emulamos una situación crónica de estrés oxidativo de manera *ex vivo*. Dicha emulación se lleva a cabo mediante la exposición de PBMCs y neutrófilos a concentraciones sostenidas de H_2O_2 producida por la enzima glucosa oxidasa (GOX). Esta enzima cataliza la conversión de glucosa, presente en el medio de cultivo, a D-glucono-δ-lactona y H_2O_2 Estas células, que provienen de pacientes con síndrome metabólico documentado, son estimuladas con dos dosis diferentes de GOX (Alta y Baja) e incubadas durante 2

horas a 37^{O}C, por lo que durante ese tiempo cada tipo celular está expuesto a dos concentraciones diferentes de H_2O_2. Concretamente, las concentraciones Alta y Baja en PBMCs se corresponden con 1 y 0,1 µg GOX/ml respectivamente. En neutrófilos, las concentraciones Alta y Baja se corresponden con 15 y 5 µg GOX/ml, respectivamente.

Observamos, en primer lugar, que la concentración de H_2O_2 en el medio de cultivo alcanza unos niveles relativamente estables a diferentes tiempos y niveles según la dosis de GOX aplicada y según el tipo celular. Los neutrófilos muestran una mayor capacidad para eliminar el H_2O_2 que las PBMCs, es decir, una mayor habilidad para igualar las tasas de generación y eliminación de H_2O_2 y alcanzar así un estado estacionario de H_2O_2 en el medio extracelular. Estas respuestas se traducen en efectos distintos sobre la producción de ROS y la modulación de enzimas antioxidantes en estas células inmunitarias provenientes de pacientes con síndrome metabólico. La exposición de células inmunes tanto a Alta como Baja dosis de GOX durante 2 horas no altera de manera significativa la expresión de genes implicados en mecanismos enzimáticos antioxidantes en neutrófilos. En PBMCs, en cambio, la respuesta antioxidante se encuentra ligeramente inhibida ya que se observa que la expresión de CuZnSOD experimenta una reducción significativa tras la exposición a Alta dosis de GOX. La expresión de otras enzimas antioxidantes en PBMCs como CAT y Mn-SOD no se ve afectada por ninguna de las dosis de GOX aplicadas. La CAT degrada el H_2O_2 a oxígeno y agua para mantener el equilibrio redox intracelular y proteger así a las células del daño inducido por un exceso de ROS. Pero, aparentemente, la expresión basal de las enzimas antioxidantes resulta suficiente como para contrarrestar el aporte extra de H_2O_2 inducido por la adición de GOX al medio de cultivo. Por tanto, la maquinaria enzimática no se ve afectada por el aporte exógeno extra de H_2O_2 y los neutrófilos parecen ser más resistentes a los efectos del mismo en términos de expresión génica.

Observamos diferencias en la capacidad de producción de H_2O_2, como forma de ROS, por parte de PBMCs y neutrófilos estimulados con patrones moleculares asociados a patógenos (PAMPs) como el LPS y ZYM. Detectamos que los tratamientos previos con H_2O_2 exógena influyen de forma dispar en PBMCs y en neutrófilos. Los neutrófilos muestran una tasa más alta de producción de ROS tras de la estimulación con LPS o ZYM que las PBMCs. Esta respuesta podría estar relacionada con las dos vías de producción de ROS en los

neutrófilos versus la única vía mitocondrial en las PBMCs. Parece ser que la producción de H_2O_2 es principalmente mitocondrial en PBMCs, pudiendo ser además lisosomal en neutrófilos [338–340]. Tanto el LPS como el ZYM son reconocidos por TLRs, desencadenando una respuesta inflamatoria y oxidativa mediante la activación del NFkB [341,342]. Los TLRs son receptores de membrana que reconoce PAMPs y desempeñan un papel importante en la inmunidad innata [343,344]. La unión de los PAMPS a los TLRs conduce a la producción de numerosas citoquinas proinflamatorias [345], a la expresión y activación de la ciclooxigenasa-2 (COX2), el retraso de la liberación de eicosanoides [346], al aumento de la producción de ROS [339,342,346] y, a su vez, a la expresión de genes antioxidantes [347,348]. El LPS ejerce su acción principalmente a través de la interacción con TLR4 [349] mientras que ZYM se une directamente a TLR2 [350]. Esta direccionalidad en las acciones del LPS y del ZYM podría asociarse con que la producción de ROS es mayor tras la exposición con ZYM que con LPS, tanto en PBMCs como en neutrófilos, indicando una mayor sensibilidad con el receptor TLR2 que con el TLR4 en ambos tipos celulares. Los tratamientos tanto con Alta como Baja dosis de GOX afectan de manera diferente a la respuesta de PBMCs y neutrófilos tras la estimulación con LPS o ZYM: el tratamiento con H_2O_2 disminuye la respuesta de las PBMC a ZYM, pero no afecta la respuesta a LPS, mientras que el tratamiento con H_2O_2 aumenta la respuesta de los neutrófilos a LPS, pero no la respuesta a ZYM. Estos efectos podrían indicar, en principio, una respuesta disimilar en la expresión de los TLRs frente a la exposición a H_2O_2 en estas células inmunitarias ya que, los tratamientos con H_2O_2 también influyen en la expresión de los genes de los receptores TLR2 y TLR4 en PBMCs y neutrófilos. Sin embargo, la pauta de los cambios inducidos por el H_2O_2 sobre la expresión de los genes no concuerda con aquellos efectos observados sobre la producción de ROS en estos tipos celulares, poniendo de manifiesto que este comportamiento probablemente no sea atribuible a una acción directa o indirecta del H_2O_2 sobre la expresión génica. Se ha señalado que en situaciones con alta tasa de producción de H_2O_2 como el ejercicio agudo puede disminuir la disponibilidad de TLR4 en la superficie de monocitos CD4, apuntando a la posibilidad de que el receptor sea internalizado en la membrana [185], y por tanto no se encuentre disponible para la interacción con PAMPs. Entonces, los efectos observados tras la estimulación con LPS y ZYM en términos de producción de ROS podrían ser consecuencia a una acción posterior a la interacción de los inmunoestimuladores con los receptores de TLR, como la activación de la vía de

NFκβ o ya a nivel mitocondrial o lisosomal [351]. Por tanto, la adición de GOX al medio de cultivo no parece influir de manera relevante sobre la producción de ROS por parte de PBMCs y neutrófilos tras una inmunestimulación *ex vivo* con H_2O_2 durante 2 horas.

En resumen, la exposición *ex vivo* durante 2 horas a Alta dosis de H_2O_2 inhibe parcialmente la respuesta antioxidante en PBMCs mientras que no afecta a neutrófilos en términos de expresión génica. Los neutrófilos producen más ROS cuando son inmunoestimulados dada la existencia de dos vías -mitocondrial y lisosomal- de producción de ROS en estas células con respecto a la vía mitocondrial en las PBMCs. Los cambios en la expresión de TLR2 y TLR4 en PBMCs y neutrófilos como consecuencia de la exposición con H_2O_2 no parecen influir de manera directa sobre la capacidad de ZYM y LPS para estimular la producción de ROS. El estudio *in vitro* de los efectos del H_2O_2, generado de forma continua y sostenida a diferentes tasas de producción, sobre la funcionalidad y expresión génica de PBMCs y neutrófilos permitirá conocer el papel dual dañino o de señalización celular de esta especie reactiva de oxígeno. La metodología desarrollada resulta útil para mantener a las células inmunitarias bajo una exposición extracelular sostenida de H_2O_2 y para alcanzar unos niveles estacionarios de H_2O_2 en los medios de cultivo durante al menos 2 horas.

2. Dinámica mitocondrial

2.1 Dinámica mitocondrial: efectos del ejercicio agudo y regular, y de la suplementación con DHA

La regulación del ciclo vital mitocondrial, desde la biogénesis de nuevas mitocondrias a la eliminación de mitocondrias disfuncionales, determina la cantidad total y, lo más importante, la calidad y función de las mitocondrias en el músculo esquelético [143,194,352], factores clave en la mejora de la función metabólica y el rendimiento físico [139,353]. Los mecanismos subyacentes que implican la regulación de la red o retículo mitocondrial son objeto de estudio constante [140]. En concreto, se ha propuesto que el ejercicio físico regular provoca una remodelación de la red mitocondrial a través de la fusión, fisión y eliminación de las mitocondrias dañadas mediante procesos de mitofagia [354–

356]. Por otra parte, se ha descrito que los ácidos grasos omega 3 ejercen mejoras sobre la función mitocondrial y el comportamiento dinámico en músculo [339], promoviendo la fusión mitocondrial en experimentos *in vivo* e *in vitro* [284]. Además, el DHA ha sido reconocido como una molécula protectora contra la inflamación y el estrés oxidativo [268,273] y que también promueve la expresión génica de enzimas clave que introducen ácidos grasos en las mitocondrias y favorecen su uso como combustible energético en la cadena respiratoria [357].

Con el objetivo de caracterizar la respuesta de los procesos de biogénesis y dinámica mitocondrial a una situación de estrés oxidativo derivado de la práctica actividad física aguda y regular, junto con la suplementación de la dieta con ácidos grasos omega 3, se determinó la expresión génica de proteínas de fusión, de fisión y de biogénesis mitocondrial en PBMCs de futbolistas jóvenes participantes en la intervención deportivo-nutricional. Ocho semanas de entrenamiento provocan aumentos en el contenido de DHA en las membranas eritrocitarias. Ello indica que las PBMCs podrían también haber incorporado la disponibilidad de este omega 3 en su membrana [308], induciéndose adaptaciones antioxidantes en PBMCs tendentes a reducir la producción mitocondrial de ROS al aumentar la expresión de las proteínas desacopladoras de la cadena respiratoria UCP2 y UCP3. Este hecho se pone de manifiesto en una reducción de la tasa de producción de ROS tras la estimulación con PMA al final del periodo de entrenamiento. Esta respuesta, está potenciada por la suplementación de DHA.

Las investigaciones en los últimos años han puesto de relieve que el PGC-1α ejerce un papel central en la regulación de la biogénesis mitocondrial frente a diversas situaciones fisiológicas [205,358,359]. A nivel molecular, el PGC-1α no presenta capacidad de unión al ADN, pero puede modular la expresión de proteínas implicadas en la biogénesis mitocondrial a través de su interacción con factores de transcripción implicados: PGC-1α coactiva la actividad transcripcional de NRF-1, e induce a su vez la expresión del NRF-1, del NRF-2 y del TFAM [358]. Esta cascada de señalización incluye eventos de fusión y fisión, que desencadenan cambios en el tamaño, forma, distribución, transporte y número de mitocondrias en la célula [202,360]. Observamos un aumento de los niveles proteicos de PGC1α, el regulador central inductor de la biogénesis mitocondrial. De hecho, este aumento en el contenido proteico de PGC1α tras

el periodo de entrenamiento es análogo al aumento observado en NRF1 y Tfam. NRF1 promueve la expresión de genes nucleares que codifican para proteínas de la cadena de transporte de electrones, y a su vez influencia la actividad transcripcional de Tfam. Por su parte, Tfam regula la expresión del mtDNA que codifica para proteínas respiratorias fundamentales y otros factores involucrados en la transcripción y replicación del mtDNA. Por tanto, PGC-1α, a través de la interacción con NRF-1 y Tfam, coordina una regulación positiva del contenido mitocondrial, favoreciendo el metabolismo y la fosforilación oxidativa, y orquestando así adaptaciones fisiológicas que mejoran el rendimiento asociado al entrenamiento [39,147]. PGC-1α también regula la actividad de la COX-IV, la última oxidasa en la cadena de transporte de electrones mitocondrial, y un posible indicador del contenido mitocondrial [361]. Los niveles proteicos de COX-IV también aparecen incrementados tras las 8 semanas de entrenamiento en PBMCs, y la administración adicional de DHA mediante la bebida enriquecida favorece, a su vez, un aumento con respecto al grupo placebo. Adicionalmente, se observa también un aumento en la expresión relativa de PGC-1α, COXIV y MitND5 – gen que codifica para la subunidad 5 de la NADH deshidrogena- tras la sesión de ejercicio agudo. El aumento de la expresión de COX-IV tras el ejercicio agudo y los niveles proteicos mayores de COXIV tras 8 semanas de entrenamiento en PBMCs, junto con una mayor expresión de MitND5 en condiciones post-ejercicio -otro posible indicador del contenido mitocondrial- pueden deberse a un mayor número de mitocondrias por célula. De hecho, está documentado el ejercicio agudo puede actuar como un inductor de la biogénesis mitocondrial a través de la activación de NF-κB y la expresión del gen PGC1α [206,362,363]. En este sentido, el entrenamiento y el ejercicio agudo podrían inducir nuevos procesos de biogénesis de mitocondrias, hecho que favorecería el metabolismo energético durante las aumentadas demandas derivadas de la práctica de ejercicio. Las señales inducidas por el estrés mecánico, como las p38 MAPK, que se activan en respuesta a la elevación del Ca^{2+} citosólico durante la contracción muscular, tienen potencial para estimular y regular la actividad y la expresión de factores de transcripción sensibles al ejercicio como el PGC-1α [359]. Por tanto, todas estas moléculas proporcionan un vínculo entre los estímulos fisiológicos y la transcripción de genes nucleares que inducen adaptaciones fisiológicas que aumentan los umbrales de tolerancia a dosis subsiguientes de factores estresantes [39]. Se evidencia, por tanto, que el entrenamiento promueve la biogénesis mitocondrial en PBMCs en términos de niveles proteicos, aunque en

este caso el DHA no contribuye a mejorar este proceso. El ejercicio agudo, por su parte, contribuye a activar la expresión de genes iniciadores de este proceso de biogénesis y otros implicados en la cadena de transporte de electrones.

Las mitocondrias forman redes interconectadas, además de experimentar continuamente variaciones en su tamaño y forma, como resultado de los procesos de fisión, fusión y movimiento a lo largo del citoesqueleto [211]. El equilibrio entre las tasas de fisión y fusión está influenciado por las condiciones metabólicas y/o patológicas en las propias mitocondrias y su entorno celular. Proteínas de la familia de las Dinaminas son los componentes clave de las maquinarias de fisión y fusión [354]. Destacan las proteínas de la membrana mitocondrial interna Mtf1/2 y OPA1. La proteína OPA1, que interviene en la regulación de la morfología mitocondrial, es degradada por OMA1 cuando las mitocondrias pierden el potencial de membrana. Dicha proteólisis inducible actúa como un mecanismo regulador para inactivar proteolíticamente a OPA1, evitando así la fusión de mitocondrias dañadas al retículo mitocondrial. En los futbolistas del estudio, los niveles proteicos de Mtf1, Mtf2, OPA1 y OMA1 también se encuentran significativamente aumentados tras las ocho semanas de entrenamiento en PBMCs. Además, los niveles de ambas mitofusinas son mayores en el grupo suplementado con DHA respecto al grupo placebo. También observamos que el ejercicio agudo promueve un incremento en los niveles proteicos de Mtf2. Por tanto, la práctica regular de ejercicio actúa como estímulo regulador de la remodelación mitocondrial. Adicionalmente, en el caso de Tfam, ambas mitofusinas y OMA1 de las PBMCs, observamos también una interacción entre los factores entrenamiento y suplementación a la hora de promover aumentos en los niveles proteicos de estas moléculas.

Tomados en su conjunto, todos estos resultados evidencian que tanto la práctica de ejercicio regular como un episodio de ejercicio agudo producen adaptaciones mitocondriales que pueden contribuir a mejorar la calidad y función mitocondrial, y que la suplementación con DHA contribuye sinérgicamente de manera positiva a dichos efectos.

2.2 Dinámica mitocondrial: efectos de un estilo de vida activo

Numerosos estudios en la literatura científica ratifican que las mitocondrias desempeñan una función crítica en la longevidad y el envejecimiento, no sólo porque constituyen el lugar más cercano a la generación y acción de los radicales libres, sino también porque intervienen en la regulación de la respuesta al estrés y a la apoptosis [364]. El hecho de que el mtDNA se halle continuamente expuesto a la acción de agentes oxidantes favorece la aparición de mutaciones en su seno, situación que se ve agravada con la edad. Las mutaciones del mtDNA dan lugar a la generación subunidades defectuosas de las enzimas respiratorias, hecho que produce un deterioro del transporte de los electrones y un incremento del daño oxidativo a la mitocondrias [365,366]. Por tanto, el envejecimiento de las personas está caracterizado por un declive progresivo de la capacidad oxidativa [367] y de la viabilidad y funcionalidad mitocondrial, especialmente en células del músculo esquelético, que presentan menores actividades enzimáticas, menores ratios de respiración y menor tasa de generación de ATP [368,369]. Por todo ello, el control efectivo de la biogénesis y recambio mitocondrial se vuelve crítico para el mantenimiento de la producción de energía, la prevención del estrés oxidativo endógeno y la promoción del envejecimiento saludable [370].

Con el objetivo de profundizar en estrategias relacionadas con el ejercicio para promover un envejecimiento saludable en términos de función mitocondrial, evaluamos el impacto y la influencia de llevar un estilo de vida activo sobre la capacidad oxidativa y el estatus mitocondrial en PBMCs de adultos mayores de 55 años. Los sujetos son clasificados de acuerdo con el grado de actividad física reportado, atendiendo a la nomenclatura de inactivos, intermedios y activos.

MitND5, como subunidad 5 de la NADH deshidrogenasa (ubiquinona), se encuentra en la membrana interna mitocondrial, y puede ser considerada una proteína mitocondrial constitutiva e indicadora del contenido mitocondrias. No hallamos diferencias significativas en la expresión relativa de MitND5 en PBMCs entre los grupos de personas mayores activas, intermedias e inactivas. Este hecho, sugiere que el retículo mitocondrial no presenta un mayor contenido estructural en hombres y mujeres mayores de 55 años activos con respecto a

los clasificados como sedentarios. Las proteínas de fusión mitofusinas 1 y 2 no exhiben cambios significativos entre grupos, mientras que la expresión de COXIV está aumentada 5 y 4 veces en mujeres y hombres activos, respectivamente, en comparación con el grupo control (mujeres inactivas). Por todo ello, no se puede sugerir la existencia de un retículo mitocondrial mayor (de acuerdo con la expresión de ARNm de MitND5 inalterada) ni la activación de la biogénesis (expresión de ARNm de Mtf1 y 2 constante), sino más bien una mejora de la capacidad respiratoria y oxidativa del *stock* preexistente de mitocondrias (advirtiendo el aumento significativo de COXIV en sujetos activos). Estos resultados sugieren una buena calidad mitocondrial en sujetos activos, pero también que un estilo de vida activo no es un estímulo suficiente, ni equivalente al entrenamiento, para promover la biogénesis mitocondrial en personas ancianas activas. Estos resultados coinciden con otros similares observados en hombres y mujeres de edad avanzada que realizaron un entrenamiento con ejercicios de resistencia durante 14 semanas, y en los que se observa un aumento significativo en COXIV pero no se observan cambios aparentes en el contenido de mtDNA [21]. Se ha establecido que las personas mayores muestran un contenido mitocondrial reducido, así como una disminución en la calidad y actividad mitocondrial y, consecuentemente, una menor capacidad oxidativa [367]. Estos eventos se asocian con el proceso normal de envejecimiento y se han correlacionado con el desarrollo de una amplia gama de enfermedades relacionadas con la edad [371]. Sin embargo, nuestros resultados indican que las actividades físicas diarias regular y el llevar un estilo de vida activo podría ralentizar esta disminución de la función mitocondrial.

En conclusión, el hecho de llevar un estilo de vida activo contribuye a la mejora de la calidad del *stock* mitocondrial en sujetos sanos y una ralentización de la función mitocondrial asociada al proceso normal de envejecimiento.

2.3 Dinámica mitocondrial: efectos de la exposición continua a H_2O_2

Al ser las mitocondrias la principal fuente de ROS [120], estos orgánulos están constantemente expuestos a la acción de radicales libres y agentes oxidantes. La evidencia acumulada sugiere que la función/disfunción

mitocondrial, los niveles de ROS y las aberraciones en la morfología mitocondrial están interconectados, aunque de manera dependiente del tipo celular y del contexto [372]. Por ello se ha planteado que las ROS están involucradas en la regulación a corto plazo de la morfología mitocondrial y la función a través de vías no transcripcionales [95]. Los mecanismos post-traduccionales de la homeostasis redox incluyen S-nitrosilaciones, puentes disulfuro y nitraciones sobre proteínas Drp1, OPA1 y PKA, entre muchas otras, que interaccionan con moléculas implicadas directamente en la dinámica mitocondrial, como las mitofusinas [222,372]. Todas estas modificaciones pueden ser inducidas por la acción del H_2O_2, entre otros ROS.

Con el objetivo de evaluar los efectos diferenciales de distintas dosis de estímulo oxidante *ex vivo* sobre la expresión de proteínas mitocondriales, exponemos a PBMCs y neutrófilos a concentraciones de H_2O_2 sostenidas, producidas por la GOX. Durante ese período de dos horas los neutrófilos y las PBMCs están a dos concentraciones diferentes de H_2O_2, lo que permite detectar respuestas dosis-dependientes. El tratamiento con Baja dosis de GOX estimula la expresión de Mtf2 y TFAM en PBMCs, poniendo de manifiesto la inducción de una remodelación mitocondrial y biogénesis tras un estímulo oxidante de intensidad tolerable. Cabe recordar que Mtf1 y Mtf2 son proteínas involucradas en la fusión y biogénesis mitocondrial [218,232] mientras que TFAM se une al ADN mitocondrial promoviendo la transcripción de las mitofusinas y otros genes mitocondriales [373,374]. Estos procesos de fusión, junto con los de fisión, regulan el remodelado del retículo mitocondrial, el cual se encuentra estrechamente vinculado al estado metabólico. Por el contrario, la dosis Alta de GOX reduce significativamente la expresión génica de Mtf1 y TFAM en PBMCs, indicando una posible inhibición de los procesos de fusión y biogénesis mitocondrial. Al ser la mitocondria uno de los principales focos de producción de ROS [58,363], la regulación y modulación de la biogénesis mitocondrial mediada por ROS podría considerarse como un mecanismo antioxidante y un proceso protector y de control de calidad de dicho orgánulo [321].

Por otra parte, detectamos que la Mtf2 exhibe una mayor expresión tras la incubación con bajas dosis de H_2O_2 en PBMCs, lo que podría señalar un papel emergente de las proteínas relacionadas con la dinámica mitocondrial en los procesos de inflamación. Este hecho se debe a que se ha descrito en macrófagos que Mtf2 interacciona con el receptor NLRP3 y juega un papel

esencial a la hora de promover la activación del inflamasoma, un complejo multiproteico responsable de la activación de los procesos inflamatorios en caso de infecciones virales o bacterianas [375]. Estas evidencias destacan la importancia de la mitocondria como *plataforma* para la activación del inflamosoma NLRP3. Las evidencias más recientes, de hecho, implican a Mtf2 en múltiples vías de señalización no restringidas exclusivamente a la regulación del remodelado del retículo mitocondrial [376].

En el caso de los neutrófilos, la expresión de las mitofusinas y TFAM se mantiene constante tras la exposición a H_2O_2, mientras que la expresión de NRF2 se encuentra inhibida tras la exposición a Alta dosis de GOX. NRF2, también llamado GABPA [377–380], funciona como un factor de transcripción que activa la expresión de algunos genes metabólicos clave que regulan el crecimiento celular y genes nucleares necesarios para la respiración mitocondrial, así como la biogénesis mitocondrial y la transcripción y replicación del mtDNA [378,381]. La disminución de la expresión de NRF2 inducida por el tratamiento con Alta dosis de H_2O_2 producida por la GOX podría repercutir en la respiración mitocondrial en los neutrófilos a la vez que inhibir la respuesta mitocondrial en términos de biogénesis y otros procesos asociados.

En conclusión, dosis leves de H_2O_2 aplicadas de manera *ex vivo* estimulan la remodelación y biogénesis mitocondrial mientras que dosis elevadas inhiben dichos procesos. También se detecta el rol emergente de las mitocondrias en procesos inflamatorios.

3. Inflamación

3.1 Inflamación: efectos de un estilo de vida activo

Las personas mayores suelen padecer de manera crónica un estado de inflamación subclínico (asintomático) de bajo grado asociado al propio proceso de envejecimiento. Está documentado que las citoquinas proinflamatorias aparecen incrementadas en adultos sanos mayores de 50 años, mientras que las citoquinas antiinflamatorias están disminuidas [245,382]. Este patrón pro/antiinflamatorio se ha asociado a la redistribución de la grasa corporal y aumentos concomitantes en los ácidos grasos circulantes que conducen a la

activación de macrófagos con un perfil proinflamatorio [383]. En este estado inflamatorio crónico de bajo grado asociado al envejecimiento (del inglés *inflammaging,* según algunos autores) [246,384–386], parece ser un componente importante de las enfermedades más comunes relacionadas con la edad, como la diabetes, la osteoporosis, las enfermedades cardiovasculares y el cáncer. El aumento del riesgo de padecer estas enfermedades crónicas se ha asociado con marcadores de inflamación elevados [387], mientras que la práctica de la actividad física ha demostrado reducir algunos de estos biomarcadores proinflamatorios como la CRP [388], TNF-α [389] o IL6 [247]. Por lo tanto, la modulación del estado inflamatorio a lo largo de la vida constituye una estrategia adecuada para lograr un envejecimiento saludable.

Con el objetivo de evaluar la composición corporal, la hipertensión y el perfil lipídico, así como el estado inflamatorio en los adultos mayores y su asociación con un estilo de vida activo, 116 mujeres y hombres mayores de 55 años son clasificados en activos e inactivos según el nivel de actividad física reportada. Los datos antropométricos obtenidos revelan que los sujetos clasificados como activos, quienes realizan más actividades diarias, presentan menor peso, masa grasa, IMC y presión arterial diastólica en comparación a los que llevan una vida sedentaria. El HDL se encuentra, a su vez, aumentado en aquellos hombres y mujeres clasificados como activos respecto a los inactivos. La leucopenia observada en los participantes activos se explica por recuentos más bajos de neutrófilos y linfocitos, y concuerda con estudios previos que reportan un cierto grado de leucopenia en respuesta a la práctica regular de la actividades físicas, hecho que a su vez se interpreta como parte de una respuesta antiinflamatoria [149]. De hecho, la práctica de actividad física diaria moderada y el llevar un estilo de vida activo se ha propuesto como un componente clave para un envejecimiento saludable [153,326,390], así que la adquisición de un perfil más antiinflamatorio contribuiría a los beneficios inducidos por el ejercicio.

Por otra parte, observamos cómo el hecho de llevar un estilo de vida más activo y realizar más actividades físicas diarias conduce a la presencia de concentraciones basales más bajas de IL-6 tanto en mujeres como en hombres, en comparación con un estilo de vida sedentario. Aunque la IL6 también puede ejercer efectos antiinflamatorios cuando se libera de forma aguda tras un episodio de ejercicio, al promover la secreción de las citoquinas

antiinflamatorias IL-10 e IL-1ra [179,188], la presencia crónica de elevadas concentraciones de IL6 pueden inducir una respuesta inmune de fase aguda [184,391]. No evidenciamos, sin embargo, cambio alguno en los niveles de TNF-α. El hecho de llevar un estilo de vida activo no es estímulo suficiente para aumentar significativamente el TNFα. Esta molécula está considerada como el regulador central del sistema inmunológico, ya que desempeña un papel importante en la propagación de la inflamación debido a la activación y el reclutamiento de células inmunitarias a través de su receptor *TNF receptor 1*. La expresión génica de otras citoquinas proinflamatorias como IL1β o el receptor proinflamatorio TLR4 en PBMCs no se ve afectada significativamente por el hecho de llevar una rutina diaria activa. Estos resultados van en consonancia con estudios previos que reportan que la IL1β no responde a diferentes grados de ejercicio, incluido el ejercicio aeróbico de baja intensidad, el ejercicio aeróbico de alta intensidad o una combinación de ejercicio aeróbico de alta intensidad y ejercicio de resistencia [189]. Aunque tampoco se observan efectos de una rutina activa en la expresión de la interleuquina con propiedades antiinflamatorias IL1ra, se observa una activación significativa de la expresión del gen de la citoquina antiinflamatoria IL10. Otros estudios han reportado niveles más altos de IL10 en respuesta a la actividad física [189,190], y este hecho se ha relacionado con una disminución de la masa grasa [190,392]. De hecho, el estado inflamatorio crónico se ha relacionado con la adiposidad, y la influencia de la actividad física sobre la composición corporal puede influir en el estado inflamatorio [393–395]. El hecho de que las concentraciones de citoquinas antiinflamatorias disminuyan en adultos sanos mayores de 55 años también se ha asociado con la redistribución de la grasa corporal [383]. Nuestros resultados actuales (menor masa grasa y mayor expresión de IL10 en sujetos activos) refuerzan el efecto antiinflamatorio de la práctica regular de actividades físicas y el llevar un estilo de vida activo en personas mayores.

También se observa una sobreexpresión significativa del factor de transcripción NFκB en los grupos físicamente activos. Este factor nuclear puede activarse mediante la acción de citoquinas proinflamatorias (como TNFα), pero también puede activarse mediante ROS [396]. Una vez activado, el NFκB migra al núcleo y puede inducir la expresión de una amplia variedad de genes, incluyendo gran variedad de citoquinas como TNFα, IL-6 e IL-1β [397,398], pero también enzimas antioxidantes como las SODs o la óxido nítrico sintasa [156,399]. Como el perfil de expresión génica en PBMCs no muestra evidencias

de un fenotipo proinflamatorio, podríamos interpretar la activación de NFκB a través de la ruta ROS en lugar de mediante una respuesta proinflamatoria. En este caso, la práctica regular actividades físicas diarias expone al organismo a una producción sostenida y continuada de bajos niveles de ROS; se ha demostrado que estos niveles bajos de ROS actúan como segundos mensajeros que conducen una respuesta antioxidante y antiinflamatoria a través de la activación de NFκB y otros muchos genes [156,399].

Referente a los receptores relacionados con la inflamación, si bien no observamos efectos de un estilo de vida activo con respecto a los niveles proteicos en de TLR4 en PBMCs, sí detectamos una disminución en ambos sexos en los niveles de proteína de TLR2 en este tipo celular. TLR2 reconoce una amplia variedad de PAMPs entre los que destacan lipoproteínas, peptidoglicanos y ZYM [344], mientras que TLR4 reconoce el LPS [341]. La activación de la vía de señalización de TLR4 estimula el aumento de citoquinas proinflamatorias como TNFα, IL1β o IL6, y se ha descrito que la actividad física puede regular a la baja la expresión de TLR4 en las células inmunes [400,401], así como de citoquinas como TNFα, IL1β e IL6 [297,402]. Aunque TLR4 suele ser el TLR más sensible en respuesta a la actividad física, observamos únicamente una regulación a la baja de TLR2 en sujetos activos, debido probablemente a que los voluntarios entrevistados no eran personas entrenadas o que realizasen ejercicios intensos diarios, sino que más bien llevaban un estilo de vida activo. TLR2 también está involucrado en la respuesta celular a los estímulos inmunes, y comparte con TLR4 su cascada de señalización. Una reciente revisión sistemática evidenció que el ejercicio moderado crónico tiene efectos antiinflamatorios en el organismo a través de la regulación negativa tanto de TLR2 como de TLR4 en los niveles de expresión génica y niveles proteicos [403], hecho que concuerda con nuestros resultados.

En resumen, a pesar del estado inflamatorio crónico subclínico asociado a la edad, un estilo de vida activo mejora los parámetros antropométricos en adultos mayores. La reducción de la masa grasa y la mejora del perfil lipídico en sujetos con un estilo de vida activo podrían contribuir de manera favorable a la atenuación de parámetros proinflamatorios en comparación a sujetos con un estilo de vida sedentario.

3.2 Inflamación: efectos de la exposición continua a H_2O_2

Las ROS, entre las que se incluye el H_2O_2, son moléculas de señalización clave que desempeñan un papel importante en la progresión de los procesos inflamatorios [334,404]. Las respuestas inflamatorias agudas son esenciales para combatir infecciones y durante la cicatrización de heridas, pero se vuelven perjudiciales si no se resuelven y se prolongan en el tiempo [405]. Hemos estudiado los efectos que diferentes dosis sostenidas de H_2O_2 ejercen sobre el carácter pro/antiinflamatorio de neutrófilos y PBMCs mediante experimentos *ex vivo* con estos tipos celulares aislados de pacientes con síndrome metabólico y sometidos a la producción contínua de H_2O_2 mediante la actividad GOX.

En términos de expresión génica, observamos en PBMCs que la expresión de las citoquinas proinflamatorias IL6, IL8 e IL1β aumenta tras 2 horas de incubación con Baja dosis de H_2O_2, aunque este estímulo de H_2O_2 no es suficiente para aumentar significativamente el TNFα, molécula central del proceso de inflamación [406]. Este patrón en PBMCs sugiere que la exposición crónica a H_2O_2 a bajas dosis es suficiente para promover la expresión de genes proinflamatorios, sugiriendo una inducción de la respuesta proinflamatoria en las células inmunes. Por otra parte, se ha señalado que la mitofusina, un mediador de la fusión mitocondrial, juega un papel clave en la activación del inflamasoma NLPR3, un complejo multiproteico implicado en la activación de procesos inflamatorios [375,376]. Mtf2 también exhibe una mayor expresión tras la incubación con H_2O_2, hecho que refuerza el emergente rol de las proteínas relacionadas con la dinámica mitocondrial, y de la mitocondria en sí, en los procesos inflamatorios. Detectamos también que el tratamiento con Alta dosis de H_2O_2 incrementa la expresión de la COX2 de neutrófilos, una enzima inducible responsable de sintetizar prostaglandinas proinflamatorias a partir de ácido araquidónico. Por tanto, se evidencia también en este tipo celular otro posible efecto proinflamatorio del H_2O_2, a pesar de que el resto de moléculas con propiedades inflamatorias no experimentan cambios en su expresión tras la exposición exógena a H_2O_2.

La inflamación crónica se asocia comúnmente a varios trastornos incluidos en el síndrome metabólico como la diabetes tipo 2, el sobrepeso y la obesidad. De hecho, este último se caracteriza por una inflamación crónica de bajo grado con un aumento permanente del estrés oxidativo [331,332,407,408]. Esta

condición inflamatoria patológica subclínica normalmente conduce a niveles bajos de adiponectina plasmática (con propiedades antiinflamatorias) y niveles altos de TNFα e IL6 (con propiedades proinflamatorias). No observamos cambios significativos en el caso de los neutrófilos en cuanto a los niveles de TNFα e IL6, a la vez que no detectamos producción de adiponectina, evidenciándose la inhabilidad de este tipo celular para producirla [409]. Investigaciones previas demuestran que la adiponectina puede reducir la secreción de marcadores implicados en la activación de NFκB, incluidos el TNF-α, IL-6, por adipocitos y macrófagos, a través de los TLR [410]. En PBMCs observamos en PBMCs un comportamiento antitético con respecto al patrón inflamatorio asociado a patologías con el estado inflamatorio alterado: la producción de IL6 se encuentra disminuida, mientras que la secreción de adiponectina aumenta, como resultado de los tratamientos con H_2O_2 en PBMCs. TNFα, por su parte, también se mantiene constante en PBMCs. Estos eventos podrían estar relacionado con propiedades antiinflamatorias directas o indirectas del H_2O_2 [411,412]. Se han documentado evidencias previas sobre la secreción de adiponectina por tejidos diferentes al tejido adiposo como por ejemplo los linfocitos [413]. Mediante esta metodología evidenciamos no sólo que las PBMCs secretan adiponectina, sino que esta producción incrementa tras los tratamientos la exposición exógena a Alta y Baja dosis H_2O_2.

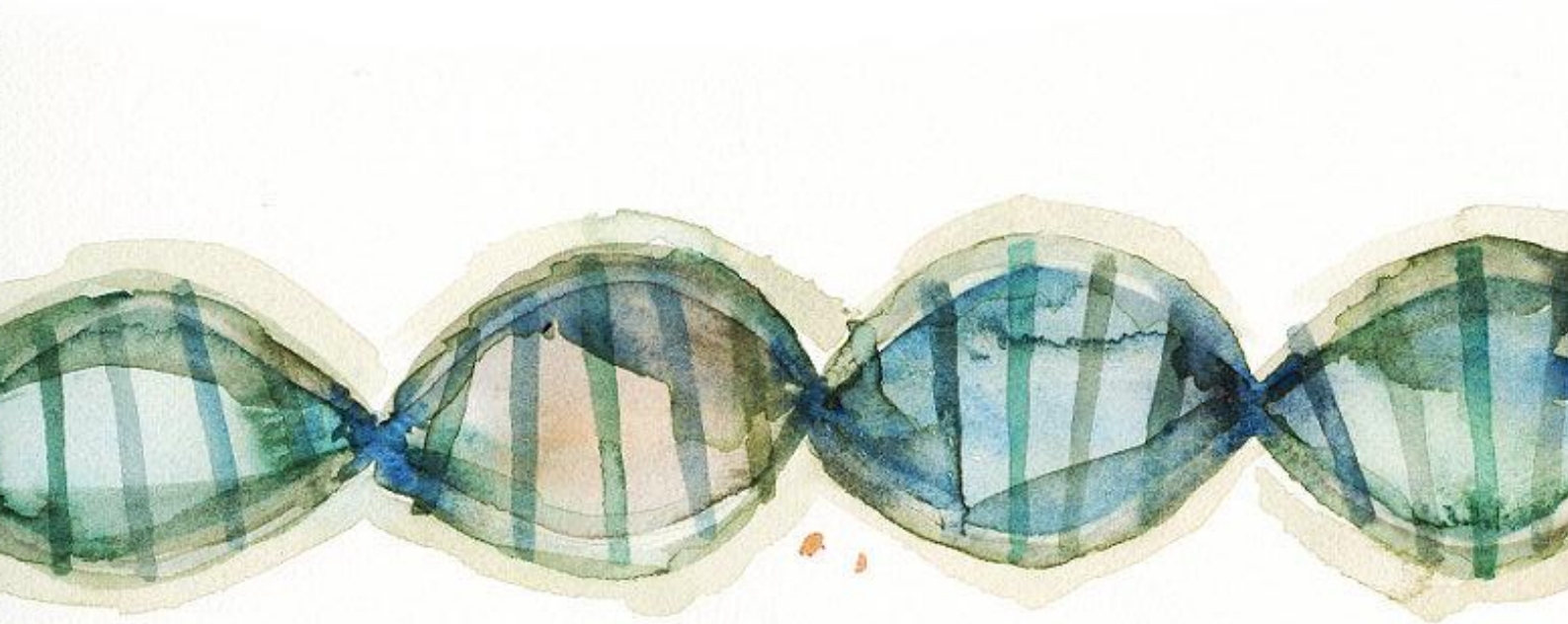
En resumen, el tratamiento *ex vivo* con H_2O_2 promueve la inducción de una respuesta antiinflamatoria en PBMCs de sujetos con síndrome metabólico, marcado por la secreción de adiponectina, pero actúa como un estimulador genético de genes proinflamatorios en dichas células. Los neutrófilos resultan ser células más resistentes a la acción del H_2O_2.

4. Reflexión final

El objetivo general de esta tesis radica en evaluar y caracterizar la respuesta diferencial antioxidante, inflamatoria y mitocondrial, frente a diferentes dosis de ejercicio físico, como estímulo con carácter prooxidante, y valorar el uso de ácido docosahexaenoico como suplemento nutricional en este contexto. Hemos puesto de manifiesto que el ejercicio físico practicado de forma aguda y de forma regular (incluyendo tanto el entrenamiento y un estilo de vida activo) resultan un buen modelo fisiológico para estudiar los mecanismos adaptativos y

de respuesta mitocondrial, pro-antiinflamatoria y pro-antioxidante y su modulación por nutrientes como los ácidos grasos poliinsaturados omega 3 en células inmunitarias. En líneas generales, el entrenamiento induce adaptaciones antioxidantes y mitocondriales en PBMCs y estas respuestas, además, están potenciada por la suplementación de DHA. Sin embargo, aunque una sesión de ejercicio induce también procesos de biogénesis mitocondrial, los mecanismos antioxidantes que también se activan no resultan suficientes para contrarrestar la aparición de una situación de estrés oxidativo en PBMCs. Por otra parte, el hecho de llevar un estilo de vida activo contribuye a la mejora de la calidad mitocondrial en sujetos sanos y una ralentización de la función mitocondrial asociada al proceso normal de envejecimiento. La mejora de los parámetros antropométricos, bioquímicos y hematológicos asociados a un estilo de vida activo contribuye de manera favorable a la adquisición de un perfil antiinflamatorio en comparación a sujetos con un estilo de vida sedentario, a pesar del estado inflamatorio crónico subclínico asociado a la edad. Por último, las dosis de H_2O_2 circulantes parecen representar una variable clave que influye significativamente en la promoción de efectos disimilares en el organismo, ya que diferentes niveles extracelulares de H_2O_2 influyen de manera hormética en el estado pro-antiinflamatorio, pro-antioxidante y mitocondrial en las células. Un control de estos niveles podría tener relevancia médica en términos de diagnóstico/pronóstico de enfermedades con estados inflamatorios y oxidativos alterados como el síndrome metabólico.

V. Conclusiones



1. El entrenamiento durante ocho semanas promueve la biogénesis mitocondrial en PBMCs al inducir aumentos en la expresión de PGC-1α y en los niveles proteicos de los factores de transcripción Tfam y NRF1, y potencia la capacidad de respiración mitocondrial incrementando los niveles de COXIV.

2. El entrenamiento durante ocho semanas intensifica la capacidad de remodelación mitocondrial incrementando los niveles proteicos de Mtf1, Mtf2, OPA1 y OMA1, moléculas implicadas en los procesos de fusión de las mitocondrias. La suplementación de la dieta con DHA durante ocho semanas contribuye de manera sinérgica a los efectos del entrenamiento sobre la remodelación mitocondrial.

3. El ejercicio agudo induce procesos de biogénesis mitocondrial en PBMCs al inducir aumentos en la expresión de PGC-1α, COXIV y MitND5, a la vez que promueve la remodelación mitocondrial al provocar incrementos en los niveles proteicos de Mtf2.

4. El entrenamiento durante ocho semanas promueve el incremento de los niveles de UCP-2 y UCP-3, y la suplementación con DHA potencia de manera sinérgica el aumento de los niveles proteicos de UCP-3 en PBMCs, reduciendo la capacidad de producción mitocondrial de especies reactivas de oxígeno.

5. El ejercicio agudo induce un aumento en los niveles de UCP2 en PBMCs reduciendo la capacidad mitocondrial de producción de peróxido de hidrogeno aunque aumenta su tasa de producción tras inmunoestimulación con PMA. Los efectos antioxidantes del ejercicio agudo no resultan suficientes para prevenir la respuesta prooxidante de las PBMCs activadas con PMA.

6. Un estilo de vida activo mejora las características antropométricas en adultos mayores de 55 años al favorecer un menor peso, masa grasa e índice de masa corporal respecto a adultos con un estilo de vida sedentario. Este efecto en la composición corporal se acompaña de una presión arterial diastólica más baja y niveles más altos de colesterol HDL circulante.

7. Un estilo de vida activo contribuye favorablemente a la atenuación de parámetros proinflamatorios, con un menor recuento de linfocitos y

neutrófilos y menores niveles de circulantes de IL6 y TLR2, y a la adquisición de un perfil antiinflamatorio al incrementar la secreción de adiponecitna y la expresión de IL10 en PBMCs.

8. Un estilo de vida activo permite una mejor respuesta antioxidante en PBMCs al disponer de mayores niveles de las proteínas antioxidantes CAT, MnSOD, GRd, GPx, TrxR1 para hacer frente al estrés oxidativo.

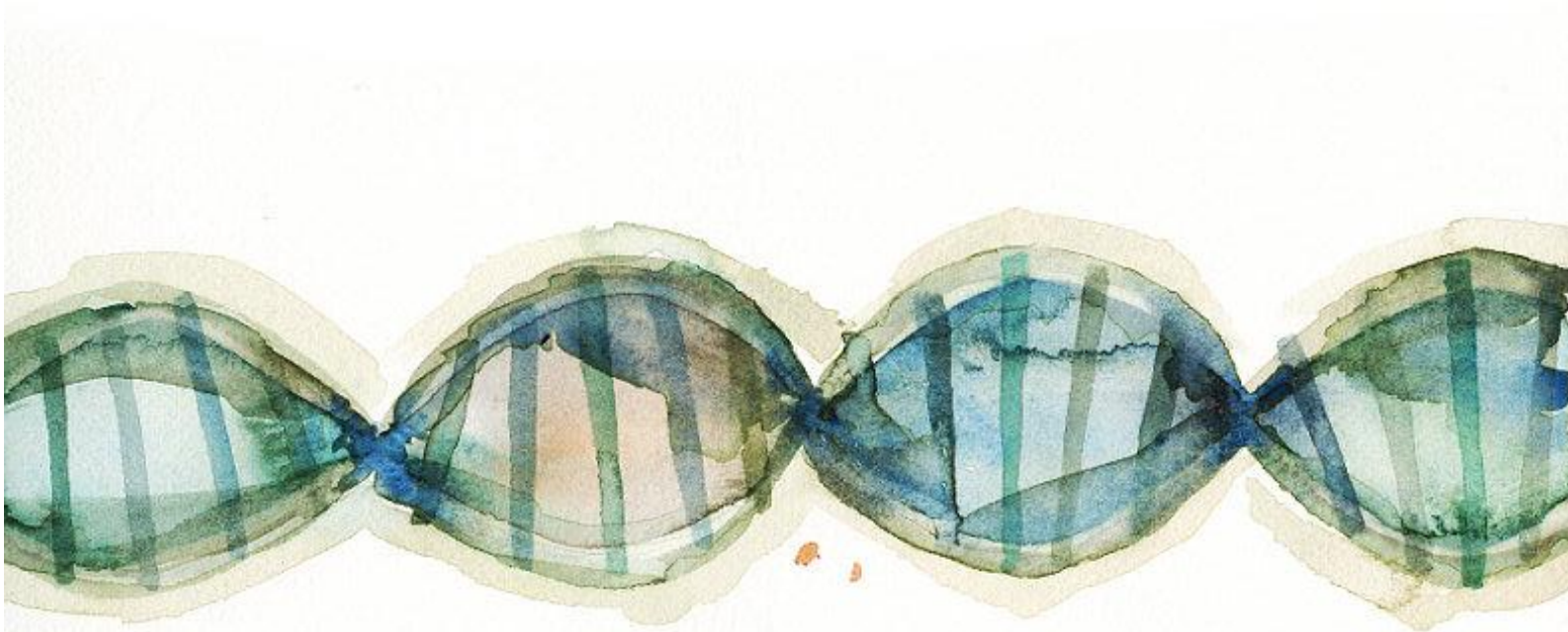
9. El entrenamiento deportivo durante ocho semanas en atletas jóvenes y un estilo d vida activo en personas mayores promueve una mejora de las adaptaciones antioxidantes celulares; sin embargo, estas situaciones no evitan que se produzcan daños de carbonilacion en proteínas en PBMCs de estos colectivos.

10. El tratamiento de PBMCs y neutrófilos con glucosa oxidasa y glucosa con una generación sostenida de diferentes niveles de H_2O_2 durante 2 horas permite simular el efecto hormético de las especies reactivas de oxígeno de manera *ex vivo* para generar una respuesta adaptativa al estrés oxidativo. Los neutrófilos precisan de mayores tasas de producción de H_2O_2 para padecer estrés oxidativo al presentar una mayor capacidad para eliminar el H_2O_2 que las PBMCs. Este hecho supone menores niveles de H_2O_2 en el medio de cultivo de los neutrófilos, lo que se traduce en efectos más limitados del H_2O_2 sobre la expresión génica y producción de ROS en estas células.

11. La exposición continua *ex vivo* de PBMCs y neutrófilos a concentraciones sostenidas de H_2O_2 estimula de manera hormética procesos implicados en la dinámica y biogénesis mitocondrial. Dosis leves de H_2O_2 estimulan la remodelación y biogénesis mitocondrial mientras que dosis elevadas inhiben dichos eventos en ambos tipos celulares.

12. El tratamiento *ex vivo* con H_2O_2 promueve una respuesta antiinflamatoria en PBMCs de sujetos con síndrome metabólico caracterizada por un incremento en la secreción de adiponectina y un decremento de IL6, estimulando de manera hormética la expresión de genes proinflamatorios en estas células. Un control de los de niveles de H_2O_2 en el organismo podría tener relevancia médica en enfermedades con estados inflamatorio y redox alterados como el síndrome metabólico.

VI. Bibliografía



[1] Sies H. Oxidative stress: A concept in redox biology and medicine. Redox Biol 2015;4:180–3. doi:10.1016/j.redox.2015.01.002.

[2] Sies H, Cadenas E. Oxidative stress: damage to intact cells and organs. Philos Trans R Soc L B Biol Sc 1985;17;35:617–31.

[3] Radák Z, Naito H, Kaneko T, Tahara S, Nakamoto H, Takahashi R, et al. Exercise training decreases DNA damage and increases DNA repair and resistance against oxidative stress of proteins in aged rat skeletal muscle. Pflugers Arch Eur J Physiol 2002;445:273–8. doi:10.1007/s00424-002-0918-6.

[4] Birben E, Murat U, Md S, Sackesen C, Erzurum S, Kalayci O. Oxidative Stress and Antioxidant Defense. WAO J 2012;5:9–19. doi:10.1097/WOX.0b013e3182439613.

[5] Cui H, Kong Y, Zhang H. Oxidative Stress, Mitochondrial Dysfunction, and Aging. J Signal Transduct 2012;2012:1–13. doi:10.1155/2012/646354.

[6] Schieber M, Chandel NS. ROS function in redox signaling and oxidative stress. Curr Biol 2014;24:1–25. doi:10.1016/j.cub.2014.03.034.ROS.

[7] Davies KJA. Oxidative stress: the paradox of aerobic life. Biochem Soc Symp 1995;61:1–31. doi:10.1042/bss0610001.

[8] Halliwell B. Letters to the Editors THE DEFINITION AND MEASUREMENT OF ANTIOXIDANTS IN. Free Radic Biol Med 1995;18:125–6.

[9] Sies H. Role of metabolic H2O2 generation: Redox signaling and oxidative stress. J Biol Chem 2014;289:8735–41. doi:10.1074/jbc.R113.544635.

[10] Wazawa T, Matsuoka A, Tajima G, Sugawara Y, Nakamura K, Shikama K. Hydrogen peroxide plays a key role in the oxidation reaction of myoglobin by molecular oxygen. A computer simulation. Biophys J 1992;63:544–50. doi:10.1016/S0006-3495(92)81608-9.

[11] Sokolov VS, Batishchev O V., Akimov SA, Galimzyanov TR, Konstantinova AN, Malingriaux E, et al. Residence time of singlet oxygen in membranes. Sci Rep 2018;8:14000. doi:10.1038/s41598-018-31901-9.

[12] Aikens J, Dix TA. Perhydroxyl radical (HOO·) initiated lipid peroxidation: The role of fatty acid hydroperoxides. J Biol Chem 1991;266:15091–8.

[13] Min L, Jian-xing X. Detoxifying function of cytochrome c against oxygen toxicity. Mitochondrion 2007;7:13–6. doi:10.1016/j.mito.2006.11.011.

[14] Singh IN, Sullivan PG, Hall ED. Peroxynitrite-Mediated Oxidative Damage to Brain Mitochondria: Protective Effects of Peroxynitrite Scavengers. J

Neurosci Res 2007;85:2216–23. doi:10.1002/jnr.

[15] Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Cell Signal 2007;19:1807–19. doi:10.1016/j.cellsig.2007.04.009.

[16] Wolin MS. Interactions of oxidants with vascular signaling systems. Arterioscler Thromb Vasc Biol 2000;20:1430–42. doi:10.1161/01.ATV.20.6.1430.

[17] Winyard CJ and PG. Redox Signaling and Regulation in Biology and Medicine. Exeter, UK: 2009.

[18] Marks IM. Empirical psychotherapeutic methods. Psychother Psychosom 1974;24:222–37. doi:10.1159/000286737.

[19] Tomanek L. Proteomic responses to environmentally induced oxidative stress. J Exp Biol 2015;218:1867–79. doi:10.1242/jeb.116475.

[20] Greene EL, Paller MS. Xanthine oxidase produces O2-. in posthypoxic injury of renal epithelial cells. Am J Physiol 1992;263:F251–5.

[21] Parise G, Brose AN, Tarnopolsky MA. Resistance exercise training decreases oxidative damage to DNA and increases cytochrome oxidase activity in older adults. Exp Gerontol 2005;40:173–80. doi:10.1016/j.exger.2004.09.002.

[22] Ferrer MD, Tauler P, Sureda A, Romaguera D, Llompart I, Palacin C, et al. Enzyme antioxidant defences and oxidative damage in red blood cells of variegate porphyria patients. Redox Rep 2009;14:69–74. doi:10.1179/135100009X392502.

[23] Sureda A, Ferrer MD, Tauler P, Tur J a, Pons A. Lymphocyte antioxidant response and H2O2 production after a swimming session: gender differences. Free Radic Res 2008;42:312–9. doi:10.1080/10715760801989926.

[24] Ferrer MD, Sureda A, Tauler P, Palacín C, Tur JA, Pons A. Impaired lymphocyte mitochondrial antioxidant defences in variegate porphyria are accompanied by more inducible reactive oxygen species production and DNA damage: Research paper. Br J Haematol 2010;149:759–67. doi:10.1111/j.1365-2141.2010.08149.x.

[25] Capó X, Martorell M, Sureda A, Llompart I, Tur J a., Pons A. Diet supplementation with DHA-enriched food in football players during training season enhances the mitochondrial antioxidant capabilities in blood mononuclear cells. Eur J Nutr 2014;54:35–49. doi:10.1007/s00394-

014-0683-2.

[26] Turner JE, Bosch JA, Drayson MT, Aldred S. Assessment of oxidative stress in lymphocytes with exercise 2011:206–11. doi:10.1152/japplphysiol.00051.2011.

[27] Sureda A, Tauler P, Aguilo A, Cases N, Tur JA, Pons A, et al. Relation between oxidative stress markers and antioxidant endogenous defences during exhaustive exercise 2005;39:1317–24. doi:10.1080/10715760500177500.

[28] Ferrer MD, Sureda A, Batle JM, Tauler P, Tur JA, Pons A. Scuba diving enhances endogenous antioxidant defenses in lymphocytes and neutrophils. Free Radic Res 2007;41:274–81. doi:10.1080/10715760601080371.

[29] Ferrer MD, Sureda A, Mestre A, Tur J a, Pons A. The Double Edge of Reactive Oxygen Species as Damaging and Signaling Molecules in HL60 Cell Culture. Cell Physiol Biochem 2010;25:241–52.

[30] Szili EJ, Harding FJ, Hong SH, Herrmann F, Voelcker NH, Short RD. The hormesis effect of plasma-elevated intracellular ROS on HaCaT cells. J Phys D Appl Phys 2015;48. doi:10.1088/0022-3727/48/49/495401.

[31] Ristow M. Unraveling the truth about antioxidants. Nat Med 2014;20:709–11. doi:10.1038/nm.3624.

[32] Calabrese EJ. Hormesis: Improving Predictions in the Low-Dose Zone. Mol Clin Environ Toxicol 2012;101:551–64.

[33] Radak Z, Chung HY, Goto S. Exercise and hormesis : oxidative stress-related adaptation for successful aging 2005:71–5. doi:10.1007/s10522-004-7386-7.

[34] Ji LL, Gomez-Cabrera M-C, Steinhafel N, Vina J. Acute exercise activates nuclear factor (NF)-kappaB signaling pathway in rat skeletal muscle. FASEB J 2004;18:1499–506. doi:10.1096/fj.04-1846com.

[35] Calabrese EJ, Baldwin LA. Defining hormesis. Hum Exp Toxicol 2002;21:91–7. doi:10.1191/0960327102ht217oa.

[36] Bhakta-Guha D, Efferth T. Hormesis: Decoding two sides of the same coin. Pharmaceuticals 2015;8:865–83. doi:10.3390/ph8040865.

[37] Nathan C, Cunningham-Bussel A. Beyond oxidative stress: An immunologist's guide to reactive oxygen species. Nat Rev Immunol 2013;13:349–61. doi:10.1038/nri3423.

[38] Yun J& TF. Mitohormesis. Cell Metab 2014;19:757–66. doi:10.1016/j.pestbp.2011.02.012.Investigations.

[39] Merry TL, Ristow M. Mitohormesis in exercise training. Free Radic Biol Med 2016;98:123–30. doi:10.1016/j.freeradbiomed.2015.11.032.

[40] Ristow M, Schmeisser K. Mitohormesis: Promoting health and lifespan by increased levels of reactive oxygen species (ROS). Dose-Response 2014;12:288–341. doi:10.2203/dose-response.13-035.Ristow.

[41] Sharma K. Mitochondrial hormesis and diabetic complications. Diabetes 2015;64:663–72. doi:10.2337/db14-0874.

[42] Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev 2014;2014. doi:10.1155/2014/360438.

[43] Tsikas D. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. Anal Biochem 2017;524:13–30. doi:10.1016/j.ab.2016.10.021.

[44] Richard D, Kefi K, Barbe U, Bausero P, Visioli F. Polyunsaturated fatty acids as antioxidants. Pharmacol Res 2008;57:451–5. doi:10.1016/j.phrs.2008.05.002.

[45] Alessio HM, Hagerman a E, Fulkerson BK, Ambrose J, Rice RE, Wiley RL. Generation of reactive oxygen species after exhaustive aerobic and isometric exercise. Med Sci Sports Exerc 2000;32:1576–81. doi:10.1097/00005768-200009000-00008.

[46] Negre-Salvayre A, Auge N, Ayala V, Basaga H, Boada J, Brenke R, et al. Pathological aspects of lipid peroxidation. Free Radic Res 2010;44:1125–71. doi:10.3109/10715762.2010.498478.

[47] Shinjyo N, Kita K. Relationship between reactive oxygen species and heme metabolism during the differentiation of Neuro2a cells. Biochem Biophys Res Commun 2007;358:130–5. doi:10.1016/j.bbrc.2007.04.071.

[48] Kwiecien S, Jasnos K, Magierowski M, Sliwowski Z, Pajdo R, Jagiellonian P. LIPID PEROXIDATION, REACTIVE OXYGEN SPECIES AND ANTIOXIDATIVE FACTORS IN THE PATHOGENESIS OF GASTRIC MUCOSAL LESIONS AND MECHANISM OF PROTECTION AGAINST OXIDATIVE STRESS - INDUCED GASTRIC INJURY 2014;65:613–22.

[49] Córdova Martínez A, Martorell Pons M, Sureda Gomila A, Tur Marí JA, Pons Biescas A. Changes in circulating cytokines and markers of muscle

damage in elite cyclists during a multi-stage competition. Clin Physiol Funct Imaging 2015;35:351–8. doi:10.1111/cpf.12170.

[50] Tauler P, Sureda A, Cases N, Aguiló A, Rodríguez-Marroyo JA, Villa G, et al. Increased lymphocyte antioxidant defences in response to exhaustive exercise do not prevent oxidative damage. J Nutr Biochem 2006;17:665–71. doi:10.1016/j.jnutbio.2005.10.013.

[51] Viña J, Gomez-Cabrera MC, Lloret A, Marquez R, Miñana JB, Pallardó F V., et al. Free radicals in exhaustive physical exercise: Mechanism of production, and protection by antioxidants. IUBMB Life 2000;50:271–7. doi:10.1080/15216540051080994.

[52] Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med 2006;10:389–406. doi:10.1111/j.1582-4934.2006.tb00407.x.

[53] Jawalekar SL, Kulkarni UJ, Surve VT, Deshmukh YA. Status of Lipid profile , MDA and protein carbonyl in patients with cardiovascular diseases 2010;2:8–14.

[54] Pirinccioglu AG, Gökalp D, Pirinccioglu M, Kizil G, Kizil M. Malondialdehyde (MDA) and protein carbonyl (PCO) levels as biomarkers of oxidative stress in subjects with familial hypercholesterolemia. Clin Biochem 2010;43:1220–4. doi:10.1016/j.clinbiochem.2010.07.022.

[55] Fedorova M, Bollineni RC, Hoffmann and R. PROTEIN CARBONYLATION AS A MAJOR HALLMARK OF OXIDATIVE DAMAGE: UPDATE OF ANALYTICAL STRATEGIES. Mass Spectrom Rev 2013. doi:10.1002/mas.

[56] Afreen A, Javarappa D. Correlation of Protein Carbonyl and Malondialdehyde in Oxidative 2014;14:0–3.

[57] Wickens AP. Ageing and the free radical theory. Respir Physiol 2001;128:379–91. doi:10.1016/S0034-5687(01)00313-9.

[58] Phaniendra A, Jestadi DB, Periyasamy L. Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. Indian J Clin Biochem 2015;30:11–26. doi:10.1007/s12291-014-0446-0.

[59] Ji LL. Modulation of skeletal muscle antioxidant defense by exercise: Role of redox signaling. Free Radic Biol Med 2008;44:142–52. doi:10.1016/j.freeradbiomed.2007.02.031.

[60] Sen C, Packer L. Antioxidant and redox regulation of gene transcription. Faseb 1996;10:709–20.

[61] Ren X, Zou L, Zhang X, Branco V, Wang J, Carvalho C, et al. Redox Signaling Mediated by Thioredoxin and Glutathione Systems in the Central Nervous System. Antioxid Redox Signal 2017;27:ars.2016.6925. doi:10.1089/ars.2016.6925.

[62] Zitka O, Skalickova S, Gumulec J, Masarik M, Adam V, Hubalek J, et al. Redox status expressed as GSH:GSSG ratio as a marker for oxidative stress in paediatric tumour patients. Oncol Lett 2012;4:1247–53. doi:10.3892/ol.2012.931.

[63] Chai Y-C. S-thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: evidence against a role for glutathione disulfide. n.d.

[64] Yoboue ED, Mougeolle A, Kaiser L, Averet N, Rigoulet M, Devin A. The role of mitochondrial biogenesis and ROS in the control of energy supply in proliferating cells. Biochim Biophys Acta 2014;1837:1093–8. doi:10.1016/j.bbabio.2014.02.023.

[65] Ludovico P, Burhans WC. Reactive oxygen species, ageing and the hormesis police. FEMS Yeast Res 2014;14:33–9. doi:10.1111/1567-1364.12070.

[66] Radak Z, Ishihara K, Tekus E, Varga C, Posa A, Balogh L, et al. Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve. Redox Biol 2017;12:285–90. doi:10.1016/j.redox.2017.02.015.

[67] Goto S, Radák Z. Hormetic effects of reactive oxygen species by exercise: A view from animal studies for successful aging in human. Dose-Response 2010;8:68–72. doi:10.2203/dose-response.09-044.Goto.

[68] D'Autréaux B, Toledano MB. ROS as signalling molecules: Mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol 2007;8:813–24. doi:10.1038/nrm2256.

[69] Turrens JF. Mitochondrial formation of reactive oxygen species. J Physiol 2003;552:335–44. doi:10.1113/jphysiol.2003.049478.

[70] H.J. Forman AB. Superoxide radical and hydrogen peroxide in mitochondria. In: Press A, editor. Free Radicals Biol., New York: 1982, p. 65–90.

[71] Forman HJ, Azzi A. On the virtual existence of superoxide anions in mitochondria: thoughts regarding its role in pathophysiology. FASEB J 1997;11:374–5. doi:10.1096/FASEBJ.11.5.9141504.

[72] Luo T, Xia Z. A small dose of hydrogen peroxide enhances tumor necrosis

factor-alpha toxicity in inducing human vascular endothelial cell apoptosis: Reversal with propofol. Anesth Analg 2006;103:110–6. doi:10.1213/01.ane.0000221183.02244.80.

[73] Antunes F, Brito PM. Quantitative biology of hydrogen peroxide signaling. Redox Biol 2017;13:1–7. doi:10.1016/j.redox.2017.04.039.

[74] Winn JS, Guille J, Gebicki JM DR. Hydrogen peroxide modulation of the respiratory burst of human neutrophils. Biochem Pharmacol 1991;41:31–6.

[75] Stöcker S, Van Laer K, Mijuskovic A, Dick TP. The Conundrum of Hydrogen Peroxide Signaling and the Emerging Role of Peroxiredoxins as Redox Relay Hubs. Antioxid Redox Signal 2017;28:ars.2017.7162. doi:10.1089/ars.2017.7162.

[76] Rhee SG, Woo HA, Kang D. The Role of Peroxiredoxins in the Transduction of H_2O_2 Signals. Antioxid Redox Signal 2017;28:ars.2017.7167. doi:10.1089/ars.2017.7167.

[77] Sureda A, Hebling U, Pons A, Mueller S. Extracellular H2O2 and not superoxide determines the compartment-specific activation of transferrin receptor by iron regulatory protein 1. Free Radic Res 2005;39:817–24. doi:10.1080/10715760500164045.

[78] Bienert GP, Schjoerring JK, Jahn TP. Membrane transport of hydrogen peroxide. Biochim Biophys Acta - Biomembr 2006;1758:994–1003. doi:10.1016/j.bbamem.2006.02.015.

[79] Bienert GP, M??ller ALB, Kristiansen KA, Schulz A, M??ller IM, Schjoerring JK, et al. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. J Biol Chem 2007;282:1183–92. doi:10.1074/jbc.M603761200.

[80] Lopez M, Petry A, Schulz R, Vina J, Winyard P, Abbas K. European contribution to the study of ROS : A summary of the findings and prospects for the future from the COST action BM1203 (EU-ROS). 2017;1203:1–17. doi:10.1016/j.redox.2017.05.007.

[81] Marinho, Susana H, Real C, Cyrne L, Soares H, Antunes F. Redox Biology Hydrogen peroxide sensing , signaling and regulation of transcription factors 2014;2:535–62. doi:10.1016/j.redox.2014.02.006.

[82] Sies H. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. Redox Biol 2017;11:613–9. doi:10.1016/j.redox.2016.12.035.

[83] Santos CX, Hafstad AD, Beretta M, Zhang M, Molenaar C, Kopec J, et al. Targeted redox inhibition of protein phosphatase 1 by Nox4 regulates eIF2 -mediated stress signaling. EMBO J 2016;35:319–34. doi:10.15252/embj.201592394.

[84] Mantzaris MD, Bellou S, Skiada V, Kitsati N, Fotsis T, Galaris D. Intracellular labile iron determines H2O2-induced apoptotic signaling via sustained activation of ASK1/JNK-p38 axis. Free Radic Biol Med 2016;97:454–65. doi:10.1016/j.freeradbiomed.2016.07.002.

[85] Nauseef W. Detection of superoxide anion and hydrogen peroxide production by cellular NADPH oxidases 2008;64:2391–404. doi:10.1038/jid.2014.371.

[86] Rhee SG, Woo HA. Multiple Functions of Peroxiredoxins: Peroxidases, Sensors and Regulators of the Intracellular Messenger H $_2$ O $_2$, and Protein Chaperones. Antioxid Redox Signal 2011;15:781–94. doi:10.1089/ars.2010.3393.

[87] Rhee SG, Woo HA, Kang D. The Role of Peroxiredoxins in the Transduction of H $_2$ O $_2$ Signals. Antioxid Redox Signal 2017;28:ars.2017.7167. doi:10.1089/ars.2017.7167.

[88] Ursini F, Maiorino M, Forman HJ. Redox homeostasis: The Golden Mean of healthy living. Redox Biol 2016;8:205–15. doi:10.1016/j.redox.2016.01.010.

[89] Sobotta MC, Liou W, Stöcker S, Talwar D, Oehler M, Ruppert T, et al. Peroxiredoxin-2 and STAT3 form a redox relay for H<inf>2</inf>O<inf>2</inf> signaling. Nat Chem Biol 2015;11:64–70. doi:10.1038/nchembio.1695.

[90] Makino N, Mise T, Sagara J ichi. Kinetics of hydrogen peroxide elimination by astrocytes and C6 glioma cells. Analysis based on a mathematical model. Biochim Biophys Acta - Gen Subj 2008;1780:927–36. doi:10.1016/j.bbagen.2008.03.010.

[91] Sasaki K, Bannai S, Makino N. Kinetics of hydrogen peroxide elimination by human umbilical vein endothelial cells in culture. Biochim Biophys Acta Gen Subj 1998;1380:275–88.

[92] Luo D, Smith SW, Anderson BD. Kinetics and mechanism of the reaction of cysteine and hydrogen peroxide in aqueous solution. J Pharm Sci 2005;94:304–16. doi:10.1002/jps.20253.

[93] Huang BK, Sikes HD. Quantifying intracellular hydrogen peroxide

perturbations in terms of concentration. Redox Biol 2014;2:955–62. doi:10.1016/j.redox.2014.08.001.

[94] Huang BK, Stein KT, Sikes HD. Modulating and Measuring Intracellular H2O2Using Genetically Encoded Tools to Study Its Toxicity to Human Cells. ACS Synth Biol 2016;5:1389–95. doi:10.1021/acssynbio.6b00120.

[95] Egea J, Fabregat I, Frapart YM, Ghezzi P, Görlach A, Kietzmann T, et al. Redox Biology European contribution to the study of ROS : A summary of the fi ndings and prospects for the future from the COST action BM1203 (EU-ROS) 2017;13:94–162. doi:10.1016/j.redox.2017.05.007.

[96] Y BH, Veronique M, Hua L. Hydrogen peroxide in the human body 2000;486:14–7.

[97] Oliveira-marques V. A Quantitative Study of NF- κ B Activation by H 2 O 2 : Relevance in Inflammation and Synergy with TNF- α 2017. doi:10.4049/jimmunol.178.6.3893.

[98] Oliveira-marques V, Silva T, Cunha F, Covas G, Marinho HS, Antunes F, et al. Redox Biology A quantitative study of the cell-type speci fi c modulation of c-Rel by hydrogen peroxide and TNF- α 2013;1:347–52. doi:10.1016/j.redox.2013.05.004.

[99] Benfeitas R, Selvaggio G, Antunes F, Coelho PMBM, Salvador A. Hydrogen peroxide metabolism and sensing in human erythrocytes: A validated kinetic model and reappraisal of the role of peroxiredoxin II. Free Radic Biol Med 2014;74:35–49. doi:10.1016/j.freeradbiomed.2014.06.007.

[100] Halliwell B, Gutterridge J. Free Radicals in Biology and Medicine. Fifth Edit. Oxford, UK: Oxford University Press; 2015.

[101] Sies H. Redox Biology Oxidative stress : a concept in redox biology and medicine. Redox Biol 2015;4:180–3. doi:10.1016/j.redox.2015.01.002.

[102] Mirończuk-Chodakowska I, Witkowska AM, Zujko ME. Endogenous non-enzymatic antioxidants in the human body. Adv Med Sci 2018;63:68–78. doi:10.1016/j.advms.2017.05.005.

[103] Sureda A, Ferrer MD, Tauler P, Romaguera D, Drobnic F, Pujol P, et al. Effects of exercise intensity on lymphocyte H2O2 production and antioxidant defences in soccer players. Br J Sports Med 2007;43:186–90. doi:10.1136/bjsm.2007.043943.

[104] Gaetani GF, Ferraris AM, Rolfo M, Mangerini R, Arena S, Kirkman HN. Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. Blood 1996;87:1595–9.

[105] Espinosa-Diez C, Miguel V, Mennerich D, Kietzmann T, Sánchez-Pérez P, Cadenas S, et al. Antioxidant responses and cellular adjustments to oxidative stress. Redox Biol 2015;6:183–97. doi:10.1016/j.redox.2015.07.008.

[106] Matés JM, Pérez-Gómez C, Castro IN de. Antioxidant Enzymes and Human Diseases 1999;32:595–603.

[107] Kulinsky VI, Kolesnichenko LS. The glutathione system. II. Other enzymes, thiol-disulfide metabolism, inflammation, and immunity, functions. Biochem Suppl Ser B Biomed Chem 2009;3:211–20. doi:10.1134/S1990750809030019.

[108] Goyal MM, Basak A. Human catalase: Looking for complete identity. Protein Cell 2010;1:888–97. doi:10.1007/s13238-010-0113-z.

[109] Mueller S, Millonig G, Waite GN. The GOX/CAT system: A novel enzymatic method to independently control hydrogen peroxide and hypoxia in cell culture. Adv Med Sci 2009;54:121–35. doi:10.2478/v10039-009-0042-3.

[110] Mueller S, Riedel HD, Stremmel W. Direct evidence for catalase as the predominant H2O2-removing enzyme in human erythrocytes. Blood 1997;90:4973–8.

[111] Cebula M, Schmidt EE, Arnér ESJ. TrxR1 as a Potent Regulator of the Nrf2-Keap1 Response System. Antioxid Redox Signal 2015;23:823–53. doi:10.1089/ars.2015.6378.

[112] Goglia F, Skulachev VP. A function for novel uncoupling proteins : antioxidant defense of mitochondrial matrix by translocating fatty acid peroxides from the inner to the outer. FASEB 2003;17:1585–91. doi:10.1096/fj.03-0159hyp.

[113] Jiang N, Zhang G, Bo H, Qu J, Ma G, Cao D, et al. Upregulation of uncoupling protein-3 in skeletal muscle during exercise: a potential antioxidant function. Free Radic Biol Med 2009;46:138–45. doi:10.1016/j.freeradbiomed.2008.09.026.

[114] Schrauwen P, Hesselink M. Uncoupling protein 3 and physical activity: the role of uncoupling protein 3 in energy metabolism revisited. Proc Nutr Soc 2003;62:635–43. doi:10.1079/PNS2003277.

[115] Shi T, Wang F, Stieren E, Tong Q. SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. J Biol Chem 2005;280:13560–7. doi:10.1074/jbc.M414670200.

[116] North BJ, Sinclair DA. Sirtuins: a conserved key unlocking AceCS activity. Trends Biochem Sci 2007;32:1–4. doi:10.1016/j.tibs.2006.11.002.

[117] Schlicker C, Gertz M, Papatheodorou P, Kachholz B, Becker CFW, Steegborn C. Substrates and Regulation Mechanisms for the Human Mitochondrial Sirtuins Sirt3 and Sirt5. J Mol Biol 2008;382:790–801. doi:10.1016/j.jmb.2008.07.048.

[118] Wong HS, Dighe PA, Mezera V, Monternier PA, Brand MD. Production of superoxide and hydrogen peroxide from specific mitochondrial sites under different bioenergetic conditions. J Biol Chem 2017;292:16804–9. doi:10.1074/jbc.R117.789271.

[119] Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009;417:1–13. doi:10.1042/BJ20081386.

[120] Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release. Physiol Rev 2014;94:909–50. doi:10.1152/physrev.00026.2013.

[121] Quinlan CL, Perevoshchikova I V., Hey-Mogensen M, Orr AL, Brand MD. Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. Redox Biol 2013;1:304–12. doi:10.1016/j.redox.2013.04.005.

[122] Sureda A. la ciencia al alcance de la mano. Sebbm Divulg 2018:0–1. doi:10.1371/journal.pone.0011273.SEBBM.

[123] Mestre-Alfaro A, Ferrer MD, Banquells M, Riera J, Drobnic F, Sureda A, et al. Body temperature modulates the antioxidant and acute immune responses to exercise. Free Radic Res 2012;46:799–808. doi:10.3109/10715762.2012.680193.

[124] Sachdev S, Davies KJA. Production, detection, and adaptive responses to free radicals in exercise. Free Radic Biol Med 2008;44:215–23. doi:10.1016/j.freeradbiomed.2007.07.019.

[125] Pinheiro CHJ, Vitzel KF, Curi R. Effect of N-acetylcysteine on markers of skeletal muscle injury after fatiguing contractile activity. Scand J Med Sci Sport 2012;22:24–33. doi:10.1111/j.1600-0838.2010.01143.x.

[126] Liu M, Timmons BW. The Effect of Acute Exercise on Neutrophil Reactive Oxygen Species Production and Inflammatory Markers in Healthy Prepubertal and Adult Males. Pediatr Exerc Sci 2016;28:55–63. doi:10.1123/pes.2014-0221.

[127] Tauler P, Aguiló A, Cases N, Sureda A, Gimenez F, Villa G, et al. Acute

phase immune response to exercise coexists with decreased neutrophil antioxidant enzyme defences. Free Radic Res 2002;36:1101–7. doi:10.1080/1071576021000028334.

[128] Tossige-Gomes R, Ottone VO, Oliveira PN, Viana DJS, Araújo TL, Gripp FJ, et al. Leukocytosis, muscle damage and increased lymphocyte proliferative response after an adventure sprint race. Brazilian J Med Biol Res 2014;47:492–8. doi:10.1590/1414-431X20143187.

[129] Sureda A, Batle JM, Tauler P, Cases N, Aguiló A, Tur JA, et al. Neutrophil tolerance to oxidative stress induced by hypoxia / reoxygeneration. Free Radic Res 2004;38:1003–9. doi:10.1080/10715760400000984.

[130] Carrera-Quintanar L, Funes L, Viudes E, Tur J, Micol V, Roche E, et al. Antioxidant effect of lemon verbena extracts in lymphocytes of university students performing aerobic training program. Scand J Med Sci Sport 2012;22:454–61. doi:10.1111/j.1600-0838.2010.01244.x.

[131] Mestre-Alfaro A, Ferrer MD, Sureda A, Tauler P, Marttínez E, Bibiloni MM, et al. Phytoestrogens enhance antioxidant enzymes after swimming exercise and modulate sex hormone plasma levels in female swimmers. Eur J Appl Physiol 2011;111:2281–94. doi:10.1007/s00421-011-1862-y.

[132] Powers S, Ji LL LC. Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. Med Sci Sport Exerc 1999;31:987–97.

[133] Tanimura Y, Shimizu K, Tanabe K, Kono I, Ajisaka R. Effects of three consecutive days exercise on lymphocyte DNA damage in young men. Eur J Appl Physiol 2010;110:307–14. doi:10.1007/s00421-010-1499-2.

[134] Azizbeigi K, Stannard SR, Atashak S, Mosalman Haghighi M. Antioxidant enzymes and oxidative stress adaptation to exercise training: Comparison of endurance, resistance, and concurrent training in untrained males. J Exerc Sci Fit 2014;12:1–6. doi:10.1016/j.jesf.2013.12.001.

[135] Turner JE, Bosch J a, Drayson MT, Aldred S. Assessment of oxidative stress in lymphocytes with exercise. J Appl Physiol 2011;111:206–11. doi:10.1152/japplphysiol.00051.2011.

[136] Turner JE, Bosch JA, Aldred S. Measurement of exercise-induced oxidative stress in lymphocytes. Biochem Soc Trans 2011;39:1299–304. doi:10.1042/BST0391299.

[137] Ferrer MD, Tauler P, Sureda A, Tur JA, Pons A. Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise. J Sports Sci 2009;27:49–58. doi:10.1080/02640410802409683.

[138] Chung HY, Cesari M, Anton S, Marzetti E, Giovannini S, Seo AY, et al. Molecular inflammation: Underpinnings of aging and age-related diseases. Ageing Res Rev 2009;8:18–30. doi:10.1016/j.arr.2008.07.002.

[139] Yan Z, Lira VA, Greene NP. Exercise training-induced regulation of mitochondrial quality. Exerc Sport Sci Rev 2012;40:159–64. doi:10.1097/JES.0b013e3182575599.

[140] Drake JC, Wilson RJ, Yan Z. Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle. FASEB J 2016;30:13–22. doi:10.1096/fj.15-276337.

[141] Urso ML, Fiatarone Singh MA, Ding W, Evans WJ, Cosmas AC, Manfredi TG. Exercise training effects on skeletal muscle plasticity and IGF-1 receptors in frail elders. Age (Omaha) 2005;27:117–25. doi:10.1007/s11357-005-1629-7.

[142] Brook MS, Wilkinson DJ, Phillips BE, Perez-Schindler J, Philp A, Smith K, et al. Skeletal muscle homeostasis and plasticity in youth and ageing: Impact of nutrition and exercise. Acta Physiol 2016;216:15–41. doi:10.1111/apha.12532.

[143] Hood D a, Irrcher I, Ljubicic V, Joseph A-M. Coordination of metabolic plasticity in skeletal muscle. J Exp Biol 2006;209:2265–75. doi:10.1242/jeb.02182.

[144] Irrcher I, Adhihetty PJ, Joseph A-M, Ljubicic V, Hood DA. Regulation of mitochondrial biogenesis in muscle by endurance exercise. Sports Med 2003;33:783–93. doi:10.2165/00007256-200333110-00001.

[145] Sureda A, Ferrer MD, Batle JM, Tauler P, Tur JA, Pons A. Scuba Diving Increases Erythrocyte and Plasma Antioxidant Defenses and Spares NO without Oxidative Damage n.d.:1271–6. doi:10.1249/MSS.0b013e3181951069.

[146] Radak Z, Taylor A, Ohno H. Adaptation to exercise-induced oxidative stress: from muscle to brain. Immunol Rev 2001.

[147] Perry CGR, Lally J, Holloway GP, Heigenhauser GJF, Bonen A, Spriet LL. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. J Physiol 2010;588:4795–810. doi:10.1113/jphysiol.2010.199448.

[148] Hollander J, Fiebig R, Gore M, Ookawara T, Ohno H, Ji L. Superoxide dismutase gene expression is activated by a single bout of exercise in rat skeletal muscle. Pflugers Arch Eur J Physiol 2001;442:426–34.

doi:10.1007/s004240100539.

[149] Moro-García Marco A, Fernández-García B, Alonso-Arias R, Rodriguez-Alonso M, Suárez García Francisco M, López- Larrea C. Effects of Maintained Intense Exercise Throughout the Life on the Adaptive Immune Response in Elderly and Young Athletes. Br J Sports Med 2013;47:e3.16–e3. doi:10.1136/bjsports-2013-092558.23.

[150] Martorell M, Capó X, Sureda A, Tur J a, Pons A. Effects of docosahexaenoic acid diet supplementation, training, and acute exercise on oxidative balance in neutrophils. Appl Physiol Nutr Metab 2014;39:446–57. doi:10.1139/apnm-2013-0331.

[151] Evans WJ, Cyr-Campbell D. Nutrition, exercise, and healthy aging. J Am Diet Assoc 1997;97:632–8. doi:10.1016/S0002-8223(97)00160-0.

[152] Stratton JR, Levy WC, Cerqueira MD, Schwartz RS, Abrass IB. Cardiovascular responses to exercise. Effects of aging and exercise training in healthy men. Circulation 1994;89:1648–55. doi:10.1161/01.CIR.89.4.1648.

[153] Knight E, Petrella RJ. Prescribing physical activity for healthy aging: Longitudinal follow-up and mixed method analysis of a primary care intervention. Phys Sportsmed 2014;42:30–8. doi:10.3810/psm.2014.11.2089.

[154] Cartee GD, Hepple RT, Bamman MM, Zierath JR, Arbor A, Physiology I, et al. Exercise Promotes Healthy Aging of Skeletal Muscle 2017;23:1034–47. doi:10.1016/j.cmet.2016.05.007.Exercise.

[155] Calabrese EJ. Hormetic mechanisms. Crit Rev Toxicol 2013;43:580–606. doi:10.3109/10408444.2013.808172.

[156] Gomez-Cabrera MC, Domenech E, Viña J. Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. Free Radic Biol Med 2008;44:126–31. doi:10.1016/j.freeradbiomed.2007.02.001.

[157] Venditti P, DiMeo S. Effect of training on antioxidant capacity, tissue damage, and endurance of adult male rats. Int J Sport Med 1997;18:497–502.

[158] Tauler P, Gimeno I, Aguiló A, Guix MP, Pons A. Regulation of erythrocyte antioxidant enzyme activities in athletes during competition and short-term recovery. Pflugers Arch Eur J Physiol 1999;438:782–7. doi:10.1007/s004240051107.

[159] Gomez-Cabrera MC, Salvador-Pascual A, Cabo H, Ferrando B, Vina J.

Redox modulation of mitochondriogenesis in exercise. Does antioxidant supplementation blunt the benefits of exercise training? Free Radic Biol Med 2015;86:37–46. doi:10.1016/j.freeradbiomed.2015.04.006.

[160] Fisher G, Schwartz DD, Quindry J, Barberio MD, Foster EB, Jones KW, et al. Lymphocyte enzymatic antioxidant responses to oxidative stress following high-intensity interval exercise. J Appl Physiol 2011;110:730–7. doi:10.1152/japplphysiol.00575.2010.

[161] Ji LL. Antioxidant enzyme response to exercise and aging. Med Sci Sports Exerc 1993;25:225–31.

[162] Ji LL. Antioxidants and Oxidative Stress in Exercise. Soc Exp Biol Med 1999:283–92.

[163] Latimer-Cheung AE, Pilutti LA, Hicks AL, Martin Ginis KA, Fenuta AM, MacKibbon KA, et al. Effects of exercise training on fitness, mobility, fatigue, and health-related quality of life among adults with multiple sclerosis: A systematic review to inform guideline development. Arch Phys Med Rehabil 2013;94:1800–28. doi:10.1016/j.apmr.2013.04.020.

[164] Bailey DM, Williams C, Betts JA, Thompson D, Hurst TL. Oxidative stress, inflammation and recovery of muscle function after damaging exercise: Effect of 6-week mixed antioxidant supplementation. Eur J Appl Physiol 2011;111:925–36. doi:10.1007/s00421-010-1718-x.

[165] Das UN. Exercise and inflammation [7]. Eur Heart J 2006;27:1385. doi:10.1093/eurheartj/ehi867.

[166] Petersen AMW, Pedersen BK. The anti-inflammatory effect of exercise. J Appl Physiol 2005;98:1154–62. doi:10.1152/japplphysiol.00164.2004.

[167] Cannon JG, Orencole SF, Fielding RA, Meydani M, Meydani SN, Fiatarone MA, Blumberg JB EW. Acute phase response in exercise: interaction of age and vitamin E. Am J Physiol 1990;259:1214–9.

[168] Pillon NJ, Bilan PJ, Fink LN, Klip A. Cross-talk between skeletal muscle and immune cells: muscle-derived mediators and metabolic implications. AJP Endocrinol Metab 2013;304:E453–65. doi:10.1152/ajpendo.00553.2012.

[169] Walsh NP, Gleeson M, Shephard RJ, Jeffrey MG, Woods A, Bishop NC, et al. Part one : Immune function and exercise 2011:6–63.

[170] Steppich B, Dayyani F, Gruber R, Lorenz R, Mack M, Ziegler-Heitbrock HWL. Selective mobilization of $CD14^{+}$ $CD16^{+}$ monocytes by exercise. Am J Physiol Physiol 2000;279:C578–86. doi:10.1152/ajpcell.2000.279.3.C578.

[171] Córdova A, Sureda A, Tur JA, Pons A. Immune response to exercise in elite sportsmen during the competitive season. J Physiol Biochem 2010;66:1–6. doi:10.1007/s13105-010-0001-2.

[172] Fry RW, Morton AR, Garcia-Webb P, Crawford GPM, Keast D. Biological responses to overload training in endurance sports. Eur J Appl Physiol Occup Physiol 1992;64:335–44. doi:10.1007/BF00636221.

[173] Natale, V.M., Brenner, I.K., Moldoveanu, A.I., Vasiliou, P., Shek, P., Shephard R. Effects of Three Different Types of Exercise on Blood Leukocyte Count During and Following Exercise. Sao Paulo Med Journal/Revista Paul Med 2003;121:9–14.

[174] Sprenger H, Jacobs C, Nain M, Gressner AM, Prinz H, Wesemann W, et al. Enhanced release of cytokines, interleukin-2 receptors, and neopterin after long-distance running. Clin Immunol Immunopathol 1992;63:188–95. doi:10.1016/0090-1229(92)90012-D.

[175] Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. J Physiol 1999;515 (Pt 1:287–91. doi:10.1111/j.1469-7793.1999.287ad.x.

[176] Haahr PM, Pedersen BK, Fomsgaard A, Tvede N, Diamant M, Klarlund K, et al. Effect of physical exercise on in vitro production of interleukin 1, interleukin 6, tumour necrosis factor-alpha, interleukin 2 and interferon-gamma. Int J Sports Med 1991;12:223–7. doi:10.1055/s-2007-1024672.

[177] Ostrowski K, Schjerling P, Pedersen BK. Physical activity and plasma interleukin-6 in humans--effect of intensity of exercise. Eur J Appl Physiol 2000;83:512–5. doi:10.1007/s004210000312.

[178] Steensberg A, Keller C, Starkie RL, Osada T, Febbraio MA, Pedersen BK. IL-6 and TNF-α expression in, and release from, contracting human skeletal muscle. Am J Physiol - Endocrinol Metab 2002;283:E1272–8. doi:10.1152/ajpendo.00255.2002.

[179] Gleeson M. Immune function in sport and exercise. J Appl Physiol 2007;103:693–9. doi:00008.2007 [pii]\r10.1152/japplphysiol.00008.2007.

[180] Brenner IKM, Natale VM, Vasiliou P, Moldoveanu AI, Shek PN, Shephard RJ. Impact of three different types of exercise on components of the inflammatory response. Eur J Appl Physiol Occup Physiol 1999;80:452–60. doi:10.1007/s004210050617.

[181] Gleeson M BN. The T cell and NK cell immune response to exercise. Ann Transplant 2005;10:43–8.

[182] Suzuki K, Nakaji S, Yamada M, Totsuka M, Sato K, Sugawara K. Systemic inflammatory response to exhaustive exercise. Cytokine kinetics. Exerc Immunol Rev 2002;8:6–48.

[183] Mastorakos G, Weber J. Recominant Interleukin-6 activates the hypothalamic-pituitary-adrenal axis in humans 2015;77:1690–4.

[184] Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: Mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol 2011;11:607–10. doi:10.1038/nri3041.

[185] Gleeson M, McFarlin B, Flynn M. Exercise and Toll-like receptors. Exerc Immunol Rev 2006;12:34–53.

[186] Bilski J, Brzozowski B, Mazur-Bialy A, Sliwowski Z, Brzozowski T. The role of physical exercise in inflammatory bowel disease. Biomed Res Int 2014;2014. doi:10.1155/2014/429031.

[187] Szostak J, Laurant P. The forgotten face of regular physical exercise: a “natural” anti-atherogenic activity. Clin Sci 2011;121:91–106. doi:10.1042/CS20100520.

[188] Steensberg A, Fischer CP, Keller C, Møller K, Pedersen BK. IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. Am J Physiol - Endocrinol Metab 2003;285:E433–7. doi:10.1152/ajpendo.00074.2003.

[189] Balducci S, Zanuso S, Nicolucci A, Fernando F, Cavallo S, Cardelli P, et al. Anti-inflammatory effect of exercise training in subjects with type 2 diabetes and the metabolic syndrome is dependent on exercise modalities and independent of weight loss. Nutr Metab Cardiovasc Dis 2010;20:608–17. doi:10.1016/j.numecd.2009.04.015.

[190] Kadoglou NPE, Iliadis F, Angelopoulou N, Perrea D, Ampatzidis G, Liapis CD, et al. The anti-inflammatory effects of exercise training in patients with type 2 diabetes mellitus. Eur J Prev Cardiol 2007;14:837–43. doi:10.1097/HJR.0b013e3282efaf50.

[191] Brandt C, Jakobsen AH, Adser H, Olesen J, Iversen N, Kristensen JM, et al. IL-6 regulates exercise and training-induced adaptations in subcutaneous adipose tissue in mice 2012:224–35. doi:10.1111/j.1748-1716.2011.02373.x.

[192] Febbraio MA, Steensberg A, Starkie RL, McConell GK, Kingwell BA. Skeletal muscle interleukin-6 and tumor necrosis factor-α release in healthy subjects and patients with type 2 diabetes at rest and during exercise.

Metabolism 2003;52:939–44. doi:10.1016/S0026-0495(03)00105-7.

[193] Gritti F. Il senso del sacro nella poetica di pasolini. XLinguae 2012;5:42–53. doi:10.1096/fj.02-0670fje.

[194] Glancy B, Hartnell LM, Malide D, Yu ZX, Combs CA, Connelly PS, et al. Mitochondrial reticulum for cellular energy distribution in muscle. Nature 2015;523:617–20. doi:10.1038/nature14614.

[195] Sukhorukov VM, Dikov D, Reichert AS, Meyer-Hermann M. Emergence of the Mitochondrial Reticulum from Fission and Fusion Dynamics. PLoS Comput Biol 2012;8. doi:10.1371/journal.pcbi.1002745.

[196] Santel a, Fuller MT. Control of mitochondrial morphology by a human mitofusin. J Cell Sci 2001;114:867–74. doi:mitochondrial fusion fzo human gtpase dynamin related protein mitofusin outer-membrane protein dynamin-related gtpase saccharomyces-cerevisiae coiled coils fusion yeast reticulum fzo1p maintenance fission.

[197] Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, et al. Mitochondrial Biogenesis in Mammals : The Role of Endogenous Nitric Oxide 2003;299:896–9.

[198] Nisoli E, Clementi E, Moncada S, Carruba MO. Mitochondrial biogenesis as a cellular signaling framework. Biochem Pharmacol 2004;67:1–15. doi:10.1016/j.bcp.2003.10.015.

[199] Fernández-silva P, Enriquez J a, Montoya J. Special Review Series – Biogenesis and Physiological Adaptation of Mitochondria Replication and transcription of mammalian mitochondrial DNA Experimental Physiology. Exp Physiol 1999;88:41–56. doi:10.1113/eph8802513.

[200] Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 1999;98:115–24. doi:10.1016/S0092-8674(00)80611-X.

[201] Palikaras K, Tavernarakis N. Mitochondrial homeostasis: The interplay between mitophagy and mitochon1. Palikaras K, Tavernarakis N. Mitochondrial homeostasis: The interplay between mitophagy and mitochondrial biogenesis. Experimental Gerontology. 2014. p. 182–8. drial biogenesis. Exp Gerontol 2014;56:182–8. doi:10.1016/j.exger.2014.01.021.

[202] Shulman FRJGI. Regulation of mitochondrial biogenesis. Essays Biochem 2010;47. doi:10.1042/bse0470069.Regulation.

[203] Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. Physiol Rev 2004;84:277–359. doi:10.1152/physrev.00015.2003.

[204] Scarpulla RC, Vega RB, Kelly DP. Transcriptional integration of mitochondrial biogenesis. Trends Endocrinol Metab 2012;23:459–66. doi:10.1016/j.tem.2012.06.006.

[205] Dominy JE, Puigserver P, Schwarz TL, Stojanovski D, Bohnert M. Mitochondrial Biogenesis through Activation of Nuclear Signaling Proteins. Cold Spring Harb Perspect Biol 2014:1–18. doi:10.1101/cshperspect.a015008.

[206] Austin S, St-Pierre J. PGC1 and mitochondrial metabolism - emerging concepts and relevance in ageing and neurodegenerative disorders. J Cell Sci 2012;125:4963–71. doi:10.1242/jcs.113662.

[207] Villena JA. New insights into PGC-1 coactivators: Redefining their role in the regulation of mitochondrial function and beyond. FEBS J 2015;282:647–72. doi:10.1111/febs.13175.

[208] Shi Y, Dierckx A, Wanrooij PH, Wanrooij S, Larsson N-G, Wilhelmsson LM, et al. Mammalian transcription factor A is a core component of the mitochondrial transcription machinery. Proc Natl Acad Sci 2012;109:16510–5. doi:10.1073/pnas.1119738109.

[209] Toda C, Diano S. Mitochondrial dynamics in the central regulation of metabolism. Best Pract Res Clin Endocrinol Metab 2014;28:757–64. doi:10.1016/j.beem.2014.02.006.

[210] Jornayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. Essays Biochem 2010;47:69–84. doi:10.1042/bse0470069.

[211] van der Bliek AM, Shen Q, Kawajiri S. Mechanisms of Mitochondrial Fission and Fusion. Cold Spring Harb Perspect Biol 2013;5:a011072–a011072. doi:10.1101/cshperspect.a011072.

[212] Youle RJ, Karbowski M. Mitochondrial fission in apoptosis. Nat Rev Mol Cell Biol 2005;6:657–63.

[213] Youle RJ, van der Bliek AM. Mitochondrial fission, fusion, and stress. Science 2012;337:1062–5. doi:10.1126/science.1219855.

[214] Kuzmicic J, Del Campo A, López-Crisosto C, Morales PE, Pennanen C, Bravo-Sagua R, et al. Dinámica mitocondrial: un potencial nuevo blanco terapéutico para la insuficiencia cardiaca. Rev Esp Cardiol 2011;64:916–23. doi:10.1016/j.recesp.2011.05.018.

[215] Chen H, Chan DC. Emerging functions of mammalian mitochondrial fusion and fission. Hum Mol Genet 2005;14:283–9. doi:10.1093/hmg/ddi270.

[216] Meeusen S, McCaffery M, Nunnari J. Mitochondrial Fusion Intermediates Revealed in vitro 2010;305:1747–52.

[217] Zamponi N, Zamponi E, Cannas SA, Billoni O V., Helguera PR, Chialvo DR. Mitochondrial network complexity emerges from fission/fusion dynamics. Sci Rep 2018;8:1–10. doi:10.1038/s41598-017-18351-5.

[218] Ishihara N. Mitofusin 1 and 2 play distinct roles in mitochondrial fusion reactions via GTPase activity. J Cell Sci 2004;117:6535–46. doi:10.1242/jcs.01565.

[219] Koshiba T, Detmer SA, Kaiser JT, Chen H, McCaffery JM, Chan DC. Structural basis of mitochondrial tethering by mitofusin complexes. Science (80-) 2004;305:858–62. doi:10.1126/science.1099793.

[220] Andrew B. Knott, Guy Perkins, Robert Schwarzenbacher and EB-W. MITOCHONDRIAL FRAGMENTATION IN NEURODEGENERATION. Nat Rev Neurosci 2008;9:505–18. doi:10.1038/nrn2417.MITOCHONDRIAL.

[221] Song Z, Chen H, Fiket M, Alexander C, Chan DC. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. J Cell Biol 2007;178:749–55. doi:10.1083/jcb.200704110.

[222] Cipolat S, de Brito OM, Dal Zilio B, Scorrano L. OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc Natl Acad Sci 2004;101:15927–32. doi:10.1073/pnas.0407043101.

[223] Twig G, Elorza A, Molina AJA, Mohamed H, Wikstrom JD, Walzer G, et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J 2008;27:433–46. doi:10.1038/sj.emboj.7601963.

[224] Patrushev. Mitochondrial fission and fusion. Essays Biochem 2010;47:85–98. doi:10.1042/bse0470085.

[225] Merz S, Hammermeister M, Altmann K, Dürr M, Westermann B. Molecular machinery of mitochondrial dynamics in yeast. Biol Chem 2007;388:917–26. doi:10.1515/BC.2007.110.

[226] Yaffe MP. The machinery of mitochondrial behavior. Science (80-) 1999;283:1493–7.

[227] Twig G, Shirihai OS. The Interplay Between Mitochondrial Dynamics and

Mitophagy. Antioxid Redox Signal 2011;14:1939–51. doi:10.1089/ars.2010.3779.

[228] Head B, Griparic L, Amiri M, Gandre-Babbe S, Van Der Bliek AM. Inducible proteolytic inactivation of OPA1 mediated by the OMA1 protease in mammalian cells. J Cell Biol 2009;187:959–66. doi:10.1083/jcb.200906083.

[229] James DI, Parone PA, Mattenberger Y, Martinou JC. hFis1, a novel component of the mammalian mitochondrial fission machinery. J Biol Chem 2003;278:36373–9. doi:10.1074/jbc.M303758200.

[230] Losón OC, Song Z, Chen H, Chan DC. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. Mol Biol Cell 2013;24:659–67. doi:10.1091/mbc.E12-10-0721.

[231] Yin F, Cadenas E. Mitochondria: The Cellular Hub of the Dynamic Coordinated Network. Antioxid Redox Signal 2015;22:961–4. doi:10.1089/ars.2015.6313.

[232] Cartoni R, Leger B, Hock MB, Praz M, Crettenand A, Pich S, et al. Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. J Physiol 2005;567:349–58. doi:10.1113/jphysiol.2005.092031.

[233] Ding WX, Yin XM. Mitophagy: Mechanisms, pathophysiological roles, and analysis. Biol Chem 2012;393:547–64. doi:10.1515/hsz-2012-0119.

[234] Kirkwood TBL. Understanding the odd science of aging. Cell 2005;120:437–47. doi:10.1016/j.cell.2005.01.027.

[235] Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. J Pathol 2007;211:144–56. doi:10.1002/path.2104.

[236] Sureda A, Del Mar Bibiloni M, Julibert A, Aparicio-Ugarriza R, Palacios-Le Blé G, Pons A, et al. Trace element contents in toenails are related to regular physical activity in older adults. PLoS One 2017;12:1–11. doi:10.1371/journal.pone.0185318.

[237] Franzke B, Neubauer O, Wagner KH. Super DNAging-New insights into DNA integrity, genome stability and telomeres in the oldest old. Mutat Res - Rev Mutat Res 2015;766:48–57. doi:10.1016/j.mrrev.2015.08.001.

[238] Goloubinoff P. Mechanisms of protein homeostasis in health, aging and disease. Swiss Med Wkly 2016;146:w14306. doi:10.4414/smw.2016.14306.

[239] Knight E, Stuckey MI, Petrella RJ. Prescribing physical activity through primary care: Does activity intensity matter? Phys Sportsmed 2014;42:78–9. doi:10.3810/psm.2014.09.2079.

[240] Ježek J, Cooper K, Strich R. Reactive Oxygen Species and Mitochondrial Dynamics: The Yin and Yang of Mitochondrial Dysfunction and Cancer Progression. Antioxidants 2018;7:13. doi:10.3390/antiox7010013.

[241] Sanz A, Stefanatos RKA. The Mitochondrial Free Radical Theory of Aging: A Critical View. Curr Aging Sci 2008;1:10–21. doi:10.2174/1874609810801010010.

[242] Loeb LA, Wallace DC, Martin GM. The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations. Proc Natl Acad Sci 2005;102:18769–70. doi:10.1073/pnas.0509776102.

[243] Jin K, Simpkins JW, Ji X, Leis M, Stambler I. The Critical Need to Promote Research of Aging and Aging-related Diseases to Improve Health and Longevity of the Elderly Population. Aging Dis 2015;6:1. doi:10.14336/AD.2014.1210.

[244] Eklund KN, Kovanen P. Immune functions of serum amyloid A. Crit Rev Immunol 2012;32:335–48.

[245] Michaud M, Balardy L, Moulis G, Gaudin C, Peyrot C, Vellas B, et al. Proinflammatory cytokines, aging, and age-related diseases. J Am Med Dir Assoc 2013;14:877–82. doi:10.1016/j.jamda.2013.05.009.

[246] Franceschi C, Garagnani P, Parini P, Giuliani C, Santoro A. Inflammaging: a new immune–metabolic viewpoint for age-related diseases. Nat Rev Endocrinol 2018;14:576–90. doi:10.1038/s41574-018-0059-4.

[247] Franceschi C, Campisi J. Chronic inflammation (Inflammaging) and its potential contribution to age-associated diseases. Journals Gerontol - Ser A Biol Sci Med Sci 2014;69:S4–9. doi:10.1093/gerona/glu057.

[248] Driver JA, Djoussé L, Logroscino G, Gaziano JM, Kurth T. Incidence of cardiovascular disease and cancer in advanced age: Prospective cohort study. Bmj 2008;337:1400–3. doi:10.1136/bmj.a2467.

[249] Cowie CC, Rust KF, Byrd-Holt DD, Eberhardt MS, Flegal KM, Engelgau MM, et al. Prevalence of diabetes and impaired fasting glucose in adults in the U.S. population: National Health and Nutrition Examination Survey 1999-2002. Diabetes Care 2006;29:1263–8. doi:10.2337/dc06-0062.

[250] Perry EK. Alzheimer's disease and vascular dementia in developing

countries: prevalence, management, and risk factors. Lancet Neurol 2008;7:812–26. doi:10.1016/S1474-4422(08)70169-8.Alzheimer.

[251] Woods J, Wilund K, Martin S, Kistler B. Exercise, inflammation and aging. Aging Dis 2012;3:130–40. doi:10.1016/j.vacuum.2008.01.038.

[252] Thompson PD, Buchner D, Piña IL, Balady GJ, Williams MA, Marcus BH, et al. Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease. Circulation 2003;107:3109–16. doi:10.1161/01.CIR.0000075572.40158.77.

[253] Teri L, Gibbons LE, Mccurry SM, Logsdon RG, Buchner DM, Barlow WE, et al. Exercise Plus Behavioral Management 2015;290:2015–22.

[254] Daley MJ, Spinks WL. Exercise, mobility and aging. Sport Med 2000;29:1–12. doi:10.2165/00007256-200029010-00001.

[255] Frankel JE, Bean JF, Frontera WR. Exercise in the Elderly: Research and Clinical Practice. Clin Geriatr Med 2006;22:239–56. doi:10.1016/j.cger.2005.12.002.

[256] Perna F, Schneiderman N, LaPerriere A. Psychological Stress, Exercise and Immunity. Int J Sports Med 1997;18:S78–83. doi:10.1055/s-2007-972703.

[257] Axelsson J, Heimbürger O, Lindholm B, Stenvinkel P. Adipose tissue and its relation to inflammation: The role of adipokines. J Ren Nutr 2005;15:131–6. doi:10.1053/j.jrn.2004.09.034.

[258] Nihi M, Manfro R, Martins C. Association between body fat, inflammation and oxidative stress in hemodialysis. J Bras … 2010:9–15.

[259] Folsom AR, Pankow JS, Tracy RP, Arnett DK, Peacock JM, Hong Y. Association of C-Reactive Protein Atherosclerotic Disease 2001;88:112–7.

[260] Short KR, Short KR, Vittone JL, Bigelow ML, Vittone JL, Bigelow ML, et al. Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. Diabetes 2003;52:1888–96. doi:10.2337/diabetes.52.8.1888.

[261] Silva LCR, de Araújo AL, Fernandes JR, Matias M de ST, Silva PR, Duarte AJS, et al. Moderate and intense exercise lifestyles attenuate the effects of aging on telomere length and the survival and composition of T cell subpopulations. Age (Omaha) 2016;38:1–16. doi:10.1007/s11357-016-9879-0.

[262] Ji LL. Exercise-induced Modulation of Antioxidant Defense. J Gastroenterol Hepatol 2002;959:82–92. doi:10.1111/j.1440-1746.2007.05180.x.

[263] Karolkiewicz J, Szczêsniak L, Deskur-Smielecka E, Nowak A, Stemplewski R, Szeklicki R. Oxidative stress and antioxidant defense system in healthy, elderly men: Relationship to physical activity. Aging Male 2003;6:100–5. doi:10.1080/tam.6.2.100.105.

[264] Valentine RC, Valentine DL. Omega-3 fatty acids in cellular membranes: A unified concept. Prog Lipid Res 2004;43:383–402. doi:10.1016/j.plipres.2004.05.004.

[265] Calder PC, Yaqoob P. Understanding Omega-3 Polyunsaturated Fatty Acids. Postgr Med 2009;121:148–57.

[266] Garneau V, Rudkowska I, Paradis A-M, Godin G, Julien P, Pérusse L, et al. Omega-3 fatty acids status in human subjects estimated using a food frequency questionnaire and plasma phospholipids levels. Nutr J 2012;11:46. doi:10.1186/1475-2891-11-46.

[267] Flock MR, Skulas-ray AC, Harris WS, Gaugler TL, Jennifer A, Kris-etherton PM. Effects of supplemental long-chain omega-3 fatty acids and erythrocyte membrane fatty acid content on circulating inflammatory markers in a randomized controlled trial of healthy adults 2015:1–21. doi:10.1016/j.plefa.2014.07.006.Effects.

[268] Massaro M, Habib A, Lubrano L, Turco SD, Lazzerini G, Bourcier T, et al. The omega-3 fatty acid docosahexaenoate attenuates endothelial cyclooxygenase-2 induction through both NADP(H) oxidase and PKC inhibition. Proc Natl Acad Sci 2006;103:15184–9. doi:10.1073/pnas.0510086103.

[269] Monmai C, Go S, Shin I-S, You S, Lee H, Kang S, et al. Immune-Enhancement and Anti-Inflammatory Activities of Fatty Acids Extracted from Halocynthia aurantium Tunic in RAW264.7 Cells. Mar Drugs 2018;16:309. doi:10.3390/md16090309.

[270] Capo X, Martorell M, Sureda A, Tur JA, Pons A. Effects of docosahexaenoic supplementation and in vitro vitamin C on the oxidative and inflammatory neutrophil response to activation. Oxid Med Cell Longev 2015;2015. doi:10.1155/2015/187849.

[271] Arterburn L, Hall E, Oken H. Distribution, interconversion, and dose response of n− 3 fatty acids in humans. Am J Cinical Nutr 2006;83:1467S – 1476S. doi:10.1093/ajcn/83.6.1467s.

[272] Martorell M, Capó X, Sureda A, Batle JM, Llompart I, Argelich E, et al. Effect of DHA on plasma fatty acid availability and oxidative stress during training season and football exercise. Food Funct 2014;5:1920–31.

doi:10.1039/C4FO00229F.

[273] Tatsumi Y, Kato A, Sango K, Himeno T, Kondo M, Kato Y, et al. Omega-3 Polyunsaturated Fatty Acids Exert Antioxidant Effects Through the Nrf2 Pathway in the Immortalized Mouse Schwann IMS32 Cells. J Diabetes Investig 2018:0–1. doi:10.1111/jdi.12931.

[274] Ishikado A, Morino K, Nishio Y, Nakagawa F, Mukose A, Sono Y, et al. 4-Hydroxy Hexenal Derived from Docosahexaenoic Acid Protects Endothelial Cells via Nrf2 Activation. PLoS One 2013;8. doi:10.1371/journal.pone.0069415.

[275] Kusunoki C, Yang L, Yoshizaki T, Nakagawa F, Ishikado A, Kondo M, et al. Omega-3 polyunsaturated fatty acid has an anti-oxidant effect via the Nrf-2/HO-1 pathway in 3T3-L1 adipocytes. Biochem Biophys Res Commun 2013;430:225–30. doi:10.1016/j.bbrc.2012.10.115.

[276] Jeukendrup AE, Aldred S. Fat supplementation, health, and endurance performance. Nutrition 2004;20:678–88. doi:10.1016/j.nut.2004.04.018.

[277] Peoples GE, McLennan PL, Howe PRC, Groeller H. Fish oil reduces heart rate and oxygen consumption during exercise. J Cardiovasc Pharmacol 2008;52:540–7. doi:10.1097/FJC.0b013e3181911913.

[278] Esteve H. Efectos de la ingesta del ácido docosahexaenoico (dha) sobre la velocidad de reacción y el bienestar psicológico en mujeres deportistas. 2012.

[279] Bentley DJ, Ackerman J, Clifford T. Acute and Chronic Supplementation on 1999:141–54.

[280] Busquets-Cortés C, Capó X, Martorell M, Tur JA, Sureda A, Pons A. Training Enhances Immune Cells Mitochondrial Biosynthesis, Fission, Fusion, and Their Antioxidant Capabilities Synergistically with Dietary Docosahexaenoic Supplementation. Oxid Med Cell Longev 2016;2016:2016:8950384. doi:10.1155/2016/8950384.

[281] Connor WE. Importance of omega 3 fatty acids in health and disease 1 – 3 2000;71:1–5.

[282] Brilla LR LT. Effect of fish oil supplementation and exercise on serum lipids and aerobic fitness. J Sport Med Phys Fitness 1990;30:173–80.

[283] Lamb PW, Melton MA, Yakel JL. Inhibition of Neuronal Nicotinic Acetylcholine Receptor Channels Expressed in Xenopus Oocytes by β-Amyloid1-42 Peptide. J Mol Neurosci 2005;27:13–22. doi:10.1385/JMN.

[284] Putti R, Sica R, Migliaccio V, Lionetti L. Diet impact on Mitochondrial Bioenergetics and Dynamics. Front Physiol 2015;6:1–7. doi:10.3389/fphys.2015.00109.

[285] Rastogi RP, Singh SP, Häder D, Sinha RP. Biochemical and Biophysical Research Communications Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2 0 , 7 0 -dichlorodihydrofluorescein diacetate in the cyanobacterium Anabaena variabilis PCC 7937. Biochem Biophys Res Commun 2010;397:603–7. doi:10.1016/j.bbrc.2010.06.006.

[286] Kaludercic N, Deshwal S, Di Lisa F. Reactive oxygen species and redox compartmentalization. Front Physiol 2014;5 JUL:1–15. doi:10.3389/fphys.2014.00285.

[287] Ferrer MD, Sureda A, Mestre A, Tur J a, Pons A. Cellular Physiology and Biochemistr y Biochemistry The Double Edge of Reactive Oxygen Species as Damaging and Signaling Molecules in HL60 Cell Culture 2010.

[288] Di Meo S, Reed TT, Venditti P, Victor VM. Role of ROS and RNS Sources in Physiological and Pathological Conditions. Oxid Med Cell Longev 2016;2016. doi:10.1155/2016/1245049.

[289] Aldosari S, Awad M, Harrington E, Sellke F, Abid M. Subcellular Reactive Oxygen Species (ROS) in Cardiovascular Pathophysiology. Antioxidants 2018;7:14. doi:10.3390/antiox7010014.

[290] Di Meo S, Reed TT, Venditti P, Victor VM. Harmful and Beneficial Role of ROS. Oxid Med Cell Longev 2016;2016:1–3. doi:10.1155/2016/7909186.

[291] Radak Z, Chung HY, Goto S. Systemic adaptation to oxidative challenge induced by regular exercise. Free Radic Biol Med 2008;44:153–9. doi:10.1016/j.freeradbiomed.2007.01.029.

[292] Radak Z, Taylor AW, Ohno H GS. Adaptation to exercise-induced oxidative stress: from muscle to brain. Exerc Immunol Rev 2001;7:90–107.

[293] Farhat F, Dupas J, Amérand A, Goanvec C, Feray A, Simon B, et al. Effect of exercise training on oxidative stress and mitochondrial function in rat heart and gastrocnemius muscle. Redox Rep 2015;20:60–8. doi:10.1179/1351000214Y.0000000105.

[294] Aparicio-ugarriza R, Luzardo-socorro R, Palacios G, Julibert A, Tur JA. Impact of physical activity and sedentarism on hydration status and liquid intake in Spanish older adults . The PHYSMED study 2016;33:4–8.

[295] Who WHO. Global recommendations on physical activity for health. Geneva World Heal Organ 2010:60. doi:10.1080/11026480410034349.

[296] Prasad D, Das B. Physical inactivity : A cardiovascular risk factor. Indian J Med Sci 2009;63:33. doi:10.4103/0019-5359.49082.

[297] Senchina DS, Kohut ML. Immunological outcomes of exercise in older adults. Clin Interv Aging 2007;2:3–16. doi:10.2147/ciia.2007.2.1.3.

[298] Li L, Kang C, Zhang Y. Exercise-induced hormesis and skeletal muscle health. Free Radic Biol Med 2016;98:113–22. doi:10.1016/j.freeradbiomed.2016.02.025.

[299] He F, Li J, Liu Z, Chuang CC, Yang W, Zuo L. Redox mechanism of reactive oxygen species in exercise. Front Physiol 2016;7:1–10. doi:10.3389/fphys.2016.00486.

[300] Powers SK, Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. 2008;88:1243–76. doi:10.1152/physrev.00031.2007.Exercise-Induced.

[301] Kazumi M, Tanabe K, Kuno S. Exercise and Reactive Oxygen Species in Elderly. Int J Sport Heal Sci 2006;4:348–59.

[302] Ru-Rong Ji, Zhen-Zhong Xu, Gary Strichartz and CNS. Emerging Roles of Resolvins in the Resolution of Inflammation and Pain. Trends Neurosci 2011;34:599–609. doi:10.1016/j.tins.2011.08.005.Emerging.

[303] Capó X, Martorell M, Busquets-Cortés C, Tejada S, Tur JA, Pons A, et al. Resolvins as proresolving inflammatory mediators in cardiovascular disease. Eur J Med Chem 2018;153. doi:10.1016/j.ejmech.2017.07.018.

[304] Arab-Tehrany E, Jacquot M, Gaiani C, Imran M, Desobry S, Linder M. Beneficial effects and oxidative stability of omega-3 long-chain polyunsaturated fatty acids. Trends Food Sci Technol 2012;25:24–33. doi:10.1016/j.tifs.2011.12.002.

[305] Fayh APT, Borges K, Cunha GS, Krause M, Rocha R, de Bittencourt PIH, et al. Effects of n-3 fatty acids and exercise on oxidative stress parameters in type 2 diabetic: A randomized clinical trial. J Int Soc Sports Nutr 2018;15:1–9. doi:10.1186/s12970-018-0222-2.

[306] Martorell M, Capó X, Sureda A, Batle JM, Llompart I, Argelich E, et al. Effect of DHA on plasma fatty acid availability and oxidative stress during training season and football exercise. Food Funct 2014;5:1920–31. doi:10.1039/c4fo00229f.

[307] Filaire E, Massart A, Portier H, Rouveix M, Rosado F, Bage AS, et al. Effect of 6 weeks of n-3 fatty-acid supplementation on oxidative stress in judo athletes. Int J Sport Nutr Exerc Metab 2010;20:496–506.

doi:10.1123/ijsnem.20.6.496.

[308] Toft AD, Thorn M, Ostrowski K, Asp S, Moller K, Iversen S, et al. N-3 polyunsaturated fatty acids do not affect cytokine response to strenuous exercise. J Appl Physiol 2000;89:2401–6. doi:10.1152/JAPPL.2000.89.6.2401.

[309] Mahjoub S, Roudsari JM. Role of oxidative stress in pathogenesis of metabolic syndrome. Casp J Intern Med 2012;3:386–96. doi:10.1016/j.psyneuen.2004.05.007.

[310] Yan L-Y, Forster M. Chemical probes for analysis of carbonylated proteins: a review. Eur Respir J 2010;879:1308–15. doi:10.1016/j.jchromb.2010.08.004.Chemical.

[311] Uchida K, Sakai K, Itakura K, Osawa T, Toyokuni S. Protein modification by lipid peroxidation products: formation of malondialdehyde-derived N(epsilon)-(2-propenol)lysine in proteins. Arch Biochem Biophys 1997;346:45–52. doi:http://dx.doi.org/10.1006/abbi.1997.0266.

[312] Suzuki YJ, Carini M, Butterfield DA. Protein Carbonylation 1 2010;12:2–5.

[313] Stadtman E, Levine R. Chemical Modification of Proteins by Reactive Oxygen Species. Redox Proteomics from Protein Modif to Cell Dysfunct Dis 2006:1–23.

[314] Kansanen E, Kuosmanen SM, Leinonen H, Levonenn AL. The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. Redox Biol 2013;1:45–9. doi:10.1016/j.redox.2012.10.001.

[315] Ferrer MD, Tauler P, Sureda A, Tur JA, Pons A. Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise. J Sports Sci 2009;27:49–58. doi:10.1080/02640410802409683.

[316] Jezek P, Holendová B, Garlid KD, Jaburek M. Mitochondrial uncoupling proteins: subtle regulators of cellular redox signaling. Antioxid Redox Signal 2018;29:ars.2017.7225. doi:10.1089/ars.2017.7225.

[317] Ježek P, Olejár T, Smolková K, Ježek J, Dlasková A. Antioxidant and Regulatory Role of Mitochondrial Uncoupling Protein UCP2 in Pancreatic β -cells 2014;63.

[318] Cha SH, Fukushima a, Sakuma K, Kagawa Y. Chronic docosahexaenoic acid intake enhances expression of the gene for uncoupling protein 3 and affects pleiotropic mRNA levels in skeletal muscle of aged C57BL/6NJcl mice. J Nutr 2001;131:2636–42.

[319] Smith M, K van der Maesen, Somera F, Sobel R. Effects of phorbol myristate acetate (PMA) on functions of macrophages and microglia in vitro. Neurochem Res 1998;23:427–34.

[320] Ahmed A, Mukherjee S, Nandi D. Intracellular concentrations of Ca2+modulate the strength of signal and alter the outcomes of cytotoxic T-lymphocyte antigen-4 (CD152)-CD80/CD86 interactions in CD4+T lymphocytes. Immunology 2009;126:363–77. doi:10.1111/j.1365-2567.2008.02902.x.

[321] Szuster-Ciesielska A, Słotwińska M, Stachura A, Marmurowska-Michałowska H, Dubas-Ślemp H, Bojarska-Junak A, et al. Accelerated apoptosis of blood leukocytes and oxidative stress in blood of patients with major depression. Prog Neuro-Psychopharmacology Biol Psychiatry 2008;32:686–94. doi:10.1016/j.pnpbp.2007.11.012.

[322] Park KA, Byun HS, Won M, Yang K-J, Shin S, Piao L, et al. Sustained activation of protein kinase C downregulates nuclear factor- B signaling by dissociation of IKK- and Hsp90 complex in human colonic epithelial cells. Carcinogenesis 2007;28:71–80. doi:10.1093/carcin/bgl094.

[323] Tada M, Ichiishi E, Saito R, Emoto N, Niwano Y. Myristic Acid, A Side Chain of Phorbol Myristate Acetate (PMA), the Society for Free Radical Research Japan 10.3164/jcbn.09-30 Original Article Kyoto, Japan JCBN jcbn09-30 Journal of Clinical Biochemistry and Nutrition 0912-0009 1880-5086 Can Activate Hum. J Clin Biochem Nutr 2009;45:309–14.

[324] Kuwabara WMT, Zhang L, Schuiki I, Curi R, Volchuk A, Alba-Loureiro TC. NADPH oxidase-dependent production of reactive oxygen species induces endoplasmatic reticulum stress in neutrophil-like HL60 cells. PLoS One 2015;10:1–15. doi:10.1371/journal.pone.0116410.

[325] Pecqueur C, Alves-Guerra C, Ricquier D, Bouillaud F. UCP2, a metabolic sensor coupling glucose oxidation to mitochondrial metabolism? IUBMB Life 2009;61:762–7. doi:10.1002/iub.188.

[326] Gremeaux V, Gayda M, Lepers R, Sosner P, Juneau M, Nigam A. Exercise and longevity. Maturitas 2012;73:312–7. doi:10.1016/j.maturitas.2012.09.012.

[327] Sallam N, Laher I. Exercise modulates oxidative stress and inflammation in aging and cardiovascular diseases. Oxid Med Cell Longev 2016;2016:46–54. doi:10.1155/2016/7239639.

[328] Zheng Q, Cui G, Chen J, Gao H, Wei Y, Uede T, Chen Z DH. Regular Exercise Enhances the Immune Response Against Microbial Antigens Through Up-

Regulation of Toll-like Receptor Signaling Pathways. Cell Physiol Biochem 2015;37:735–46. doi:10.1159/000430391.

[329] Williams S. Healthy ageing. vol. 11 (24). 1997.

[330] Gomez-Cabrera MC, Ferrando B, Brioche T, Sanchis-Gomar F, Viña J. Exercise and antioxidant supplements in the elderly. J Sport Heal Sci 2013;2:94–100. doi:10.1016/j.jshs.2013.03.007.

[331] Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity & inflammation: The linking mechanism & the complications. Arch Med Sci 2017;13:851–63. doi:10.5114/aoms.2016.58928.

[332] Marseglia L, Manti S, D'Angelo G, Nicotera A, Parisi E, Di Rosa G, et al. Oxidative stress in obesity: A critical component in human diseases. Int J Mol Sci 2015;16:378–400. doi:10.3390/ijms16010378.

[333] McMurray F, Patten DA, Harper ME. Reactive Oxygen Species and Oxidative Stress in Obesity—Recent Findings and Empirical Approaches. Obesity 2016;24:2301–10. doi:10.1002/oby.21654.

[334] Mittal M, Siddiqui M, Tran K, Reddy S, Malik A. Reactive Oxygen Species in Inflammation and Tissue Injury. Antioxid Redox Signal 2014;20:1126–67. doi:10.1089/ars.2012.5149.

[335] Di Marzo N, Chisci E, Giovannoni R, Di Marzo N, Chisci E, Giovannoni R. The Role of Hydrogen Peroxide in Redox-Dependent Signaling: Homeostatic and Pathological Responses in Mammalian Cells. Cells 2018;7:156. doi:10.3390/cells7100156.

[336] Castro JP, Grune T, Speckmann B. The two faces of reactive oxygen species (ROS) in adipocyte function and dysfunction. Biol Chem 2016;397:709–24. doi:10.1515/hsz-2015-0305.

[337] Zielonka J, Kalyanaraman B. Small-molecule luminescent probes for the detection of cellular oxidizing and nitrating species. Free Radic Biol Med 2018;128:3–22. doi:10.1016/j.freeradbiomed.2018.03.032.

[338] Nguyen GT, Green ER, Mecsas J. Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. Front Cell Infect Microbiol 2017;7. doi:10.3389/fcimb.2017.00373.

[339] Makni-Maalej K, Chiandotto M, Hurtado-Nedelec M, Bedouhene S, Gougerot-Pocidalo MA, Dang PMC, et al. Zymosan induces NADPH oxidase activation in human neutrophils by inducing the phosphorylation of p47phox and the activation of Rac2: Involvement of protein tyrosine kinases, PI3Kinase, PKC, ERK1/2 and p38MAPkinase. Biochem Pharmacol

2013;85:92–100. doi:10.1016/j.bcp.2012.10.010.

[340] Smirnov A, Daily KP, Criss AK. Assembly of NADPH oxidase in human neutrophils is modulated by the opacity-associated protein expression state of Neisseria gonorrhoeae. Infect Immun 2014;82:1036–44. doi:10.1128/IAI.00881-13.

[341] Kawasaki T, Kawai T. Toll-like receptor signaling pathways. Front Immunol 2014;5:461. doi:10.3389/fimmu.2014.00461.

[342] Frantz S, Kelly RA, Bourcier T. Role of TLR-2 in the Activation of Nuclear Factor ??B by Oxidative Stress in Cardiac Myocytes. J Biol Chem 2001;276:5197–203. doi:10.1074/jbc.M009160200.

[343] Takeuchi O Hoshino K KTSHTHOTTKAS. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999;11:443–51.

[344] Carl VS, Brown-Steinke K, Nicklin MJH, Smith MF. Toll-like receptor 2 and 4 (TLR2 and TLR4) agonists differentially regulate secretory interleukin-1 receptor antagonist gene expression in macrophages. J Biol Chem 2002;277:17448–56. doi:10.1074/jbc.M111847200.

[345] Capó X, Martorell M, Sureda A, Batle JM, Tur JA, Pons A. Docosahexaenoic diet supplementation, exercise and temperature affect cytokine production by lipopolysaccharide-stimulated mononuclear cells. J Physiol Biochem 2016;72(3):421–34. doi:10.1007/s13105-016-0490-8.

[346] Buczynski MW, Stephens DL, Bowers-Gentry RC, Grkovich A, Deems RA, Dennis EA. TLR-4 and sustained calcium agonists synergistically produce eicosanoids independent of protein synthesis in RAW264.7 cells. J Biol Chem 2007;282:22834–47. doi:10.1074/jbc.M701831200.

[347] Pushpakumar S, Ren L, Kundu S, Gamon A, Tyagi SC, Sen U. Toll-like Receptor 4 Deficiency Reduces Oxidative Stress and Macrophage Mediated Inflammation in Hypertensive Kidney. Sci Rep 2017;7:1–15. doi:10.1038/s41598-017-06484-6.

[348] Karwaciak I, Gorzkiewicz M, Bartosz G, Pulaski L. TLR2 activation induces antioxidant defence in human monocyte-macrophage cell line models. Oncotarget 2017;8:54243–64. doi:10.18632/oncotarget.17342.

[349] Pålsson-McDermott EM, O'Neill LAJ. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology 2004;113:153–62. doi:10.1111/j.1365-2567.2004.01976.x.

[350] Sato M, Sano H, Iwaki D, Kudo K, Konishi M, Takahashi H, et al. Direct

Binding of Toll-Like Receptor 2 to Zymosan, and Zymosan-Induced NF- B Activation and TNF- Secretion Are Down-Regulated by Lung Collectin Surfactant Protein A. J Immunol 2003;171:417–25. doi:10.4049/jimmunol.171.1.417.

[351] Tanaka SI, Isoda F, Ishihara Y, Kimura M, Yamakawa T. T lymphopaenia in relation to body mass index and TNF-α in human obesity: Adequate weight reduction can be corrective. Clin Endocrinol (Oxf) 2001;54:347–54. doi:10.1046/j.1365-2265.2001.1139/cn2155.x.

[352] Iversen N, Krustrup P, Rasmussen HN, Rasmussen UF, Saltin B, Pilegaard H. Mitochondrial biogenesis and angiogenesis in skeletal muscle of the elderly. Exp Gerontol 2011;46:670–8. doi:10.1016/j.exger.2011.03.004.

[353] Groennebaek T, Vissing K. Impact of resistance training on skeletal muscle mitochondrial biogenesis, content, and function. Front Physiol 2017;8. doi:10.3389/fphys.2017.00713.

[354] Scott I, Youle RJ. Mitochondrial fission and fusion. Essays Biochem 2010;47:85–98. doi:10.1042/bse0470085.

[355] Zhang J. Autophagy and mitophagy in cellular damage control. Redox Biol 2013;1:19–23. doi:10.1016/j.redox.2012.11.008.

[356] Babbar M, Sheikh MS. Metabolic Stress and Disorders Related to Alterations in Mitochondrial Fission or Fusion. Mol Cell Pharmacol 2013;5:109–33. doi:10.4255/mcpharmacol.13.11.

[357] López A, Macaya C. Efectos antitrombóticos y antiinflamatorios de los ácidos grasos omega-3 2006:31–7.

[358] Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. Mechanisms Controlling Mitochondrial Biogenesis and Respiration through the Thermogenic Coactivator PGC-1 1999;98:115–24.

[359] Safdar A, Little JP, Stokl AJ, Hettinga BP, Akhtar M, Tarnopolsky MA. Exercise increases mitochondrial PGC-1α content and promotes nuclear-mitochondrial cross-talk to coordinate mitochondrial biogenesis. J Biol Chem 2011;286:10605–17. doi:10.1074/jbc.M110.211466.

[360] Dabrowska A, Venero JL, Iwasawa R, Hankir M khair, Rahman S, Boobis A, et al. PGC-1a controls mitochondrial biogenesis and dynamics in leadinduced neurotoxicity. Aging (Albany NY) 2015;7:629–47. doi:10.18632/aging.100837.

[361] Samjoo IA, Safdar A, Hamadeh MJ, Glover AW, Mocellin NJ, Santana J, et al. Markers of Skeletal Muscle Mitochondrial Function and Lipid

Accumulation Are Moderately Associated with the Homeostasis Model Assessment Index of Insulin Resistance in Obese Men. PLoS One 2013;8. doi:10.1371/journal.pone.0066322.

[362] Vider J, Laaksonen DE, Kilk ANN, Atalay M, Lehtmaa J, Zilmer M, et al. Physical Exercise Induces Activation of NF-kB in Human Peripheral Blood Lymphocytes. Antioxid Redox Signal 2001;3:1131–7.

[363] Dan Dunn J, Alvarez LAJ, Zhang X, Soldati T. Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. Redox Biol 2015;6:472–85. doi:10.1016/j.redox.2015.09.005.

[364] Marcos Becerro JF, Miquel J. Mitocondrias, envejecimiento, músculo y ejercicio. Arch Med Del Deport 2002;19:231–44.

[365] Stress O, Aging H. M IN1 REV1 EW Oxidative Stress and Mitochondrial DNA Mutations 2015.

[366] Van Remmen H, Hamilton ML, Richardson A. Oxidative damage to DNA and aging. Exerc Sport Sci Rev 2003;31:149–53. doi:10.1097/00003677-200307000-00009.

[367] Conley KE, Jubrias SA, Esselman PC. Oxidative capacity and ageing in human muscle. J Physiol 2000;526:203–10. doi:10.1111/j.1469-7793.2000.t01-1-00203.x.

[368] Brierleyl EJ, Johnson MA, James OFW, Turnbull DM, Brierley EJ, Johnson MA, et al. Effects of physical activity and age on mitochondrial function. Qjm 1996;89:251–8. doi:10.1093/qjmed/89.4.251.

[369] Larsen RG, Callahan DM, Foulis SA, Kent-braun JA. Age-related changes in oxidative capacity differ between Appl Physiol Nutr Metab 2013;37:88–99. doi:10.1139/h11-135.Age-related.

[370] Sun N, Youle RJ, Finkel T. The Mitochondrial Basis of Aging. Mol Cell 2016;61:654–66. doi:10.1016/j.molcel.2016.01.028.

[371] Lopez-Lluch G, Irusta P, Navas P, Cabo R de. MITOCHONDRIAL BIOGENESIS AND HEALTHY AGING Guillermo. Exp Gerontol 2009;43:813–9. doi:10.1016/j.exger.2008.06.014.MITOCHONDRIAL.

[372] Willems PHGM, Rossignol R, Dieteren CEJ, Murphy MP, Koopman WJH. Redox Homeostasis and Mitochondrial Dynamics. Cell Metab 2015;22:207–18. doi:10.1016/j.cmet.2015.06.006.

[373] Alcolea MP, Colom B, Llad?? I, Gianotti M, Garc??a-Palmer FJ. Mitochondrial transcription factor A (TFAM) is increased in rat embryo

during placentation and associated with mitochondrial differentiation. Cell Physiol Biochem 2006;17:79–88. doi:10.1159/000091466.

[374] Picca A, Lezza AMS. Regulation of mitochondrial biogenesis through TFAM-mitochondrial DNA interactions. Useful insights from aging and calorie restriction studies. Mitochondrion 2015;25:67–75. doi:10.1016/j.mito.2015.10.001.

[375] Ichinohe T, Yamazaki T, Koshiba T, Yanagi Y. Mitochondrial protein mitofusin 2 is required for NLRP3 inflammasome activation after RNA virus infection. Proc Natl Acad Sci 2013;110:17963–8. doi:10.1073/pnas.1312571110.

[376] de Brito OM, Scorrano L. Mitofusin 2: A Mitochondria-Shaping Protein with Signaling Roles Beyond Fusion. Antioxid Redox Signal 2008;10:621–34. doi:10.1089/ars.2007.1934.

[377] Rosmarin AG, Resendes KK, Yang Z, McMillan JN, Fleming SL. GA-binding protein transcription factor: A review of GABP as an integrator of intracellular signaling and protein-protein interactions. Blood Cells, Mol Dis 2004;32:143–54. doi:10.1016/j.bcmd.2003.09.005.

[378] Yang Z-F, Drumea K, Mott S, Wang J, Rosmarin AG. GABP Transcription Factor (Nuclear Respiratory Factor 2) Is Required for Mitochondrial Biogenesis. Mol Cell Biol 2014;34:3194–201. doi:10.1128/MCB.00492-12.

[379] Priya A, Johar K, Wong-Riley MTT. Nuclear respiratory factor 2 regulates the expression of the same NMDA receptor subunit genes as NRF-1: Both factors act by a concurrent and parallel mechanism to couple energy metabolism and synaptic transmission. Biochim Biophys Acta - Mol Cell Res 2013;1833:48–58. doi:10.1016/j.bbamcr.2012.10.014.

[380] Baldelli S, Aquilano K, Ciriolo MR. Punctum on two different transcription factors regulated by PGC-1α: Nuclear factor erythroid-derived 2-like 2 and nuclear respiratory factor 2. Biochim Biophys Acta - Gen Subj 2013;1830:4137–46. doi:10.1016/j.bbagen.2013.04.006.

[381] Bruni F, Polosa PL, Gadaleta MN, Cantatore P, Roberti M. Nuclear respiratory factor 2 induces the expression of many but not all human proteins acting in mitochondrial DNA transcription and replication. J Biol Chem 2010;285:3939–48. doi:10.1074/jbc.M109.044305.

[382] Brüünsgaard H, Pedersen BK. Age-related inflammatory cytokines and disease. Immunol Allergy Clin North Am 2003;23:15–39. doi:10.1016/S0889-8561(02)00056-5.

[383] Pararasa C, Bailey CJ, Griffiths HR. Ageing, adipose tissue, fatty acids and inflammation. Biogerontology 2015;16:235–48. doi:10.1007/s10522-014-9536-x.

[384] Yu HT, Park S, Shin E-C, Lee W-W. T cell senescence and cardiovascular diseases. Clin Exp Med 2016;16:257–63. doi:10.1007/s10238-015-0376-z.

[385] Xia S, Zhang X, Zheng S, Khanabdali R, Kalionis B, Wu J, et al. An Update on Inflamm-Aging: Mechanisms, Prevention, and Treatment. J Immunol Res 2016;2016. doi:10.1155/2016/8426874.

[386] Ventura MT, Casciaro M, Gangemi S, Buquicchio R. Immunosenescence in aging: Between immune cells depletion and cytokines up-regulation. Clin Mol Allergy 2017;15:1–8. doi:10.1186/s12948-017-0077-0.

[387] Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. Eur Cytokine Netw 2006;17:4–12. doi:10.5551/jat.12.295.

[388] Castaneda C, Gordon PL, Parker RC, Uhlin KL, Roubenoff R, Levey AS. Resistance Training to Reduce the Malnutrition-Inflammation Complex Syndrome of Chronic Kidney Disease. Am J Kidney Dis 2004;43:607–16. doi:10.1053/j.ajkd.2003.12.025.

[389] Adamopoulos S, Parissis J, Karatzas D, Kroupis C, Georgiadis M, Karavolias G, et al. Physical training modulates proinflammatory cytokines and the soluble Fas/soluble Fas ligand system in patients with chronic heart failure. J Am Coll Cardiol 2002;39:653–63. doi:10.1016/S0735-1097(01)01795-8.

[390] Khoo S, Müller M, Khoo S, Müller M, Activity P, Geri- LTN. Physical Activity for Health and Longevity To cite this version : HAL Id : hal-01097695 2014. doi:10.5281/zenodo.13333.

[391] Panagiotakos DB, Pitsavos C, Chrysohoou C, Kavouras S, Stefanadis C. The associations between leisure-time physical activity and inflammatory and coagulation markers related to cardiovascular disease: The ATTICA Study. Prev Med (Baltim) 2005;40:432–7. doi:10.1016/j.ypmed.2004.07.010.

[392] Kantor ED, Lampe JW, Kratz M, White E. Lifestyle Factors and Inflammation: Associations by Body Mass Index. PLoS One 2013;8. doi:10.1371/journal.pone.0067833.

[393] Visser M, Pahor M, Taaffe DR, Goodpaster BH, Simonsick EM, Newman AB, et al. Relationship of interleukin-6 and tumor necrosis factor-α with muscle mass and muscle strength in elderly men and women: The health

ABC study. Journals Gerontol - Ser A Biol Sci Med Sci 2002;57:326–32. doi:10.1093/gerona/57.5.M326.

[394] Fried SK, Bunkin DA, Greenberg AS. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: Depot difference and regulation by glucocorticoid. J Clin Endocrinol Metab 1998;83:847–50. doi:10.1210/jc.83.3.847.

[395] del Mar Bibiloni M, Julibert A, Argelich E, Aparicio-Ugarriza R, Palacios G, Pons A, et al. Western and mediterranean dietary patterns and physical activity and fitness among Spanish older adults. Nutrients 2017;9. doi:10.3390/nu9070704.

[396] Hoesel B, Schmid JA. The complexity of NF-κB signaling in inflammation and cancer. Mol Cancer 2013;12:1. doi:10.1186/1476-4598-12-86.

[397] Khansari N, Shakiba Y, Mahmoudi M. Chronic Inflammation and Oxidative Stress as a Major Cause of Age- Related Diseases and Cancer. Recent Pat Inflamm Allergy Drug Discov 2009;3:73–80. doi:10.2174/187221309787158371.

[398] Mendes KL, Lelis D de F, Santos SHS. Nuclear sirtuins and inflammatory signaling pathways. Cytokine Growth Factor Rev 2017;38:98–105. doi:10.1016/j.cytogfr.2017.11.001.

[399] Ji LL, Gomez-Cabrera M-C, Vina J. Role of nuclear factor κB and mitogen-activated protein kinase signaling in exercise-induced antioxidant enzyme adaptation. Appl Physiol Nutr Metab 2007;32:930–5. doi:10.1139/H07-098.

[400] Flynn MG, McFarlin BK, Phillips MD, Stewart LK, Timmerman KL. Toll-like receptor 4 and CD14 mRNA expression are lower in resistive exercise-trained elderly women. J Appl Physiol 2003;95:1833–42. doi:10.1152/japplphysiol.00359.2003.

[401] Stewart LK, Flynn MG, Campbell WW, Craig BA, Robinson JP, McFarlin BK, et al. Influence of exercise training and age on CD14+ cell-surface expression of toll-like receptor 2 and 4. Brain Behav Immun 2005;19:389–97. doi:10.1016/j.bbi.2005.04.003.

[402] Kohut ML, McCann DA, Russell DW, Konopka DN, Cunnick JE, Franke WD, et al. Aerobic exercise, but not flexibility/resistance exercise, reduces serum IL-18, CRP, and IL-6 independent of β-blockers, BMI, and psychosocial factors in older adults. Brain Behav Immun 2006;20:201–9. doi:10.1016/j.bbi.2005.12.002.

[403] Cavalcante PAM, Gregnani MF, Henrique JS, Ornellas FH, Araújo RC. Aerobic but not Resistance Exercise Can Induce Inflammatory Pathways via Toll-Like 2 and 4: a Systematic Review. Sport Med - Open 2017;3. doi:10.1186/s40798-017-0111-2.

[404] Fischer R, Maier O. Review Article Interrelation of Oxidative Stress and Inflammation in Neurodegenerative Disease : Role of TNF 2015;2015.

[405] Ingram S, Brighton MD. Reactive oxygen species : rapid fire in inflammation 2017:30–3.

[406] Blaser H, Dostert C, Mak TW, Brenner D. TNF and ROS Crosstalk in Inflammation. Trends Cell Biol 2016;26:249–61. doi:10.1016/j.tcb.2015.12.002.

[407] Monteiro R, Azevedo I. Chronic inflammation in obesity and the metabolic syndrome. Mediators Inflamm 2010;2010. doi:10.1155/2010/289645.

[408] Saltiel AR, Olefsky JM. Inflammatory mechanisms linking obesity and metabolic disease. J Clin Investig 2017;127:4–7. doi:10.1172/JCI92035.systems.

[409] Upadhyaya S, Kadamkode V, Mahammed R, Doraiswami C, Banerjee G. Adiponectin and IL-6: Mediators of inflammation in progression of healthy to type 2 diabetes in Indian population. Adipocyte 2014;3:39–45. doi:10.4161/adip.26553.

[410] Sargolzaei J, Chamani E, Kazemi T, Fallah S, Soori H. The role of adiponectin and adipolin as anti-inflammatory adipokines in the formation of macrophage foam cells and their association with cardiovascular diseases. Clin Biochem 2018;54:1–10. doi:10.1016/j.clinbiochem.2018.02.008.

[411] Banerjee S, Zmijewski JW, Lorne E, Liu G, Sha Y, Abraham E. Modulation of SCFβ-TrCP-dependent IκBα ubiquitination by hydrogen peroxide. J Biol Chem 2010;285:2665–75. doi:10.1074/jbc.M109.060822.

[412] Zmijewski JW, Lorne E, Zhao X, Tsuruta Y, Sha Y, Liu G, et al. Antiinflammatory effects of hydrogen peroxide in neutrophil activation and acute lung injury. Am J Respir Crit Care Med 2009;179:694–704. doi:10.1164/rccm.200806-851OC.

[413] Crawford LJA, Peake R, Price S, Morris TCM, Irvine AE. Adiponectin is produced by lymphocytes and is a negative regulator of granulopoiesis. J Leukoc Biol 2010;88:807–11. doi:10.1189/jlb.1109723.

VII. Anexo.
Publicaciones complementarias

Manuscript VI

Effects of dietary almond- and olive oil-based docosahexaenoic acid- and vitamin E-enriched beverage supplementation on athletic performance and oxidative stress markers

Xavier Capó, Miquel Martorell, Carla-Busquets Cortés, Antoni Sureda, Joan Riera, Franchek Drobnic, Josep A. Tur, Antoni Sureda, Antoni Pons.

Food Funct. 2016 Dec 7;7(12):4920-4934.

DOI: 10.1039/c6fo00758a.

Food & Function

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: X. Capó, M. Martorell, C. Busquets-Cortés, A. Sureda, J. Riera, F. Drobnic, J. A. Tur and A. Pons, *Food Funct.*, 2016, DOI: 10.1039/C6FO00758A.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/food-function

Food & Function

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/c6fo00758a

Effects of dietary almond- and olive oil-based docosahexaenoic acid- and vitamin E-enriched beverage supplementation on athletic performance and oxidative stress markers

X. Capó,[a,b] M. Martorell,[a,c] C. Busquets-Cortés,[a,b] A. Sureda,[a,b] J. Riera,[d] F. Drobnic,[d] J. A. Tur[a,b] and A. Pons*[a,b]

Received 23rd May 2016,
Accepted 20th October 2016

DOI: 10.1039/c6fo00758a

www.rsc.org/foodfunction

Functional beverages based on almonds and olive oil and enriched with α-tocopherol and docosahexaenoic acid (DHA) could be useful in modulating oxidative stress and enhancing physical performance in sportsmen. The aim of this work was to evaluate the effects of supplementation with functional beverages on physical performance, plasma and erythrocyte fatty acids' and polyphenol handling, oxidative and nitrative damage, and antioxidant and mitochondrial gene expression in young and senior athletes. Athletes performed maximal exercise tests before and after one month of dietary supplementation and blood samples were taken immediately before and one hour after each test. The beverages did not alter performance parameters during maximal exercise. Supplementation increased polyunsaturated and reduced saturated plasma fatty acids while increasing the DHA erythrocyte content; it maintained basal plasma and blood polyphenol levels, but increased the blood cell polyphenol concentration in senior athletes. Supplementation protects against oxidative damage although it enhances nitrative damage in young athletes. The beverages enhance the gene expression of antioxidant enzymes in peripheral blood mononuclear cells after exercise in young athletes.

Introduction

The strategies to obtain optimal endurance training effects include certain training methods, but also nutritional approaches. Physical activity, athletic performance, and recovery from exercise are enhanced by optimal nutrition.[1] Supplements containing omega 3 fatty acids, polyphenols, antioxidants and vitamins are widely consumed for the purpose of improving health and athletic achievement.[2] The enhancing effects of dietary supplementation with omega 3 polyunsaturated fatty acids (n3-PUFA) on exercise performance[3] have been described alongside oxidative balance during physical activity.[4] Docosahexaenoic (DHA) and eicosapentaenoic acid[5] diet supplementation modulates erythrocyte membrane deformability and the capacity of O_2 transport, and adapts mitochondria to use fatty acids as a fuel increasing energy efficiency.[3] Foods enriched with n3-PUFA often need to be protected from oxidation by the addition of antioxidants such as vitamin E and, for this reason, functional foods enriched with PUFA are usually enriched with vitamin E. Recently, how suitable it is to supplement the diet of athletes with isolated vitamin C and E supplements in order to ameliorate physical performance has come into question. The effects of antioxidants on physical performance are not yet entirely clear, with studies reporting positive, negative or no effects.[6] In this sense, chronic and elevated intakes of most antioxidants have been considered as having harmful effects on performance,[7] but moderate vitamin C and E dietary supplementation prevents neutrophil protein oxidation without affecting the adaptive response to exercise.[8] Polyphenol supplementation has been tested as a countermeasure against oxidative stress induced by exercise training.[9] Almonds and olive oil are representative products of the Mediterranean diet, which can be used as a source to enrich food with polyphenols. A Mediterranean diet supplemented with olive oil or nuts increases the total polyphenol intake and also correlates with a rise in plasma nitric oxide (NO) and a reduction in

[a]Research Group on Community Nutrition and Oxidative Stress, Science Laboratory of Physical Activity, Department of Fundamental Biology and Health Sciences, University of Balearic Islands, 07122 Palma de Mallorca, Spain
[b]CIBER: CB12/03/30038 Fisiopatología de la Obesidad la Nutrición, CIBEROBN, Instituto de Salud Carlos III (ISCIII), University of Balearic Islands, 07122 Palma de Mallorca, Spain. E-mail: antonipons@uib.es; Fax: +34 971 173 184; Tel: +34 971 173 171
[c]Departamento de Nutrición y Dietética, Facultad de Farmacia, Universidad de Concepción, 4070386 Concepción, Chile
[d]Sports Physiology Dept. CAR, Sant Cugat del Valles, GIRSANE, Barcelona 08174, Spain

systolic and diastolic blood pressure.[10] Almonds are an important source of essential nutrients such as arginine, calcium, potassium, niacin, α-tocopherol, fibre, monounsaturated fatty acids and polyphenols.[11] A diet supplemented with almonds exerts protective effects against oxidative stress.[11] A functional beverage based on almond and olive oil enriched with α-tocopherol acetate and DHA may be a good vehicle to supplement the diet of athletes in order to counteract oxidative damage and enhance physical performance. However, the efficacy of such a functional food in ameliorating exercise-induced physiological and oxidative stress and as an enhancer for athletic performance needs to be evaluated. New information related to this topic could contribute to the clarification of the controversy on the need for antioxidant use in well-trained athletes to improve physical performance and the body redox status.[12]

The oxidative stress associated with intense exercise in a warm environment is a good human model for studying the possible functional effects of consuming foods enriched with n3-PUFA, vitamin E and polyphenols.[13] Ageing is an additional factor influencing physical performance and oxidative stress that could influence the demands of n3-PUFA and vitamins.[14,15] It has been reported that elderly people are more susceptible than young people to oxidative damage in muscles after exercising.[15] In fact, n3-PUFAs are now identified as potential key nutrients, safe and effective in the treatment and prevention of several negative consequences of ageing.[16]

The aim of the present study was to evaluate the effects of diet supplementation with an almond and olive oil based beverage enriched with DHA and vitamin E on physical performance, plasma and erythrocyte fatty acids' and polyphenol handling, oxidative and nitrative damage and antioxidant gene expression induced by acute exercise in a warm environment in young and senior athletes. The main hypothesis was based on polyunsaturated fatty acids, given their pro-oxidative character and also due to their action on transcription factors together with vitamin E and other polyphenols which can modulate the antioxidant response and oxidative and nitrative damage induced by physical activity. The overall objective of the study is to establish situations improving athletic performance and study the oxidative balance by manipulation of feeding patterns and consumption of nutritional supplements and investigate the involved mechanisms.

Materials and methods

Subjects and anthropometric characteristics

Ten young male taekwondo athletes and eight well-trained senior athletes related to sports competitions (trainers and sport medical doctors) volunteered to participate in this study. All the subjects were informed of its purpose, requirements and possible risks before giving their written consent to take part. Before being accepted to participate in the research, each subject underwent a complete medical examination, which included a medical history and resting ECG, to prevent any medical problem that would contraindicate participation in the study. The protocol complied with the Declaration of Helsinki for research on human subjects, and was approved by the Ethical Committee for Clinical Research at the Direcció General de l'Esport of the Catalonian Sports Council. The study was registered at ClinicalTrials.gov (NCT02177383). The participants were not acclimatised to heat and the study was conducted in the months of May and June with an average temperature of 18.2 °C and 22.8 °C respectively. The participants were split into two groups depending on their age. All participants began the nutritional diet trial, but only five young and five senior athletes completed it. The reason for abandoning the nutritional trial was participation in sporting competitions.

Beverage composition

The nutritional intervention consisted of daily supplementation of the diet with one liter of almond and olive oil based beverage enriched with a DHA functional beverage five days a week. Beverages were isotonic (278 mOsm kg^{-1}) and made up of 3.0% almond and 0.8% sucrose and the rest was water, flavour, and the added oils and α-tocopherol acetate (vitamin E). The added oils were 0.6% olive oil and 0.2% (wt%) DHA-S (DSM, Columbia, USA). DHA-S is a nutritional oil derived from the marine algae, *Schizochytrium* sp., a rich source of DHA with soy lecithin and rosemary (*Rosmarinus officinalis*) extract as the flavour, and tocopherols and ascorbyl palmitate as antioxidants. The procedure for obtaining the beverage was bleaching of the almonds; crushing of the almonds in water; centrifuging of the mixture to eliminate insoluble materials; and the addition of cinnamon and lemon natural flavours, sucrose, vitamin E, and olive oil plus DHA-S. Finally, the beverage was sterilized and packed. The functional beverage was elaborated by Liquats Vegetals S.A. (Girona, Spain).

The fatty acid composition of the beverage was determined following the same procedure used to determine the erythrocyte fatty acid composition as described below. Similarly, the polyphenol content of the beverage was determined following the same procedure used to determine the polyphenol content of plasma, erythrocytes and blood described below. The total fat content of the functional beverage was 2.6%, taking into account 60% of the fat content of the almonds, olive oil and DHA-S added.

Nutritional intervention

The nutritional intervention comprised ingesting one litre per day (five days per week) of the isotonic functional beverage instead of water for five weeks. Half of the beverage was taken in the morning and the other half before the daily training session. The participants maintained their nutritional and exercise habits alongside the study. Each subject performed two exercise tests separated by a 5-week period during which they all took the nutritional supplement. Before the first exercise test athletes take water habitually; this fact was changed during the nutritional intervention when athletes consumed the functional beverage until the second exercise test. At the

end of the nutritional intervention, all participants performed the same exercise test as at the beginning of the intervention. The results obtained after the nutritional intervention (supplemented groups) were compared with those obtained at the beginning of the intervention (control groups).

Experimental procedure

Two weeks before the first test, each subject performed an incremental maximal test until exhaustion on a motorised treadmill (EG2, Vitoria, Spain) to determine their maximal oxygen consumption (VO_2max) using a computerised metabolic chart (Master Screen CPX, Erich Jaeger, Würzburg, Germany). The velocity corresponding to 60% (V60), 70% (V70), 80% (V80) and 90% (V90), of their VO_2max was calculated by linear interpolation of data from the maximal exercise test. On day 1 of the study, subjects arrived at the laboratory at 9:00 AM after an overnight fast and having drunk a minimum of 500 cc of water since wakening. Dry nude body weight was measured before and after the experiment after the subjects had emptied their urinary bladder. The subjects equipped with a heart rate transmitter and skin thermistors entered into the climatic chamber set at 30 °C temperature and 70% humidity; after 10 minutes the baseline core temperature, skin temperature and heart rate (HR) values were collected. Subjects continuously ran on the treadmill at the speed of V60 for 5 minutes, V70 for 5 minutes and V80 for 5 minutes for three consecutive bouts with two minutes of recovery between bouts. Finally, the subjects ran at V90 until exhaustion, and this time was measured as a quantity of exercise performance. Subjects were required to wear the same clothes and shoes in the two exercise sessions. Water was provided *ad libitum* in 50 cl bottles at room temperature and the amount of water consumed was measured. The percentage of dehydration was calculated from the weight difference corrected by drinking water during the test. The Polar® heart watch system (Polar Electro Inc., Kempele, Finland) was used to measure the basal HR every 5 minutes during the test and after 5 minutes of recovery time. A microsample of blood (20 μl) was taken from the ear lobe to measure lactate concentration. The Borg scale was used to assess subjective perception of effort at minutes 15, 32, 49 and after concluding the test.[17]

Venous blood samples were obtained from the antecubital vein of participants with vacutainers containing EDTA (ethylenediaminetetraacetic acid) as an anticoagulant for blood count analyses (2 mL), to obtain erythrocytes and plasma (6 mL) and purify peripheral blood mononuclear cells (PBMCs) (6 mL). Venous blood samples were obtained after 12 hours, overnight, under fasting conditions (basal sample), and 2 hours after finishing training, which is consonant with the increased circulating immune cells and significant changes in antioxidant enzyme activities and in markers for oxidative damage.

The erythrocyte fraction was obtained after centrifugation at 900*g*, 30 min, 4 °C. Then, erythrocytes were washed with phosphate buffered saline (PBS), centrifuged at 900*g*, 20 min, 4 °C and lysed with distilled water at the initial blood volume. Cell lysates were stored at −80 °C until biochemical analyses thereof.

PBMCs were obtained by following a method previously described.[18] Blood was carefully introduced on Ficoll in a proportion of 1.5 : 1 and was then centrifuged at 9009*g*, at 4 °C for 30 min. The PBMC layer was carefully removed. The plasma and the Ficoll phases were discarded. The PBMC slurry was then washed twice with PBS and centrifuged for 10 min at 1000*g*, 4 °C. This process was performed in triplicate, with one of the samples used to obtain RNA, and another being lysed with distilled water. Cell lysates were stored at −80 °C until biochemical analyses thereof.

Fatty acid determination

Erythrocyte, plasma and beverage fatty acids were extracted in duplicate with chloroform/methanol (2 : 1 v/v) by a modified method of Folch,[19,20] containing 0.01% butylated hydroxyanisole as the antioxidant and 20 μL of *n*-heptadecanoic acid (15 mM) as the internal standard. The resultant organic phase was evaporated under a nitrogen stream at 55 °C. The dry residue was dissolved in 225 μL of *n*-hexane and 25 μL of Meth-Prep™ II (Grace Davison Discovery Sciences, Columbia, MD, USA) and the derivatization reagent was added. The gas chromatograph was an Agilent 5890 model (Agilent Technologies, Santa Clara, CA, USA) with a flame ionization detector (FID) and the column was a Supelcowax® 10 Capillary GC column, 30 m × 0.53 mm × 0.50 μm (Supelco, Bellefonte, PA, USA).

Plasma triglyceride determination

Plasma triglycerides were determined in duplicate using an enzymatic colorimetric assay kit (Spinreact®) following the manufacturer's instructions for use. The intra-assay coefficient of variation was 1.57% and the inter-assay coefficient of variation 3.15%.

Malondialdehyde determination

Malondialdehyde (MDA) as a marker of lipid peroxidation was analyzed in duplicate in 1/100 diluted erythrocytes and in plasma using a colorimetric assay kit (Calbiochem®). Briefly, samples and standards were placed in 1.5 mL tubes containing *n*-methyl-2-phenylindole (10.3 mM) in acetonitrile/methanol (3 : 1, by vol). 12 N HCl was added and the samples were incubated for 1 h at 45 °C. The absorbance was measured at 586 nm. The method used is specific for MDA determination.[20]

Nitrotyrosine and protein carbonyl determination

Protein carbonyl derivatives and nitrotyrosine (N-Tyr) in proteins were determined in duplicate by immunological methods using the OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs, Inc.) and OxiSelect™ Nitrotyrosine Immunoblot Kit (Cell Biolabs, Inc.) following the manufacturer's instructions. Total protein concentrations were measured by a method previously described.[18,20] The light was visualized and detected by short exposure to a Chemidoc XRS densitometer (Bio-Rad Laboratories). Image analysis was

performed using Quantity One-1D analysis software (Bio-Rad Laboratories). The coefficient of variation has been calculated to be 10% for the carbonyl index and 12% for the nitrotyrosine index.

Nitrite and nitrate determination

Duplicate serum samples were centrifuged (15 000*g*, 30 min, 4 °C) in 10 K filters (Amicon® Ultra; Millipore) to remove proteins. The 10 K filters were previously cleaned twice with distilled water to eliminate nitrite and nitrate residues in the filters. The supernatants were recovered and used to measure nitrite and nitrate concentrations by detecting the synthesized NO in the gas phase chemiluminescence reaction with ozone, using a NO analyzer (NOA 280i; Sievers, GE Power & Water, Boulder, CO).[10]

Lipoperoxide determination

Plasma lipid peroxide was determined in duplicate using the human lipid peroxide (LPO) ELISA kit from Cusabio® following the manufacturer's instructions for use. The intra-assay coefficient of variation was calculated to be lower than 8% and the inter-assay coefficient of variation was lower than 10.0%.

Vitamin E determination

Total vitamin E determination for the functional beverage was attained by extracting the liposoluble vitamins using *n*-hexane after deproteinization with ethanol containing 0.2% butylated hydroxytoluene (BHT). The vitamin E concentration was determined after drying the samples under a nitrogen current and dissolving them with ethanol. The mobile phase consisted of acetonitrile–tetrahydrofuran–water (550 : 370 : 80, v/v/v). The HPLC was a Shimadzu (Canby, OR, USA) equipped with a diode array detector and a Nova Pak C_{18}, 3.9 × 150 mm column, and α-tocopherol was determined at 290 nm. Quantification was performed with an external standard (Sigma-Aldrich®).

Polyphenol determination

The total polyphenol content of the functional beverage, plasma and washed blood cells was determined through the Folin–Ciocalteu method[10] in the supernatants of deproteinized samples with cold acetone (1 : 1.2) using L-tyrosine as the standard. The results are expressed as mmoles of L-tyrosine per L. The blood concentration of polyphenols was calculated from the plasma and blood cell concentration, and haematocrit (Hm) values following the formula:

$$\text{Blood concentration} = \text{Plasma concentration} \times (100 - \text{Hm}/100) + \text{Blood cells concentration} \times (\text{Hm}/100)$$

PBMCs' RNA extraction and real-time PCR assay

Catalase, Mn-superoxide dismutase (Mn-SOD), uncoupling protein-3 (UCP3), hemoxigenase-1 (HO1), glutathione reductase (GRd), glutathione peroxidase (GPx), Cu/Zn-superoxide dismutase (Cu/Zn-SOD), mitofusin-1 (Mtf1), mitofusin-2 (Mtf2), solute carrier family 2 facilitated glucose transporter member 4 (SLC), mitochondrial NADH dehydrogenase subunit 5 (MitND5), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PCG1α) and cytochrome c oxidase subunit IV (COXIV) mRNA expression were determined by multiplex real-time PCR based on the incorporation of a fluorescent reporter dye and using human 18S rRNA as the reference. For this purpose, total RNA was isolated from PBMCs by Tripure extraction (Roche Diagnostics, Germany). RNA (1 μg) from each sample was reverse transcribed using 50 U of Expand Reverse Transcriptase (Roche Diagnostics, Germany) and 20 pmol oligo (dT) for 60 min at 37 °C in a 10 μL final volume, according to the manufacturer's instructions. The resulting cDNA (2.5 μL) was amplified using the LightCycler FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Germany). Amplification was performed at 55 °C and 45 cycles. The relative quantification was performed by standard calculations considering $2^{(\Delta\Delta Ct)}$. Antioxidant enzyme levels before and after the season were normalized to the invariant control 18S rRNA. mRNA levels at the basal young control group were arbitrarily referred to as 1. The primers used are listed in Table 1.

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS v.21.0 for Windows). Results are expressed as mean ± SEM and $p < 0.05$ was considered statistically significant. A Kolmogorov–Smirnov test was previously applied to assess the normal distribution of the data. The statistical significance of the data was assessed by a three-way analysis of variance (ANOVA). The statistical factors analysed were beverage supplementation (S), ageing (A) and exercise (E). For the sets of data where there was a significant SxExA, SxE, SxA, AxE interactions were tested by the ANOVA one-way test.

Results

There were no differences in the anthropometric characteristics and physical activity capabilities between the young and senior groups (Table 2) except for the mean age of the young (22.8 ± 1.6 years old) and senior (45.6 ± 3.8 years old) groups. The mean weight, height, % body fat, % fat-free mass, body surface area, VO_2max and the VO_2max kg^{-1} and maximal rate attained during the exercise test for athletes from the young group were similar to athletes from the senior group. These values did not change after five weeks of nutritional intervention with the functional beverage (data not shown).

Effects on exercise performance

The exercise test significantly increased the values of skin and central body temperature, blood lactate and the Borg index but significantly decreased heat storage (ΔHS) (Fig. 1A–D) in a similar way, both for the control and functional beverage supplement and in the young and senior groups. Functional beverage diet supplementation did not influence any of the measured parameters, whereas age significantly decreased

Table 1 Primer sequences and conditions

Gene	Primer		Conditions	
18S	Fw:	5′-ATG TGA AGT CAC TGT GCC AG-3′	95 °C	10 s
	Rv:	5′-GTG TAA TCC GTC TCC ACA GA-3′	60 °C	10 s
			72 °C	12 s
CAT	Fw:	5′-TTT GGC TAC TTT GAG GTC AC-3′	95 °C	10 s
	Rv:	5′-TCC CCA TTT GCA TTA ACC AG-3′	60 °C	10 s
			72 °C	15 s
CuZn-SOD	Fw:	5′-TCA GGA GAC CAT TGC ATC ATT-3′	95 °C	10 s
	Rv:	5′-CGC TTT CCT GTC TTT GTA CTT TCT TC-3′	63 °C	10 s
			72 °C	15 s
GPx	Fw:	5′-TTC CCG TGC AAC CAG TTT G-3′	94 °C	10 s
	Rv:	5′-TTC ACC TCG CAC TTC TCG AA-3′	63 °C	10 s
			72 °C	15 s
UCP3	Fw:	5′-CGT GGT GAT GTT CAT AAC CTA TG-3′	95 °C	5 s
	Rv:	5′-CGG TGA TTC CCG TAA CAT CTG-3′	60 °C	7 s
			72 °C	10 s
HO-1	Fw:	5′-CCA GCG GGC CAG CAA CAA AGT GC-3′	95 °C	10 s
	Rv:	5′-AAG CCT TCA GTG CCC ACG GTA AGG-3′	60 °C	5 s
			72 °C	10 s
Mn-SOD	Fw:	5′-GAG AAG TAC CAG GAG GCG TTG-3′	95 °C	10 s
	Rv:	5′-CAA GCC AAC CCC AAC CTG AGC-3′	64 °C	10 s
			72 °C	15 s
GRd	Fw:	5′-TCA CGC AGT TAC CAA AAG GAA A-3′	95 °C	10 s
	Rv:	5′-CAC ACC CAA GTC CCC TGC ATA T-3′	64 °C	10 s
			72 °C	15 s
COX IV	Fw:	5′-AGAAGCACTATGTGTACGGCCC-3	95 °C	10 s
	Rv:	5′-GGTTCACCTTCATGTCCAGCAT-3′	60 °C	10 s
			72 °C	15 s
MitDN5	Fw:	5′-CGGCTGAGAGGGCGTAGG-3′	95 °C	10 s
	Rv:	5′-GATGAAACCGATATCCGGCCGA-3′	60 °C	10 s
			72 °C	15 s
Mfn1	Fw:	5′-TGTTTTGGTCGCAAACTCTG-3′	95 °C	10 s
	Rv:	5′-CTGTCTGCGTACGTCTTCCA-3′	60 °C	10 s
			72 °C	15 s
Mfn2	Fw:	5′-ATGCATCCCCACTTAAGCAC-3′	95 °C	10 s
	Rv:	5′-CCAGAGGGCAGAACTTTGTC-3′	60 °C	10 s
			72 °C	15 s
SLC2A4	Fw:	5′-CTGCTCCTGGCCTCACAG-3′	95 °C	10 s
	Rv:	5′-CCCCTCGAGATTCTGGATGAT-3′	64 °C	10 s
			72 °C	15 s
PGC1α	Fw:	5′-CACTTACAAGCCAAACCAACAACT-3′	95 °C	10 s
	Rv:	5′-CAATAGTCTTGTTCTCAAATGGGGA-3′	60 °C	10 s
			72 °C	15 s

fatigue perception during the exercise test in senior athletes compared to the young athletes (Fig. 1D). Dietary functional beverage supplementation did not influence the duration of exercise at a speed representing a 90% value of VO_2max until exhaustion; nor does the age of the athletes influence this parameter.

Functional beverage composition

The composition of the functional beverage is shown in Table 3. The functional beverage contained around 2.6% (wt%) fat, 2.85 ± 0.29 mM (51 ± 5 mg per 100 mL as L-tyrosine equivalents) of polyphenols and 4.6 ± 2.8 mg per 100 mL of vitamin E. The daily intake of one litre of beverage five days a week implies a daily supplement to diet with a mean of 18.6 g day^{-1} of fat (of which 820 mg day^{-1} is DHA), 32.6 mg day^{-1} vitamin E, and 36.4 mg day^{-1} polyphenols. The fatty acid content of the beverage was mainly monounsaturated (51.7 ± 5.0%) and polyunsaturated (38.3 ± 4.4%) with a low percentage of saturated fatty acids (9.90 ± 1.15%). The most abundant

Table 2 Subject's anthropometric characteristics and physical activity time

	Young	Senior
Age (years)	22.8 ± 3.8$	45.6 ± 1.6
Weight (kg)	71.0 ± 4.8	76.1 ± 2.9
Height (cm)	176 ± 3.8	177 ± 3.8
Body mass index (BMI, kg m^{-2})	22.9 ± 0.5	24.3 ± 0.9
Fat mass (%)	47.0 ± 1.6	44.8 ± 1.1
Fat-free mass (%)	9.50 ± 1.1	11.6 ± 1.0
Body surface (m^2)	1.86 ± 0.05	1.93 ± 0.07
Max speed (km h^{-1})	16.1 ± 0.6	14.9 ± 0.9
VO_2 (l min^{-1})	4029 ± 255	3819 ± 329
VO_2 max (l min^{-1} kg^{-1})	58.8 ± 2.5	50.4 ± 3.4

Statistical analysis: two-way ANOVA, $p < 0.05$. $ Differences between young and master groups. Results are the mean ± sem.

Table 3 Beverage fatty acid composition

Fatty acid	Concentration
C16:0 (μM)	3607 ± 620
C16:1 (μM)	591 ± 115
C18:0 (μM)	1928 ± 423
C18:1 (μM)	22 503 ± 5605
C18:2 (μM)	12 177 ± 2996
C18:3n6 (μM)	298 ± 152
C18:3n3 (μM)	455 ± 44
C20:0 (μM)	116 ± 7
C20:1 (μM)	35.9 ± 2.3
C20:2 (μM)	282 ± 14
C20:3 (μM)	20.9 ± 2.2
C20:4n6 (μM)	140 ± 9
C22:0 (μM)	75.6 ± 1.7
C22:5 (μM)	1715 ± 70
C22:6n3 (μM)	3457 ± 117
Total fatty acids (μM)	47 400 ± 9586
SFA (%)	9.90 ± 1.15
MUFA (%)	51.7 ± 5.0
PUFA (%)	38.3 ± 4.4
Vitamin E (mg L^{-1})	45.7 ± 27.7
Polyphenols (mM)	2.85 ± 0.29

Values are the average of 6 samples of the beverage.

fatty acids were C18:1 and C18:2 followed by C16:0, C22:6, C18:0 and C22:5, whereas C18:3n3, C18:3n6, C20:2, C20:0, C20:4 and C22:0 represented under 1% of the total fat content of the functional beverage.

Effects on plasma triglycerides and plasma and erythrocyte fatty acid composition

Dietary supplementation with the functional beverage influenced the composition of plasma fatty acids (Table 4) for age but not acute exercise. The young group had significantly higher plasma MUFA levels than the senior group after exercising in both the placebo and supplemented group. Dietary functional beverage supplementation significantly decreased C16:0, C16:1 C18:3n3 and C18:3n6 but significantly increased C20:3, C20:4n6 and C22:6n3 in plasma. The senior group had more C22:6n3 and lower C18:1n9 and C18:3n3 in their plasma than the young group.

As with plasma, dietary supplementation with the functional beverage influenced the fatty acid composition of erythrocytes (Table 5) for age but not acute exercise. The percentages of erythrocytes C16:0, C16:1, C18:0, C18:1, C18:2, C18:3n6, C20:4, SFAs, MUFAs, and PUFAs were not impacted by functional beverage dietary supplementation, age and acute exercise. Nevertheless, the percentages of C18:3n3 and

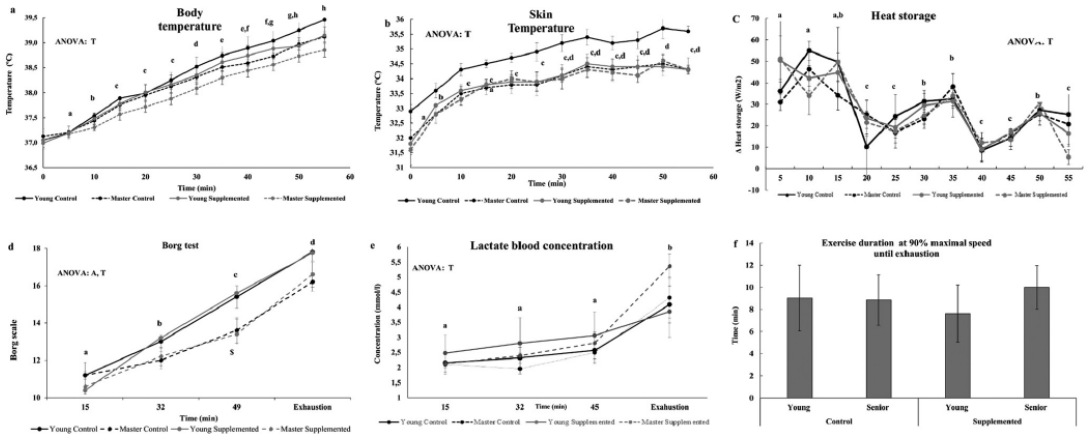


Fig. 1 Statistical analysis: three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, T, stress test effect, SxA, interaction between supplementation and age effects, SxT, interaction between supplementation and stress test effects, TxA, interaction between stress test and age effects, AxTxS, effects of interaction between three factors. S, A, T, SxA, TxA, SxT or AxTxS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation plasma levels; different letters reveal significant differences during the stress test; $ indicates significant differences between the young and senior groups. Different letters reveal significant differences during the stress test.

Table 4 Effects of supplementation with the functional beverage, acute exercise and age on plasma fatty acid content

		Pre-exercise		Post-exercise		
		Young	Senior	Young	Senior	ANOVA
C16:0 (%)	Control	20.9 ± 0.72	21.2 ± 0.73	19.7 ± 0.43	21.3 ± 1.23	
	Supplemented	19.3 ± 0.66	20.4 ± 3.08	20.1 ± 1.63	18.4 ± 1.03	
C16:1 (%)	Control	4.80 ± 0.09	4.39 ± 0.53	4.91 ± 0.42	4.93 ± 0.64	S
	Supplemented	3.18 ± 0.44*	2.83 ± 0.63	4.39 ± 0.89	3.23 ± 0.53*	
C18:0 (%)	Control	14.9 ± 1.49	13.5 ± 0.90	13.2 ± 1.19	13.7 ± 0.63	
	Supplemented	12.9 ± 0.53	14.6 ± 0.71	13.0 ± 0.87	13.4 ± 0.51	
C18:1 (%)	Control	17.8 ± 0.63	14.1 ± 0.67	19.1 ± 1.39	15.4 ± 1.37	A
	Supplemented	15.8 ± 1.68	15.4 ± 0.85	20.4 ± 1.52	14.9 ± 1.29$	
C18:2 (%)	Control	19.9 ± 1.69	23.9 ± 2.34	22.7 ± 2.14	22.6 ± 1.35	S
	Supplemented	24.7 ± 1.97*	26.4 ± 1.38	23.9 ± 1.09	24.0 ± 1.38	
C18:3n6 (%)	Control	1.80 ± 0.37	1.63 ± 0.17	1.87 ± 0.13	1.94 ± 0.26	S
	Supplemented	1.25 ± 0.31	1.11 ± 0.13	1.24 ± 0.38	0.78 ± 0.12*	
C18:3n3 (%)	Control	2.30 ± 0.80	0.96 ± 0.19$	1.57 ± 0.15	1.21 ± 0.15	S, A
	Supplemented	0.98 ± 0.08*	0.63 ± 0.18	0.88 ± 0.28	0.78 ± 0.10	
C20:3 (%)	Control	1.97 ± 0.19	1.70 ± 0.29	1.96 ± 0.17	1.71 ± 0.18	S
	Supplemented	2.23 ± 0.26	2.15 ± 0.27	2.15 ± 0.28	2.19 ± 0.18	
C20:4 (%)	Control	11.5 ± 1.75	13.4 ± 1.72	12.1 ± 0.78	12.9 ± 1.39	S
	Supplemented	14.8 ± 0.77*	15.4 ± 1.11	13.7 ± 0.44	14.6 ± 0.57	
C22:6 (%)	Control	4.09 ± 0.71	5.69 ± 2.31	2.93 ± 0.32	4.30 ± 0.98	S
	Supplemented	6.73 ± 1.36	8.22 ± 0.22	6.35 ± 1.07*	7.71 ± 0.75*	
Triglycerides (mg dL^{-1})	Control	306 ± 109[a]	111 ± 26.2[b]	110 ± 26.4[b]	186 ± 27.8[a,b]	SxExA
	Supplemented	109 ± 35.0[b]	143 ± 26.6[b]	189 ± 42.7[a,b]	118 ± 27.7[b]	
SFA (%)	Control	36.4 ± 2.13	35.3 ± 0.29	33.4 ± 1.53	35.7 ± 1.62	
	Supplemented	32.2 ± 0.75	35.4 ± 3.67	33.0 ± 2.32	32.0 ± 1.02	
MUFA (%)	Control	22.6 ± 0.66	18.5 ± 0.87	23.9 ± 1.54$	20.3 ± 1.44	A
	Supplemented	19.0 ± 2.09	18.2 ± 1.22	24.8 ± 2.33$	18.1 ± 1.60	
PUFA (%)	Control	41.6 ± 1.74	46.8 ± 0.91	43.0 ± 2.23	44.6 ± 0.86	
	Supplemented	48.9 ± 1.81	46.8 ± 4.62	42.1 ± 4.10	50.2 ± 1.78	
TOTAL (μM)	Control	100 ± 19.2	100 ± 9.30	100 ± 19.1	100 ± 8.49	
	Supplemented	100 ± 32.0	100 ± 31.3	100 ± 41.0	100 ± 22.2	

Results are the mean ± sem. Statistical analysis: three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, E, exercise effect, SxA, interaction between supplementation and age effects, SxE, interaction between supplementation and exercise effects, ExA, interaction between exercise and age effects, AxExS, effects of interaction between three factors. S, A, E, SxA, ExA, SxE or AxExS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation; # indicates significant differences between pre-exercise and post-exercise; $ indicates significant differences between the young and senior groups. Where interaction exists between different statistical factors, different letters reveal significant differences.

C22:6n3 in erythrocytes were significantly higher after dietary supplementation with the functional beverage than in the control situation, mainly in the young group after exercise for the C18:3n3 value. Furthermore, a significant age effect was observed in the percentage of C22:0 and C20:3, which was significantly higher in the senior than in the young group after exercising in the control situation.

An interaction between the functional beverage, acute exercise and age influenced plasma triglyceride levels. Pre-exercise levels of the young group in the control situation were significantly higher than all groups and situations; no differences were observed in plasma triglyceride values attributable to functional beverage dietary supplementation, acute exercise or age except for the significantly higher values of the young group in the pre-exercise and control situations.

Effects on blood polyphenol levels, haematological parameters, and plasma NOx

Blood haemoglobin is affected by the three analysed factors; haematocrit is influenced only by age and the number of

View Article Online
Paper Food & Function

Table 5 Effects of supplementation with the functional beverage, acute exercise and age on erythrocyte fatty acid content

		Pre-exercise		Post-exercise		
		Young	Senior	Young	Senior	ANOVA
C16:0 (%)	Control	14.7 ± 0.5	14.7 ± 0.5	14.6 ± 0.5	14.7 ± 0.8	
	Supplemented	14.7 ± 0.7	14.8 ± 0.8	14.5 ± 0.2	14.9 ± 0.8	
C16:1	Control	0.81 ± 0.12	0.79 ± 0.06	0.75 ± 0.10	0.80 ± 0.15	
	Supplemented	0.56 ± 0.03	0.80 ± 0.15	0.63 ± 0.03	0.82 ± 0.15	
C18:0 (%)	Control	19.4 ± 0.5	19.2 ± 0.1	19.6 ± 0.5	19.3 ± 0.3	
	Supplemented	20.5 ± 0.6	19.3 ± 0.2	19.8 ± 0.3	19.7 ± 0.7	
C18:1 (%)	Control	11.9 ± 0.2	11.4 ± 0.3	11.4 ± 0.4	11.2 ± 0.4	
	Supplemented	11.7 ± 0.4	11.5 ± 0.3	12.0 ± 0.3	11.2 ± 0.3	
C18:2 (%)	Control	11.6 ± 0.4	11.8 ± 0.4	13.0 ± 1.2	12.4 ± 0.7	
	Supplemented	11.6 ± 0.5	11.9 ± 0.3	12.0 ± 1.4	11.7 ± 0.6	
C18:3n6 (%)	Control	0.80 ± 0.13	0.54 ± 0.12	0.64 ± 0.03	0.49 ± 0.06	
	Supplemented	0.85 ± 0.18	0.62 ± 0.50	0.55 ± 0.12	0.64 ± 0.08	
C18:3n3 (%)	Control	0.39 ± 0.03	0.41 ± 0.04	0.44 ± 0.03	0.43 ± 0.04	S
	Supplemented	0.49 ± 0.03	0.40 ± 0.05	0.77 ± 0.19*	0.53 ± 0.10	
C20:3 (%)	Control	1.72 ± 0.11	1.84 ± 0.07	1.69 ± 0.13	1.79 ± 0.09$	A
	Supplemented	1.69 ± 0.17	1.52 ± 0.07	1.78 ± 0.17	1.51 ± 0.05	
C20:4 (%)	Control	28.6 ± 0.7	28.4 ± 1.2	28.2 ± 0.9	27.0 ± 0.7	
	Supplemented	28.3 ± 0.8	26.5 ± 1.4	28.3 ± 0.5	25.9 ± 1.4	
C22:0 (%)	Control	0.61 ± 0.12	0.68 ± 0.09	0.48 ± 0.09	0.73 ± 0.07$	A
	Supplemented	0.50 ± 0.04	0.64 ± 0.04	0.45 ± 0.08	0.61 ± 0.06	
C22:6 (%)	Control	9.73 ± 0.5	9.27 ± 0.3	8.81 ± 1.3	9.09 ± 1.0	S
	Supplemented	11.2 ± 1.1	12.1 ± 1.5	9.32 ± 0.7	12.7 ± 1.9	
SFA (%)	Control	34.7 ± 0.6	34.6 ± 0.5	34.7 ± 0.7	34.7 ± 0.8	
	Supplemented	35.6 ± 1.0	34.7 ± 0.9	34.8 ± 0.2	35.2 ± 1.1	
MUFA (%)	Control	12.7 ± 0.2	12.2 ± 0.3	12.2 ± 0.5	12.0 ± 0.5	
	Supplemented	12.1 ± 0.3	12.3 ± 0.3	12.6 ± 0.4	12.1 ± 0.4	
PUFA (%)	Control	52.6 ± 0.7	53.2 ± 0.6	53.1 ± 0.4	53.3 ± 1.4	
	Supplemented	52.3 ± 1.2	53.0 ± 1.0	52.6 ± 0.6	52.7 ± 1.1	

Results are the mean ± sem. Statistical analysis: three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, E, exercise effect, SxA, interaction between supplementation and age effects, SxE, interaction between supplementation and exercise effects, ExA, interaction between exercise and age effects, AxExS, effects of interaction between three factors. S, A, E, SxA, ExA, SxE or AxExS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation; # indicates significant differences between pre-exercise and post-exercise; $ indicates significant differences between the young and senior groups. Where interaction exists between different statistical factors, different letters reveal significant differences.

circulating erythrocytes was influenced in all of them (Table 6). The senior group had significantly lower haematocrit and blood haemoglobin levels than the young group. Both acute exercise and functional beverage dietary supplementation significantly increased blood haemoglobin levels.

Age and acute exercise and interaction between these two factors influenced the plasma and blood polyphenol levels (Table 6). Pre-exercise plasma and blood polyphenol levels were significantly higher in the senior group than in the young one, both under the control and supplemented conditions, and acute exercise decreased plasma and blood polyphenol levels in the senior group. No differences between the senior and young groups were evidenced after acute exercise under both control and supplemented conditions. Exercise had a similar influence on blood cell polyphenol, lowering post-exercise levels; age and dietary beverage supplementation interactions also affect this parameter. The dietary supplementation with the functional beverage increased pre-exercise polyphenol levels in blood cells of the senior group but not of the young one, reaching significantly higher values.

Nitrate and nitrite are used as markers of nitric oxide production, in addition to their use as a marker of nitrate intake. Plasma nitrite levels were not altered by acute exercise, age or functional beverage dietary supplementation (Table 5). In contrast to this, plasma nitrate levels were influenced by exercise and by an interaction between age, exercise and functional

Table 6 Effects of supplementation with the functional beverage, acute exercise and age on polyphenol levels, haematological parameters, NOx plasma levels, plasma and erythrocyte oxidative stress markers

		Pre-exercise		Post-exercise		
		Young	Senior	Young	Senior	ANOVA
Haematological parameters						
Erythrocytes (10^6 cells per µl)	Control	4.85 ± 0.27	4.60 ± 0.18	4.84 ± 0.13	4.63 ± 0.17	
	Supplemented	4.96 ± 0.16	4.44 ± 0.14	4.96 ± 0.21	4.36 ± 0.11	
Haemoglobin (g per 100 mL)	Control	15.1 ± 0.2	14.5 ± 0.4	15.7 ± 0.2	$14.6 \pm 0.3^{\$}$	E, A, S
	Supplemented	15.8 ± 0.1	$14.5 \pm 0.2^{\$}$	16.4 ± 0.3	$15.2 \pm 0.3^{\$}$	
Haematocrit (%)	Control	44.8 ± 0.6	42.9 ± 1.4	46.6 ± 0.6	43.3 ± 1.1	A
	Supplemented	45.7 ± 0.9	41.6 ± 0.7	47.1 ± 0.5	42.6 ± 0.8	
Polyphenol levels						
Blood polyphenols (µM)	Control	454 ± 45^{a}	1007 ± 313^{b}	551 ± 103^{a}	552 ± 69^{a}	
	Supplemented	486 ± 66^{a}	940 ± 177^{b}	429 ± 54^{a}	456 ± 54^{a}	E, A, ExA
Plasma polyphenols (µM)	Control	513 ± 49^{a}	1532 ± 531^{c}	$814 \pm 211^{a,b}$	779 ± 96^{a}	A, ExA
	Supplemented	607 ± 77^{a}	$1230 \pm 237^{b,c}$	597 ± 100^{a}	488 ± 35^{a}	
Blood cell polyphenols (µM)	Control	$377 \pm 48^{a,b}$	289 ± 58^{b}	257 ± 43^{b}	262 ± 47^{b}	E, AxS
	Supplemented	341 ± 51^{b}	525 ± 114^{a}	240 ± 26^{b}	$364 \pm 72^{a,b}$	
NOx plasma levels						
Nitrite (nM)	Control	41.2 ± 3.0	48.7 ± 3.4	40.5 ± 4.0	42.7 ± 3.9	
	Supplemented	40.5 ± 3.0	37.3 ± 2.4	33.8 ± 2.8	43.5 ± 3.0	
Nitrate (µM)	Control	30.0 ± 3.5^{a}	$35.3 \pm 2.1^{a,b}$	$42.4 \pm 3.8^{b,c}$	$36.0 \pm 2.7^{a,b}$	E, A*E*S
	Supplemented	32.8 ± 2.0^{a}	30.6 ± 3.5^{a}	$38.2 \pm 2.5^{a,b,c}$	44.8 ± 3.0^{c}	
Plasma oxidative damage markers						
MDA concentration (µM)	Control	2.93 ± 1.67	4.24 ± 2.31	3.41 ± 1.09	1.79 ± 0.64	S
	Supplemented	1.40 ± 1.13	0.53 ± 0.30*	1.92 ± 0.62	0.77 ± 0.26	
Carbonyl index (%)	Control	100 ± 5.0	98.0 ± 7.1	93.4 ± 5.0	$125 \pm 12.0^{\$}$	E, A
	Supplemented	78.6 ± 7.5	88.3 ± 10.9	88.1 ± 7.4	97.9 ± 8.8#	
Nitro-tyrosine (%)	Control	100 ± 7^{a}	105 ± 6^{a}	77.7 ± 9.3^{b}	93.4 ± 5.6^{a}	S*A
	Supplemented	$95.6 \pm 6.2^{a,b}$	95.1 ± 10.5^{a}	103 ± 3^{a}	111 ± 6^{a}	
Lipoperoxide (ng ml^{-1})	Control	13.1 ± 6.0	17.1 ± 1.2	19.3 ± 7.4	11.3 ± 3.1	
	Supplemented	24.9 ± 9.9	5.6 ± 10.4	19.0 ± 5.4	13.5 ± 5.4	
Erythrocytes oxidative damage markers						
MDA (µmoles × 10^6 erythrocytes)	Control	20.3 ± 0.5	19.1 ± 0.8	23.4 ± 4.2	17.7 ± 1.1	
	Supplemented	19.2 ± 1.2	18.3 ± 1.3	18.4 ± 0.5	17.4 ± 2.4	
Carbonyl index (%)	Control	100 ± 5	103 ± 7	108 ± 12	99.7 ± 5.2	
	Supplemented	113 ± 4	113 ± 8	101 ± 7	107 ± 9	
Nitro-tyrosine (%)	Control	100 ± 15	95.7 ± 13.7	75.8 ± 10.0	$128 \pm 23^{\$}$	E
	Supplemented	106 ± 19	124 ± 18	80.8 ± 6.6	$140 \pm 28^{\$}$	

Results are the mean ± sem. Statistical analysis: three-way ANOVA, $p < 0.05$. S, supplementation effect; A, age effect, E, exercise effect, SxA, interaction between supplementation and age effects, SxE, interaction between supplementation and exercise effects, ExA, interaction between exercise and age effects, AxExS, effects of interaction between three factors. S, A, E, SxA, ExA, SxE or AxExS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation; # indicates significant differences between pre-exercise and post-exercise; $ indicates significant differences between the young and senior groups. Where interaction exists between different statistical factors, different letters reveal significant differences.

beverage dietary supplementation. Acute exercise increased plasma nitrate levels with regard to pre-exercise levels, mainly in the young control group and in the senior supplemented group. The highest plasma nitrate levels were reported in the senior and supplemented groups after exercise.

Effects on oxidative and nitrative damage markers

Table 6 shows the effects of supplementation with the functional beverage, acute exercise and age on plasma and erythrocyte oxidative stress markers. The oxidative damage markers of

erythrocytes were not influenced by age, acute exercise or functional beverage dietary supplementation with the exception of the nitrative damage of erythrocyte proteins that was influenced by acute exercise. Nitrotyrosine levels increased significantly after acute exercise in the senior group but not in the young group both under control and supplemented conditions. Nitrotyrosine serves as a marker for peroxynitrite action. Nitric oxide reacts with superoxide to form peroxynitrite, which in turn nitrates tyrosine residues in proteins. Functional beverage dietary supplementation significantly decreased MDA plasma levels (Table 7), mainly in the senior group under pre-exercise conditions. Acute exercise or age did not influence MDA plasma levels. No effects of acute exercise, age or dietary functional beverage supplementation were observed in lipoperoxide levels. However, acute exercise and age significantly influenced the levels of the carbonyl index, mainly in the senior group. The plasma carbonyl index was significantly higher after acute exercise than at pre-exercise in the senior group after supplementation. In addition, the plasma carbonyl index was significantly higher in the senior group than in the young group after exercise in the control situation. No effects of functional beverage dietary supplementation were observed in the marker for protein carbonyl modification in plasma. Nitrotyrosine plasma levels were not affected by age, acute exercise or the functional beverage but an interaction between age and functional beverage dietary supplementation was observed. The plasma nitrotyrosine levels were significantly lower in the young group after acute exercise in the control situation than all other groups and situations studied.

Table 7 Effects of supplementation with the functional beverage, acute exercise and age on antioxidant and mitochondrial PBMC gene expression

		Pre-exercise		Post-exercise		
		Young	Senior	Young	Senior	ANOVA
Mn SOD	Control	1.00 ± 0.33	1.16 ± 0.37	1.22 ± 0.38	1.39 ± 0.32	
	Supplemented	1.24 ± 0.39	1.25 ± 0.42	2.62 ± 1.36	0.70 ± 0.06	
GPx	Control	1.00 ± 0.13^{a}	1.19 ± 0.25^{a}	1.21 ± 0.28^{a}	0.98 ± 0.10^{a}	S (0.080) E*A (0.068)
	Supplemented	1.23 ± 0.28^{a}	1.56 ± 0.52^{a,b}	2.33 ± 0.89^{b}	1.12 ± 0.17^{a}	
CAT	Control	1.00 ± 0.23^{a}	0.94 ± 0.17^{a}	0.80 ± 0.07^{a}	0.88 ± 0.17^{a}	S*E*A (0.066)
	Supplemented	0.88 ± 0.17^{a}	1.50 ± 0.46^{a,b}	1.94 ± 0.69^{b}	0.82 ± 0.11^{a}	
GRd	Control	1.00 ± 0.22	1.09 ± 0.31	1.17 ± 0.25	0.96 ± 0.16	S (0.099)
	Supplemented	1.68 ± 0.67	1.74 ± 0.72	1.97 ± 0.69	0.96 ± 0.13	
UCP-3	Control	1.00 ± 0.13	1.16 ± 0.28	1.86 ± 0.59	1.26 ± 0.29	
	Supplemented	2.36 ± 1.23	1.46 ± 0.44	2.96 ± 1.49	1.08 ± 0.16	
HO-1	Control	1.00 ± 0.15	1.12 ± 0.28	1.49 ± 0.48	1.11 ± 0.24	
	Supplemented	1.63 ± 0.59	1.86 ± 0.66	1.88 ± 0.62	1.01 ± 0.15	
Cu–Zn SOD	Control	1.00 ± 0.13	1.01 ± 0.17	1.02 ± 0.15	1.91 ± 0.59	E (0.08)
	Supplemented	1.08 ± 0.18	1.06 ± 0.21	3.42 ± 1.81#	1.23 ± 0.22	
COXIV	Control	1.00 ± 0.14	2.64 ± 1.15	1.35 ± 0.44	2.05 ± 0.69	S*A (0.067)
	Supplemented	2.22 ± 0.94	1.33 ± 0.35	1.86 ± 0.94	1.32 ± 0.44	
Mtf1	Control	1.00 ± 0.24	2.72 ± 1.30	2.67 ± 1.45	1.33 ± 0.42	
	Supplemented	1.41 ± 0.50	1.49 ± 0.61	1.20 ± 0.52	1.47 ± 0.59	
Mtf2	Control	1.00 ± 0.28	1.72 ± 0.65	1.65 ± 0.75	1.05 ± 0.34	
	Supplemented	1.07 ± 0.29	2.24 ± 1.16	1.75 ± 0.62	1.32 ± 0.49	
SLC	Control	1.00 ± 0.28	2.56 ± 1.30	0.82 ± 0.18	0.99 ± 0.24	
	Supplemented	1.21 ± 0.43	1.44 ± 0.57	1.13 ± 0.34	0.68 ± 0.12	
MitND5	Control	1.00 ± 0.45	1.04 ± 0.49	0.64 ± 0.22	0.64 ± 0.26	
	Supplemented	0.48 ± 0.19	0.34 ± 0.08	0.54 ± 0.17	1.07 ± 0.66	
PGC1α	Control	1.00 ± 0.40	1.17 ± 0.42	0.52 ± 0.07	1.06 ± 0.50	
	Supplemented	2.78 ± 1.53	1.14 ± 0.40	0.85 ± 0.21	1.49 ± 0.64	

Results are the mean ± sem. Statistical analysis: three-way ANOVA, $p < 0.1$. S, supplementation effect; A, age effect, E, exercise effect, SxA, interaction between supplementation and age effects, SxE, interaction between supplementation and exercise effects, ExA, interaction between exercise and age effects, AxExS, effects of interaction between three factors. S, A, E, SxA, ExA, SxE or AxExS indicates a significant effect of each statistical factor. * indicates significant differences between the dietary control and functional beverage dietary supplementation; # indicates significant differences between pre-exercise and post-exercise; $ indicates significant differences between the young and senior groups. Where interaction exists between different statistical factors, different letters reveal significant differences.

Effects on antioxidant and mitochondrial turnover genes in PBMCs

Table 7 shows the effects of the functional beverage, age and acute exercise on antioxidant and mitochondrial PBMC gene expression. Functional beverage dietary supplementation, acute exercise and age did not influence the gene expression of Mn-SOD, UCP3, HO1, Mtf1, Mtf2, SLC2A4, MitND and PCG1α. However, the dietary supplementation significantly ($p < 0.1$) increased GRd and GPx gene expression; furthermore an interaction between exercise and age influenced the GPx expression ($p < 0.1$). GPx significantly increased in the young group after exercise in the supplemented group whereas the senior group maintained pre-exercise levels. Acute exercise increased Cu/Zn-SOD gene expression, mainly in the young group in the functional supplementation situation. The catalase gene expression was influenced by an interaction between the three analysed factors. Similar to the GRd, GPx and Cu/Zn-SOD expression, the CAT expression was significantly ($p < 0.1$) higher in the young group after exercise under the functional beverage dietary supplementation conditions than all other groups and situations. Dietary supplementation interacts with age influencing the COXIV gene expression. COXIV expression was higher in the senior group than the young group in the control situation, both under pre- and post-exercise conditions. Functional beverage dietary supplementation increased COXIV expression in the young group whereas it decreased COXIV expression in the senior group, both in pre-exercise and post-exercise situations.

Discussion

The composition of the functional beverage allows supplementing the diet mainly with monounsaturated and polyunsaturated fatty acids, including DHA, polyphenols and vitamin E. The nutritional intervention regime (1 L day^{-1} for five days per week) provides a 2.6× higher dose than the recommended dietary vitamin E allowance for the general population; a polyphenol dose similar to that provided by orange juice,[21] but lower than commercial polyphenol-rich juices; an additional 17% to the total fat ingested by athletes in the Balearic Islands[20] and a 3.2× higher dose of DHA than recommended for the general population.[22] The estimated polyphenol daily intake is variable depending on the age, gender and country.[23] The beverage supplementation provides a dietary polyphenol intake of around 20% more than the total polyphenol intake for populations in European countries.[23]

Effects on physical performance parameters

Heat storage, body and skin temperature, blood lactate, time spent until exhaustion at 90% VO_2max and fatigue perception (Borg index) during a maximal exercise test in a hot environment are considered markers of physical performance. It has been evidenced that heat tolerance tests are feasible measures of physiological strain.[24] During exercise, the vast majority of the chemical energy of food is converted to heat in contracting muscles increasing the body heat content. These changes are modulated by the rate of relative heat production, and represent the rate of change in body heat storage, which in turn reflects the balance between metabolic heat production, heat absorbed from the environment and total body heat loss.[25] The excess of heat production that is reflected in the central body temperature during exercise in a hot environment is an important determinant of behaviour and, therefore, performance.[26] The increase in the body heat content and, thus, core temperature will activate autonomic heat-loss responses including skin vasodilation and sweating,[27] but also will reduce the rate of metabolic heat production by behavioural means.[26] The heat storage, body and skin temperature, and blood lactate measured during exercise tests are influenced by exercise but not by age or dietary supplementation. The exercise test increased both the skin and core body temperature and decreased heat storage change in a similar way in the young and senior groups, irrespective of their control or dietary supplemented conditions. Similarly, neither nutritional intervention with the functional beverage nor the age of athletes influenced the transition to the anaerobic phase of exercise or lactate accumulation during the exercise test in heat. Nonetheless, the perceived exertion Borg index was influenced by both the exercise test and age. The perceived exertion may be the ultimate modulator of behaviour during self-paced exercise in heat.[28] The results reveal the existence of an age-related fatigue perception resistance in accordance with a previous study,[29] although no differences in the rating of perceived exertion to exercise immediately after a maximal exercise test at 25 °C between a young group of athletes and a senior group of athletes have been noted.[30]

The lack of influence of beverage dietary supplementation on exercise performance contrasts with other studies that pointed out the enhancing effects of dietary n3-PUFA supplementation on exercise performance.[3] At the same time, our results contrast with a reduction in endurance performance induced by chronic (more than a week) high fat consumption.[31] The antioxidant content of the functional beverage, in terms of vitamin E and polyphenols, also induces no effect on the physical performance. Although, it has been reported that the chronic intake of most antioxidants can block physiological adaptations to training[7] some other studies evidenced a positive or non-significant effect.[6]

Effects of the functional beverage, exercise and age on circulating polyphenols

The functional beverage is rich in polyphenols and vitamin E (also a polyphenol). The functional effects of the almond and olive oil-based beverage are mediated by the bioavailability of their active components as polyphenols and vitamin E. Polyphenol bioavailability studies involve feeding volunteers a single supplement and monitoring the levels of polyphenol in plasma and urine over a 24-hour period.[32] Diet supplementation with the functional beverage does not influence basal plasma and blood levels of total polyphenols but the senior athletes did see an increase in the total polyphenol

concentration in blood cells as a result of functional beverage supplementation. It has been stated that the consumption of nuts (rich in polyphenols) increases postprandial plasma concentration and total antioxidant capacity, and reduces plasma lipid peroxidation.[33] In general, consuming phenolic-rich fruits increases the post-prandial antioxidant capacity of blood and when they are consumed with high fat and carbohydrates, as 'pro-oxidant and pro-inflammatory' meals, they may counterbalance their negative effects. We evidenced that increasing the dietary polyphenol intake also increases the basal blood cell polyphenol levels, mainly in senior athletes. To date, there are no studies on the polyphenol content of blood cells, although these cells could transport polyphenols into the blood. Circulating polyphenol levels are influenced by age and acute exercise. Senior athletes have higher basal polyphenol levels than young athletes in their blood, plasma and blood cells, but in the latter case only after dietary supplementation with the functional beverage. Acute exercise decreased polyphenol levels in all compartments in senior athletes but maintained basal levels in young athletes. This picture is compatible with polyphenol use as an antioxidant[34] to counteract higher exercise-induced oxidative damage in senior rather than young athletes.

Effects on plasma and erythrocytes lipid profile

Functional beverage dietary supplementation changed plasma fatty acid composition, increasing polyunsaturated and lowering saturated fatty acids. Dietary fatty acid consumption influences human plasma fatty acid profiles.[19] The plasma fatty acid profile of asymptomatic men and women with moderate hypercholesterolemia changed after supplementation with a Mediterranean-type diet for four weeks, increasing plasma MUFA and PUFA.[35] Despite the effects of dietary supplementation on the lipid profile, there is no correlation between the amount of individual fatty acids supplemented with the functional beverage and the increased individual fatty acid concentration in plasma, probably due to the selective use and purpose of dietary fatty acids for oxidation or phospholipid or triacylglyceride incorporation.[36] In addition, the response of the plasma fatty acid profile to dietary functional beverage supplementation is age-dependent; senior group plasma fatty acids were more polyunsaturated and less monounsaturated than in the young group. It has been pointed out that plasma non-esterified SFA, PUFA and MUFA increase with age; senior people have more plasma non-esterified DHA than young people.[36] The different plasma fatty acid profile is most likely to be a consequence of altered cellular uptake, metabolism and biosynthesis associated with ageing. In fact, it has been confirmed that methylation of the fatty acid elongase 2 gene is altered during ageing[37] and delta 6 desaturase activity also declines with age.[36]

Dietary supplementation influenced the erythrocyte fatty acid profile in a different way than the plasma fatty acid profile. The supplementation only altered the percentage of C18:3 and DHA in the erythrocyte membranes. The dietary fatty acids are incorporated at a different rate into different cellular and molecular pools;[20] a selective process has to occur in the incorporation of dietary fatty acids into erythrocytes. The increased DHA content in erythrocytes after dietary functional beverage supplementation is similar to what has been observed in other studies.[20] This increase indicates that athletes followed the prescribed beverage intake during the trial and it was effective in incorporating C18:3 and DHA into erythrocytes. The fatty acid composition of erythrocyte membranes is slightly age-dependent because the senior group had a higher proportion of C20:3 and C22:0 than the younger group, although these fatty acids are in a very low proportion in the erythrocyte membrane and the difference disappears after dietary supplementation with the beverage.

Effects on oxidative damage and antioxidant status

Dietary functional beverage supplementation for one month makes erythrocytes and plasma fatty acids more unsaturated and they could be more susceptible to oxidation. However, lipid oxidation measured as plasma MDA (a product of unsaturated fatty acid peroxidation) reported lower levels in supplemented than in control groups. Previous dietary supplementation trials with this functional beverage analysing lipid oxidative damage in lymphocytes[18] or consuming normal or high polyphenol concentrations in orange juice are in line with a protective role of the supplement against lipid peroxidation.[21] The protective direct or indirect antioxidant effects of functional beverage dietary supplementation could be attributed to DHA, vitamin E or polyphenol.[38,39] However, the DHA dietary supplementation for eight weeks did not alter the basal plasma and erythrocyte MDA levels in trained footballers.[20] It reinforces the role of vitamin E and polyphenols as antioxidants in the functional beverage. In fact, supplementing the diet with vitamins E and C reduces oxidative damage markers in exercising athletes.[8] Similarly, it has been stated that the high polyphenol content of orange juice is responsible for protection against DNA damage and lipid peroxidation, modifying several antioxidant enzymes in overweight or obese non-smoking adults.[21] In our study, the physical test was performed under fasting conditions and the functional beverage consumption had no effect on basal plasma polyphenol levels but did lower lipid oxidative damage.

Dietary supplementation protected plasma proteins against oxidative damage induced by both acute exercise and age. Senior athletes are more susceptible to plasma protein oxidative damage than younger athletes are, in accordance with several studies that demonstrated the existence of a positive correlation between age and protein-carbonyl levels.[34] Similarly, acute exercise damaged plasma proteins, according to another study,[40] but the dietary consumption of the functional beverage avoided protein oxidation. Protein carbonylation occurs with the reaction between MDA or glyoxal and the amino groups of proteins;[41] one could speculate that functional beverage dietary supplementation reduces MDA or glyoxal production or their reaction with plasma proteins induced by acute exercise and ageing.

Erythrocytes are more protected than plasma from oxidative damage because acute exercise increases protein oxidative damage markers in the latter but not in the former. Whereas plasma is more protected than erythrocytes from the nitrative damage of protein, exercise increased nitrative protein damage in the erythrocytes of senior athletes but not in young athletes, irrespective of functional beverage supplementation. Acute exercise enhances nitric oxide and superoxide anion production, and nitrite levels in erythrocytes;[10] protein nitration results from the reaction between protein-tyrosine and peroxynitrite (a product of the reaction between nitric oxide and a superoxide anion). In this sense, the nitrotyrosine index could reflect peroxynitrite production. This suggests that the senior group has a lower erythrocyte capacity for avoiding peroxynitrite production than the younger group during exercise. This situation was not affected by the dietary supplementation. However, dietary functional beverage supplementation influences the nitrative damage of plasma proteins in an age-dependent way. Whereas young athletes presented a lower plasma nitrotyrosine index than senior athletes after acute exercise, these differences disappeared after dietary functional beverage supplementation. In this sense, functional beverage consumption could enhance the nitrative damage of plasma proteins induced by an exercise test in young athletes. One can speculate that dietary functional beverage supplementation enhances peroxynitrite formation in young athletes during exercise. Growing evidence shows that dietary polyphenol enhances endothelial synthesis and the plasma levels of nitric oxide, and reduces systolic and diastolic blood pressure protecting the heart and blood cells.[10] The nitrative damage of plasma proteins in young athletes during exercise and after dietary supplementation with the functional beverage could reflect the higher plasma nitric oxide and superoxide anion production induced by exercise in a situation of higher polyphenol intake.

Effects on antioxidant and mitochondrial turnover gene expression

The suitability of supplementing the diet of athletes with antioxidants such as vitamins C and E or polyphenols in order to ameliorate physical adaptations to training has been scarcely questioned.[6,7,9] Although our results did not show differences in antioxidant gene expression, this fact could be explained because our experimental models are highly trained athletes who are adapted to high intensity training seasons. Furthermore, the intake of the functional beverage with antioxidants could eliminate the production of ROS, blocking their activity as cellular messengers for important cellular functions related to antioxidant defences, cellular proliferation and differentiation. However, there exist studies where variations in antioxidant gene expression on PBMCs due to exercise and supplementation are observed.[42,43] In a similar way, we did not observe significant changes in mitochondrial turnover gene expression in PBMCs due to exercise or supplementation beverage, however there exist studies where these effects were evidenced, but not in PBMCs.[44,45] Neither acute exercise nor age or functional beverage diet supplementation influenced the expression of mitochondrial turnover and antioxidant genes. However, our results pointed to an enhanced role of dietary functional beverage supplementation rich in antioxidants in the gene expression of antioxidant enzymes induced by exercise, albeit age-dependent. The gene expression of PBMC glutathione-dependent antioxidant enzymes (GPX and GRd) and antioxidant enzymes eliminating a superoxide anion and hydrogen peroxide (Mn-SOD, Cu–Zn SOD, and CAT) is enhanced after acute exercise only in the young group supplemented with the dietary functional beverage. Moderate antioxidant supplementation prevents neutrophil protein oxidation without affecting the adaptive response to exercise.[8] Dietary functional beverage supplementation avoids plasma oxidative damage and enhances adaptive PBMC antioxidant response to exercise in young athletes but does not affect senior athletes.

Conclusion

In summary, the almond- and olive oil-based, DHA and vitamin E-enriched beverage enables dietary supplementation mainly with monounsaturated and polyunsaturated fatty acids, including DHA, polyphenols and vitamin E. The dietary functional beverage supplementation for one month did not alter the performance parameters such as heat storage, body and skin temperature, blood lactate and the Borg scale for fatigue during a maximal exercise test with regard to a control non-supplemented situation. The supplementation did not influence the basal plasma and blood levels of total polyphenols but in senior athletes did increase the basal total polyphenol concentration in blood cells. Senior athletes have higher basal polyphenol levels than young athletes in blood plasma and in blood cells, but in the latter instance only after dietary supplementation with the functional beverage. The functional beverage changed the plasma fatty acid composition with higher individual polyunsaturated and lower individual saturated fatty acid levels after one month of dietary supplementation. These changes are age-dependent as the senior group plasma fatty acids were less monounsaturated than in the young group. The functional beverage only increased the percentage of both C18:3 and DHA in erythrocytes in contrast to the changes observed in plasma. Dietary functional beverage supplementation for one month protected plasma lipid oxidative damage, although it can enhance nitrative damage in young athlete erythrocytes after exercise. The gene expression of PBMC antioxidant enzymes was enhanced after acute exercise only in the young group supplemented with the functional beverage. Dietary functional beverage supplementation evaded plasma oxidative damage and enhanced the adaptive PBMC antioxidant response to exercise in young athletes but had no effect for senior athletes.

Conflict of interest disclosure

The authors declare no conflict of interest.

Acknowledgements

Acción Estratégica en Salud del Ministerio de Ciencia e Innovación DPS2008-07033-C03-03, Programme of Promotion of Biomedical Research and Health Sciences, Projects 11/01791, Red Predimed-RETIC RD06/0045/1004, CIBEROBN CB12/03/30038 and Balearic Island Government and FEDER funds (35/2011 and 23/2012). We hereby acknowledge the PhD grant provided by the University of the Balearic Islands.

References

1 N. R. Rodriguez, N. M. DiMarco and S. Langley, Position of the American Dietetic Association, Dietitians of Canada, and the American College of Sports Medicine: Nutrition and athletic performance, *J. Am. Diet. Assoc.*, 2009, **109**, 509–527.

2 A. Petroczi, D. P. Naughton, J. Mazanov, A. Holloway and J. Bingham, Performance enhancement with supplements: incongruence between rationale and practice, *J. Int. Soc. Sports Nutr.*, 2007, **4**, 19.

3 T. R. Figueira, M. H. Barros, A. A. Camargo, R. F. Castilho, J. C. Ferreira, A. J. Kowaltowski, F. E. Sluse, N. C. Souza-Pinto and A. E. Vercesi, Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health, *Antioxid. Redox Signaling*, 2013, **18**, 2029–2074.

4 M. Martorell, X. Capo, A. Sureda, J. A. Tur and A. Pons, Effects of docosahexaenoic acid diet supplementation, training, and acute exercise on oxidative balance in neutrophils, *Appl. Physiol., Nutr., Metab.*, 2014, **39**, 446–457.

5 M. Martorell, X. Capó, A. Sureda, J. Tur and A. Pons, Chromatographic and Enzymatic Method to Quantify Individual Plasma Free and Triacylglycerol Fatty Acids, *Chromatographia*, 2015, **78**, 259–266.

6 G. Paulsen, K. T. Cumming, G. Holden, J. Hallen, B. R. Ronnestad, O. Sveen, A. Skaug, I. Paur, N. E. Bastani, H. N. Ostgaard, C. Buer, M. Midttun, F. Freuchen, H. Wiig, E. T. Ulseth, I. Garthe, R. Blomhoff, H. B. Benestad and T. Raastad, Vitamin C and E supplementation hampers cellular adaptation to endurance training in humans: a double-blind, randomised, controlled trial, *J. Physiol.*, 2014, **592**, 1887–1901.

7 A. J. Braakhuis and W. G. Hopkins, Impact of Dietary Antioxidants on Sport Performance: A Review, *Sports Med.*, 2015, **45**, 939–955.

8 A. Sureda, M. D. Ferrer, A. Mestre, J. A. Tur and A. Pons, Prevention of neutrophil protein oxidation with vitamins C and E diet supplementation without affecting the adaptive response to exercise, *Int. J. Sport Nutr. Exercise Metab.*, 2013, **23**, 31–39.

9 D. C. Nieman, N. D. Gillitt, A. M. Knab, R. A. Shanely, K. L. Pappan, F. Jin and M. A. Lila, Influence of a polyphenol-enriched protein powder on exercise-induced inflammation and oxidative stress in athletes: a randomized trial using a metabolomics approach, *PLoS One*, 2013, **8**, e72215.

10 A. Medina-Remon, A. Tresserra-Rimbau, A. Pons, J. A. Tur, M. Martorell, E. Ros, P. Buil-Cosiales, E. Sacanella, M. I. Covas, D. Corella, J. Salas-Salvado, E. Gomez-Gracia, V. Ruiz-Gutierrez, M. Ortega-Calvo, M. Garcia-Valdueza, F. Aros, G. T. Saez, L. Serra-Majem, X. Pinto, E. Vinyoles, R. Estruch and R. M. Lamuela-Raventos, Effects of total dietary polyphenols on plasma nitric oxide and blood pressure in a high cardiovascular risk cohort. The PREDIMED randomized trial, *Nutr., Metab. Cardiovasc. Dis.*, 2015, **25**, 60–67.

11 N. Li, X. Jia, C. Y. Chen, J. B. Blumberg, Y. Song, W. Zhang, X. Zhang, G. Ma and J. Chen, Almond consumption reduces oxidative DNA damage and lipid peroxidation in male smokers, *J. Nutr.*, 2007, **137**, 2717–2722.

12 M. G. Nikolaidis, C. M. Kerksick, M. Lamprecht and S. R. McAnulty, Does vitamin C and E supplementation impair the favorable adaptations of regular exercise?, *Oxid. Med. Cell. Longevity*, 2012, **2012**, 707941.

13 A. Mestre-Alfaro, M. D. Ferrer, M. Banquells, J. Riera, F. Drobnic, A. Sureda, J. A. Tur and A. Pons, Body temperature modulates the antioxidant and acute immune responses to exercise, *Free Radical Res.*, 2012, **46**, 799–808.

14 M. Meydani, Vitamin E requirement in relation to dietary fish oil and oxidative stress in elderly, *EXS*, 1992, **62**, 411–418.

15 D. M. Bailey, J. McEneny, O. Mathieu-Costello, R. R. Henry, P. E. James, J. M. McCord, S. Pietri, I. S. Young and R. S. Richardson, Sedentary aging increases resting and exercise-induced intramuscular free radical formation, *J. Appl. Physiol.*, 2010, **109**, 449–456.

16 A. Molfino, G. Gioia, F. Rossi Fanelli and M. Muscaritoli, The role for dietary omega-3 fatty acids supplementation in older adults, *Nutrients*, 2014, **6**, 4058–4073.

17 G. Borg, Perceived exertion as an indicator of somatic stress, *Scand. J. Rehabil. Med.*, 1970, **2**, 92–98.

18 X. Capó, M. Martorell, A. Sureda, I. Llompart, J. A. Tur and A. Pons, Diet supplementation with DHA-enriched food in football players during training season enhances the mitochondrial antioxidant capabilities in blood mononuclear cells, *Eur. J. Nutr.*, 2015, **54**(1), 35–49.

19 M. Martorell, X. Capo, A. Sureda, J. M. Batle, I. Llompart, E. Argelich, J. A. Tur and A. Pons, Effect of DHA on plasma fatty acid availability and oxidative stress during training season and football exercise, *Food Funct.*, 2014, **5**, 1920–1931.

20 M. Martorell, X. Capó, M. M. Bibiloni, A. Sureda, A. Mestre-Alfaro, J. M. Batle, I. Llompart, J. A. Tur and A. Pons, Docosahexaenoic acid supplementation promotes erythrocyte antioxidant defense and reduces protein nitrosative damage in male athletes, *Lipids*, 2015, **50**(2), 131–148.

21 O. D. Rangel-Huerta, C. M. Aguilera, M. V. Martin, M. J. Soto, M. C. Rico, F. Vallejo, F. Tomas-Barberan, A. J. Perez-de-la-Cruz, A. Gil and M. D. Mesa, Normal or High Polyphenol Concentration in Orange Juice Affects

Antioxidant Activity, Blood Pressure, and Body Weight in Obese or Overweight Adults, *J. Nutr.*, 2015, **145**, 1808–1816.

22 E. Ros, J. Lopez-Miranda, C. Pico, M. A. Rubio, N. Babio, A. Sala-Vila, F. Perez-Jimenez, E. Escrich, M. Bullo, M. Solanas, A. Gil Hernandez and J. Salas-Salvado, Consensus on fats and oils in the diet of S Ish adults; position paper of the Spanish federation of food, nutrition and dietetics societies, *Nutr. Hosp.*, 2015, **32**, 435–477.

23 R. Zamora-Ros, V. Knaze, J. A. Rothwell, B. Hémon, A. Moskal, K. Overvad, A. Tjønneland, C. Kyrø, G. Fagherazzi, M. C. Boutron-Ruault, M. Touillaud, V. Katzke, T. Kühn, H. Boeing, J. Förster, A. Trichopoulou, E. Valanou, E. Peppa, D. Palli, C. Agnoli, F. Ricceri, R. Tumino, M. S. de Magistris, P. H. Peeters, H. B. Bueno-de-Mesquita, D. Engeset, G. Skeie, A. Hjartåker, V. Menéndez, A. Agudo, E. Molina-Montes, J. M. Huerta, A. Barricarte, P. Amiano, E. Sonestedt, L. M. Nilsson, R. Landberg, T. J. Key, K. T. Khaw, N. J. Wareham, Y. Lu, N. Slimani, I. Romieu, E. Riboli and A. Scalbert, Dietary polyphenol intake in Europe: the European Prospective Investigation into Cancer and Nutrition (EPIC) study, *Eur. J. Nutr.*, 2016, 55(4), 1359–1375.

24 J. A. Mee, J. Doust and N. S. Maxwell, Repeatability of a running heat tolerance test, *J. Therm. Biol.*, 2015, **49–50**, 91–97.

25 M. N. Cramer and O. Jay, Selecting the correct exercise intensity for unbiased comparisons of thermoregulatory responses between groups of different mass and surface area, *J. Appl. Physiol.*, 2014, **116**, 1123–1132.

26 A. D. Flouris, M. P. Poirier, A. Bravi, H. E. Wright-Beatty, C. Herry, A. J. Seely and G. P. Kenny, Changes in heart rate variability during the induction and decay of heat acclimation, *Eur. J. Appl. Physiol.*, 2014, **114**, 2119–2128.

27 A. D. Flouris and S. S. Cheung, Thermometry and calorimetry assessment of sweat response during exercise in the heat, *Eur. J. Appl. Physiol.*, 2010, **108**, 905–911.

28 Z. J. Schlader, S. R. Stannard and T. Mundel, Human thermoregulatory behavior during rest and exercise - a prospective review, *Physiol. Behav.*, 2010, **99**, 269–275.

29 Y. Yassierli and M. A. Nussbaum, Muscle fatigue during intermittent isokinetic shoulder abduction: age effects and utility of electromyographic measures, *Ergonomics*, 2007, **50**, 1110–1126.

30 U. Dimkpa and K. Ibhazehiebo, Assessment of the influence of age on the rate of heart rate decline after maximal exercise in non-athletic adult males, *Clin. Physiol. Funct. Imaging*, 2009, **29**, 68–73.

31 R. D. Starling, T. A. Trappe, A. C. Parcell, C. G. Kerr, W. J. Fink and D. L. Costill, Effects of diet on muscle triglyceride and endurance performance, *J. Appl. Physiol.*, 1997, **82**, 1185–1189.

32 D. Del Rio, G. Borges and A. Crozier, Berry flavonoids and phenolics: bioavailability and evidence of protective effects, *Brit. J. Nutr.*, 2010, **104**(Suppl 3), S67–S90.

33 S. Torabian, E. Haddad, S. Rajaram, J. Banta and J. Sabate, Acute effect of nut consumption on plasma total polyphenols, antioxidant capacity and lipid peroxidation, *J. Hum. Nutr. Diet.*, 2009, **22**, 64–71.

34 L. Gil, W. Siems, B. Mazurek, J. Gross, P. Schroeder, P. Voss and T. Grune, Age-associated analysis of oxidative stress parameters in human plasma and erythrocytes, *Free Radical Res.*, 2006, **40**, 495–505.

35 N. R. Damasceno, A. Perez-Heras, M. Serra, M. Cofan, A. Sala-Vila, J. Salas-Salvado and E. Ros, Crossover study of diets enriched with virgin olive oil, walnuts or almonds. Effects on lipids and other cardiovascular risk markers, *Nutr., Metab. Cardiovasc. Dis.*, 2011, **21**(Suppl 1), S14–S20.

36 C. Pararasa, J. Ikwuobe, S. Shigdar, A. Boukouvalas, I. T. Nabney, J. E. Brown, A. Devitt, C. J. Bailey, S. J. Bennett and H. R. Griffiths, Age-associated changes in long-chain fatty acid profile during healthy aging promote pro-inflammatory monocyte polarization via PPARγ, *Aging Cell*, 2016, **15**(1), 128–139.

37 P. Garagnani, M. G. Bacalini, C. Pirazzini, D. Gori, C. Giuliani, D. Mari, A. M. Di Blasio, D. Gentilini, G. Vitale, S. Collino, S. Rezzi, G. Castellani, M. Capri, S. Salvioli and C. Franceschi, Methylation of ELOVL2 gene as a new epigenetic marker of age, *Aging Cell*, 2012, **11**, 1132–1134.

38 A. Sureda, P. Tauler, A. Aguilo, N. Cases, I. Llompart, J. A. Tur and A. Pons, Influence of an antioxidant vitamin-enriched drink on pre- and post-exercise lymphocyte antioxidant system, *Ann. Nutr. Metab.*, 2008, **52**, 233–240.

39 I. Zararsiz, M. F. Sonmez, H. R. Yilmaz, U. Tas, I. Kus, A. Kavakli and M. Sarsilmaz, Effects of omega-3 essential fatty acids against formaldehyde-induced nephropathy in rats, *Toxicol. Ind. Health*, 2006, **22**, 223–229.

40 P. Tauler, M. D. Ferrer, D. Romaguera, A. Sureda, A. Aguilo, J. Tur and A. Pons, Antioxidant response and oxidative damage induced by a swimming session: influence of gender, *J. Sports Sci.*, 2008, **26**, 1303–1311.

41 R. S. Rao and I. M. Moller, Pattern of occurrence and occupancy of carbonylation sites in proteins, *Proteomics*, 2011, **11**, 4166–4173.

42 M. D. Ferrer, P. Tauler, A. Sureda, J. A. Tur and A. Pons, Antioxidant regulatory mechanisms in neutrophils and lymphocytes after intense exercise, *J. Sports Sci.*, 2009, **27**, 49–58.

43 A. Mestre-Alfaro, M. D. Ferrer, A. Sureda, P. Tauler, E. Martinez, M. M. Bibiloni, V. Micol, J. A. Tur and A. Pons, Phytoestrogens enhance antioxidant enzymes after swimming exercise and modulate sex hormone plasma levels in female swimmers, *Eur. J. Appl. Physiol.*, 2011, **111**, 2281–2294.

44 J. C. Drake, R. J. Wilson and Z. Yan, Molecular mechanisms for mitochondrial adaptation to exercise training in skeletal muscle, *FASEB J.*, 2016, **30**, 13–22.

45 R. Putti, R. Sica, V. Migliaccio and L. Lionetti, Diet impact on mitochondrial bioenergetics and dynamics, *Front. Physiol.*, 2015, **6**, 109.

Manuscript VII

Resolvins as proresolving inflammatory mediators in cardiovascular disease

Xavier Capó, Miquel Martorell, Carla-Busquets Cortés, Silvia Tejada, Josep A. Tur, Antoni Pons, Antoni Sureda.

Eur J Med Chem. 2018 Jun 10;153:123-130.
doi: 10.1016/j.ejmech.2017.07.018. Epub 2017 Jul 13.

European Journal of Medicinal Chemistry 153 (2018) 123–130

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Resolvins as proresolving inflammatory mediators in cardiovascular disease

Xavier Capó [a], Miquel Martorell [b], Carla Busquets-Cortés [a], Silvia Tejada [c], Josep A. Tur [a, d], Antoni Pons [a, d], Antoni Sureda [a, d, *]

[a] Research Group on Community Nutrition and Oxidative Stress, University of Balearic Islands, E-07122 Palma de Mallorca, Balearic Islands, Spain
[b] Nutrition and Dietetics Department, School of Pharmacy, University of Concepción, 4070386 Concepción, VIII – Bio Bio Region, Chile
[c] Experimental Laboratory, Research Unit, Son Llàtzer Hospital, IUNICS, Ctra. Manacor km 4, E-07198 Palma de Mallorca, Balearic Islands, Spain
[d] CIBEROBN, Physiopathology of Obesity and Nutrition, E-07122 Palma de Mallorca, Balearic Islands, Spain

ARTICLE INFO

Article history:
Received 12 April 2017
Received in revised form
17 June 2017
Accepted 12 July 2017
Available online 13 July 2017

Keywords:
Atherosclerosis
Coronary artery disease
Docosahexaenoic acid
Inflammation
Lipid mediators
Resolution

ABSTRACT

Cardiovascular disease (CVD) represents a global burden with a prevalence that continues increasing progressively. CVD comprises a group of disorders of the heart and blood vessels including coronary heart disease, cerebrovascular disease and peripheral arterial disease. This group of disorders is associated with an inflammatory process which can participate in the pathophysiology of these diseases. Inflammation resolution is an active process involving the participation of pro-resolving mediators such as lipoxins, resolvins, protectins and maresins. Pro-resolving mediators are bioactive molecules generated from omega-3 polyunsaturated fatty acids (PUFAs); among these eicosapentaenoic acid (EPA; C20:5n3) and docosahexaenoic acid (DHA; C22:6n3) are the precursors of resolvins. Pro-resolving mediators orchestrate the correct resolution of inflammation and also stimulate tissue regeneration. Their deregulation can lead to chronic inflammation involving CVD. The discovery of these novel lipid mediators opens a new range of possibilities for the design of anti-inflammatory agents with therapeutic potential for a wide variety of diseases. The present work summarizes the available data about the general characteristics, structure and biosynthesis of resolvins and their relation as protective compounds in CVD.

1. Introduction

Effective immune response pathways against pathogens constitute an essential requirement for survival living, and the functional factors that regulate key metabolic and immune processes in higher organisms have evolved from common ancestral structures, being evolutionarily conserved throughout species [35]. As a result, immune response is highly integrated with the metabolic regulation, and the proper function of each is intimately dependent on the other. For this reason, metabolic disorders are crucially linked to inflammatory events. This interface is considered a pivotal central homeostatic mechanism, dysfunction of which can lead to the development of a cluster of chronic metabolic disorders, concretely obesity, type 2 diabetes and cardiovascular disease. Currently, these diseases represent the greatest threat to the worldwide human health and welfare; in fact, coronary artery disease (CAD) remains to be the leading cause of death in the world [77]. There is extensive epidemiologic literature supporting the association between inflammation and CAD [18,77,102,105]. Current evidence supports that inflammatory cells and proteins, and inflammatory responses from vascular cells play a major role in the development and propagation of CAD, since they are major forces underlying the onset of coronary plaques, their unstable progression and eventual rupture.

The concept of inflammation and some of its satellite terms (such as acute and chronic) can be defined as a protective response of living tissues to local lesions. Inflammatory events include molecular, cellular and vascular phenomena addressed towards physical, chemical or biological aggressions [24,38,46]. Inflammation is characterized by cellular migration (extravasation) into the injured site that will trigger off the elimination or inhibition of the

* Corresponding author. Research Group on Community Nutrition and Oxidative Stress, University of Balearic Islands, Ctra. Valldemossa km 7.5, E-07122 Palma de Mallorca, Balearic Islands, Spain.
E-mail address: tosugo@hotmail.com (A. Sureda).

http://dx.doi.org/10.1016/j.ejmech.2017.07.018
0223-5234/

primary cause of damage, the clearance of necrotic cells and the initiation of the tissue repair. Both vasodilatation of closer blood vessels and an increased permeability facilitate the arrival of cellular and molecular mediators to the harmed spot [24,46]. Acute inflammation is the first response after a damaging stimulus. It takes place among toll-like receptors (TLR)-mediated infections, managed by the innate immune system, and it involves plasma and leukocytes (especially granulocytes) liberation into the injured tissue. Macrophages and mastocytes -resident in the injured tissue- are able to release soluble compounds that will eventually exert their effects on vascular permeability permitting the massive arrival of leukocytes (mainly neutrophils) and other molecules involved in inflammatory response. Afterwards, pathogent-contact or cytokines present in the scenario activated the neutrophils. A central point in the inflammatory process is the necessity to be actively finished when is no longer needed and this is accomplished by different mechanisms according with the affected tissue in order to avoid collateral damage [46]. Otherwise, an incorrect or inexistent resolution of inflammation can turn into a perpetuation of damage tissue and necrosis, which can result in chronic inflammation and developing a sustained pathological state [63]. Different biochemical events propagate and mature the inflammatory response, and multiple mediators are released, among which the following ones stand out: vaso-active amines and peptides (histamine, serotonin), fragments of the complement system (anafilotoxins), inflammatory cytokines (tumor necrosis factor alpha -TNFα-, interleukins -IL- 1 and 6), chemokines (IL-8), proteolytic enzymes (matalloproteinases, elastase) and lipid mediators (prostaglandins, thromboxanes, leukotrienes, lipoxins).

Lipids are potent signalling molecules that play an important role in homeostasis and host defence [5]; when inflammation occurs, phospholipids of membrane from activated cells of the immune system are metabolized to generate a wide variety of lipid substances which mediate the inflammatory response [86]. Stimuli such as injury, microorganisms or interleukin 8 activate Phospholipase A_2, which in turn generates free arachidonic acid (AA), a 20-carbon fatty acid and a leading precursor of lipid mediators. AA is easily metabolized via two principal pathways to form a family of oxygenated products called eicosanoids [49,94]. In addition, prostaglandins (PGs) and thromboxanes (TRX) are produced by ciclooxigenases (COX); leukotrienes (LTs) and lipoxines (LXs) are formed by lipooxigenases (LOXs). In recent years, several molecules derived from COX-2 oxidation of omega 3 fatty acids have been elucidated [88,89], and postulated to exert powerful resolving effect on inflammation. Resolvins, derived from the "resolution phase interaction products", are endogenous bioactive lipid mediators that have been found to promote resolution of acute inflammation through interaction with high-affinity surface membrane receptors in human polymorphonuclear leukocytes [14,37,38,48].

2. Chemical structure of resolvins

As it has been introduced previously, resolvins are dihydroxy and trihydroxy metabolites of omega-3 fatty acids (Table 1). The characteristics and properties of a fatty acid depend on the length of the carbon chain but also on the presence or not of unsaturations. Omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) have multiple double bonds, with the first ones being on the carbon number 3 and 6, respectively, from the methyl end (omega carbon) of the hydrogen carbonate chain [95]. Mammals cannot synthesize all fatty acids because they cannot insert double bonds before carbon 9 in oleic acid (C18:1n9). More specifically, mammals

Table 1
Resolvins E and D classes and precursors. IUPAC names.

Resolvins E class precursors	
EPA	(5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaenoic acid
18R-HEPE	(5Z,8Z,11Z,14Z,16E,18R)-18-hydroxyicosa-5,8,11,14,16-pentaenoic acid
5S-Hp-18R-HEPE	(5S,6E,8Z,11Z,14Z,16E,18R)-5-hydroperoxy-18-hydroxyicosa-6,8,11,14,16-pentaenoic acid
18S-HEPE	(5Z,8Z,11Z,14Z,16E,18S)-18-hydroxyicosa-5,8,11,14,16-pentaenoic acid
5S-Hp-18S-HEPE	(5S,6E,8Z,11Z,14Z,16E,18S)-5-hydroperoxy-18-hydroxyicosa-6,8,11,14,16-pentaenoic acid
18S-resolvins E class	
18S-RvE1	(5S,6Z,8E,10E,12R,14Z,16E,18S)-5,12,18-trihydroxyicosa-6,8,10,14,16-pentaenoic acid
18S-RvE2	(5S,6E,8Z,11Z,14Z,16E,18S)-5,18-dihydroxyicosa-6,8,11,14,16-pentaenoic acid
18S-Rv3	(5Z,8Z,11Z,13E,15E,17R,18S)-17,18-dihydroxyicosa-5,8,11,13,15-pentaenoic acid
18R-resolvins E class	
18R-RvE1	(5S,6Z,8E,10E,12R,14Z,16E,18R)-5,12,18-trihydroxyicosa-6,8,10,14,16-pentaenoic acid
18R-RvE2	(5S,6E,8Z,11Z,14Z,16E,18R)-5,18-dihydroxyicosa-6,8,11,14,16-pentaenoic acid
18R-RvE3	(5Z,8Z,11Z,13E,15E,17R,18R)-17,18-dihydroxyicosa-5,8,11,13,15-pentaenoic acid
Resolvins D class precursors	
DHA	(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid
17S-HpDHA	(4Z,7Z,10Z,13Z,15E,17S,19Z)-17-hydroperoxydocosa-4,7,10,13,15,19-hexaenoic acid
17S-HDHA	(4Z,7Z,10Z,13Z,15E,17S,19Z)-17-hydroxydocosa-4,7,10,13,15,19-hexaenoic acid
17R-HpDHA	(4Z,7Z,10Z,13Z,15E,17R,19Z)-17-hydroperoxydocosa-4,7,10,13,15,19-hexaenoic acid
17R-HDHA	(4Z,7Z,10Z,13Z,15E,17R,19Z)-17-hydroxydocosa-4,7,10,13,15,19-hexaenoic acid
17S-resolvins D class	
17S-RvD1	(4Z,7S,8R,9E,11E,13Z,15E,17S,19Z)-7,8,17-trihydroxydocosa-4,9,11,13,15,19-hexaenoic acid
17S-RvD2	(4Z,7S,8E,10Z,12E,14E,16R,17S,19Z)-7,16,17-trihydroxydocosa-4,8,10,12,14,19-hexaenoic acid
17S-RvD3	(4S,5E,7E,9E,13Z,15E,17S,19Z)-4,11,17-trihydroxydocosa-5,7,9,13,15,19-hexaenoic acid
17S-RvD4	(4S,6E,8E,10E,13E,15Z,17S,19Z)-4,5,17-trihydroxydocosa-6,8,10,13,15,19-hexaenoic acid
17R-resolvins D class or (aspirin-triggered; AT) resolvins D class	
17R-RvD1 or AT-RvD1	(4Z,7S,8R,9E,11E,13Z,15E,17R,19Z)-7,8,17-trihydroxydocosa-4,9,11,13,15,19-hexaenoic acid
17R-RvD2 or AT-RvD2	(4Z,7S,8E,10Z,12E,14E,16R,17R,19Z)-7,16,17-trihydroxydocosa-4,8,10,12,14,19-hexaenoic acid
17R-RvD3 or AT-RvD3	(4S,5E,7E,9E,13Z,15E,17R,19Z)-4,11,17-trihydroxydocosa-5,7,9,13,15,19-hexaenoic acid
17R-RvD4 or AT-RvD4	(4S,6E,8E,10E,13E,15Z,17R,19Z)-4,5,17-trihydroxydocosa-6,8,10,13,15,19-hexaenoic acid

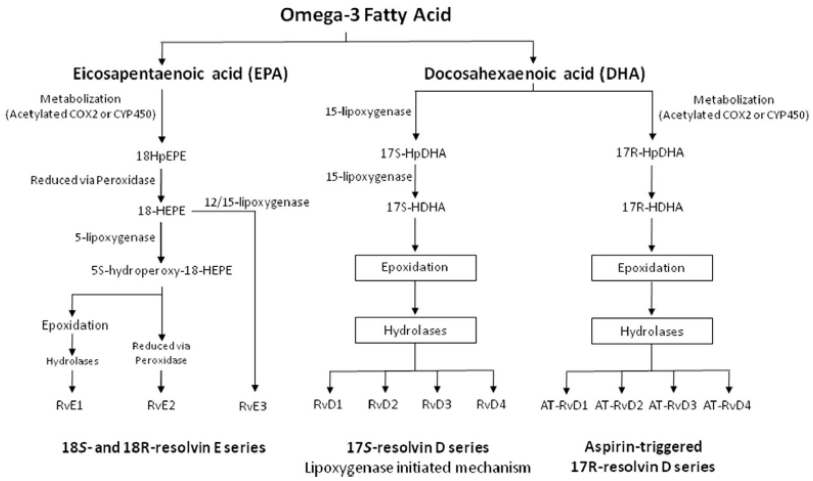


Fig. 1. Synthesis route of resolvins (resolvin E series, D series and aspirin-triggered resolvin D series) from omega-3 fatty acids.

cannot convert oleic acid into linoleic acid (LA; C18:2n6) or LA into alpha-linolenic acid (ALA; C18:3n3) because they lack desaturases [52], as Δ-15 desaturase which is present in plants and convert LA into ALA [104]. For that reason, these two fatty acids take the name of essential fatty acids. The 18-carbon chain and double *cis*-bonds of LA and ALA can be converted into fatty acids with more carbon atoms and more unsaturations though the processes of desaturation and elongation. However, human body can also synthesize small amounts of eicosapentaenoic acid (EPA; C20:5n3) and docosahexaenoic acid (DHA; C22:6n3) using ALA, abundant in soy beans, flaxseed, canola linseed and walnuts oils, such precursor [59]. EPA and DHA are mainly obtained from seafood consumption [57,92] and the intake of these omega-3 fatty acids is important because both are essential for the development and body function [95]. For these reasons, EPA and DHA have been suggested to be termed as "conditionally essential" [43]. The fatty acid profile intake changes the profile of the phospholipids of cell membranes and the fatty acids composition of different cells and tissues, affecting the availability of different fatty acids as well as the metabolic properties of the cells and tissues [8,45,53]. Once EPA and DHA are incorporated into cell composition they are proposed to be involved in the inflammatory cell response [45]. Inflammation resolution involves a decrease in the number of inflammatory cells and pro-inflammatory cytokines levels, and eicosanoids "switch" from inflammatory in nature (prostaglandins, thromboxanes, leukotrienes, etc) to anti-inflammatory or pro-resolving mediators such as lipoxins, resolvins, protectins and maresins [87]. The term resolvins or resolution–phase interaction products refer to endogenous bioactive mediators biosynthesized from the major omega-3 PUFAs EPA and DHA [87].

The families of pro-resolving lipid mediators from EPA and DHA have been identified, characterized and explained by means of lipidomics approach [84,90]. Omega-3 PUFAs, EPA and DHA are precursors of resolvins, protectins and maresins. Resolvins can be classified into two classes, the E-series (RvE) derived from EPA, and D-series (RvD) derived from DHA (Table 1) [45,89]. Moreover, DHA is precursor of protectin D1 and maresins [45,90]. The synthesis of resolvins is a complex process that occurs during the resolution phase of inflammation and involves the acetylated cyclooxygenase-2 (COX-2) and/or lipoxygenases [83,101] (Fig. 1).

RvE was the first EPA metabolite identified, its biosynthesis occurs mainly when endothelial cells interact with leukocytes during the process of inflammation [82]. At present, three subtypes of RvE has been described: 18-S-R-resolvin E1 (RvE1), 18-S-resolvin E2 (RvE2), and 17,18-diHEPE-resolvin E3 (RvE3) [36,82] (Fig. 2A). In the first step, the EPA metabolization is catalyzed by acetylated COX-2 (formed in the presence of acetylsalicylic acid) or via cytochrome P450 (CYP450), and is formed 18-(hydroperoxy)eicosapentaenoic acid (18HpEPE) which is reduced via peroxidase to 18R-HEPE [3,91]. Then, 18R-HEPE is metabolized by lipoxygenases. In the presence of 5-lipoxygenase, 5S-Hp-18R-HEPE is formed; and in the presence of 12/15-lipoxygenase, 17,18S-dihydroxy-5Z,8Z,11Z,13E,15EEPE (18S-Rv3) and 17,18R-dihydroxy-5Z,8Z,11Z,13E,15EEPE (18R-RvE3) are produced [36]. The product of 5-lipoxygenase, 5S-hydroperoxy-18R-HEPE, may be reduced to form RvE2 via peroxidase or may be transformed to an epoxide which is then hydrolyzed to produce RvE1 [82]. Alternatively at this process, 5-lipoxygenase can metabolize EPA to produce 18S-RvE1 [3,90,91]. Therefore, the formation process of RvE1 leads to both 18R-RvE1 and 18SRvE1, and these compounds share the same sites of action [67]. After supplementation with EPA without acetylsalicylic acid, the main 18-HEPE detectable in human blood was 18R-HEPE; moreover, acetylsalicylic acid increased total 18-HEPE formation and also shifted the R/S ratio towards 18S-HEPE [67,101].

DHA constitutes the origin for the D-series resolvins, which are produced during the resolution phase of the inflammatory process. DHA is hydroxylated to 17S-HpDHA via catalysis by 15-lipoxygenase, and then a second lipoxygenation by the same enzyme produces an intermediate peroxide (17S-HDHA) which is transformed to 7S,8S-epoxide [4,82,89,93]. Finally, this product is hydroxylated to yield trihydroxy compounds called resolvins D1, D2, D3, and D4 (RvD1-D4), all of these lipid mediators share the 17S-configuration and are called 17S-resolvin D class (Fig. 2B) [82,101]. Alternatively, there is evidence that acetylsalicylic acid also affects RvD biosynthesis. In the presence of acetylsalicylic acid, DHA is oxidised via acetylated COX-2 or CYP450 to form 17R-HpDHA which is further oxygenated by 5-lipoxygenase producing 17-*epi*-RvD, also known as (aspirin-triggered) resolvins D1-D4 (AT-RvD1-D4) and as 17R-resolvin D class [45,85,101]. 15-

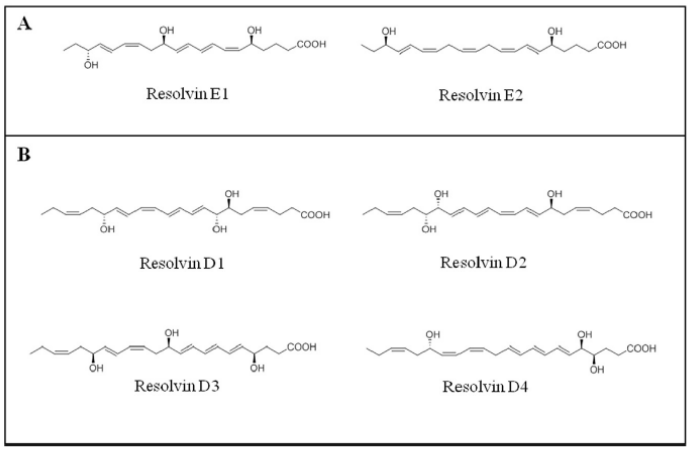


Fig. 2. Chemical structure of Resolvins E series and D series.

lipoxygenase dependent (S—) and acetylsalicylic acid triggered (R-) resolvins differ in the stereochemical configuration of their hydroxyl groups at C17; 17S-, and 17R-resolvins D1 and D2 are formed from 17S-HpDHA or 17R-HpDHA precursors, respectively, by epoxidation and 5-lipoxygenase action via 7S-hydroperoxy or 7S,(8)-epoxy or intermediates [101]. Epoxidation and lipoxygenase action via 4S-hydroperoxy or 4S,(5)-epoxy or intermediates lead to the corresponding S— and R-resolvins D3 and D4 [101]. Diastereomers 17S-RvD1 and 17R-RvD1 present very similar bioactivity, both showing equipotent reduction of leukocyte infiltration in murine inflammatory exudates [93].

Resolvins have highly potent anti-inflammatory activity and can be led to develop novel anti-inflammatory drugs. However, they are unstable in the presence of oxygen (autoxidation) due to their characteristic polyunsaturated structures and this will be a drawback to design resolvins as a drug prototype [22]. The oxidative instability of resolvins is often due to their 1,4-diene (skipped diene) structures [72]. In order to solve this instability problem, Fukuda and colleges designed and synthetized cyclopropane congeners of RvE2, α-CP-RvE2 and β-CP-RvE2; these are stable equivalents with the same or higher biological activity than RvE2 [22]. The use of cyclopropane ring for bioisosteric stabilization of 1,4-diene structure allows to increase the half-life of these compounds, RvE2 has 1.5 h while α-CP-RvE2 and β-CP-RvE2 have 35 h of stability in air atmosphere at room temperature [22].

Resolvin D5 (RvD5; (5Z,7S,8E,10Z,13Z,15E,17S,19Z)-7,17-dihydroxydocosa-5,8,10,13,15,19-hexaenoic acid) is another metabolite of DHA with anti-inflammatory activity but low biological availability. It was originally detected in brain, glial cells and leukocytes [33,34], and later in patient and animal models [12,25,56]. Moreover, RvD5 activates the host defence system in mice during bacterial infection [11]. Taking into account these reasons, RvD5 synthesis has been achieved [66,75] as an attractive novel lipid mediator available for further biological investigations. Since resolvins are particularly attractive synthetic targets, it has been developed the total synthesis of resolvin D6 (RvD6; (4S,5E,7Z,10Z,13Z,15E,17S,19Z)-4,17-dihydroxydocosa-5,7,10,13,15,19-hexaenoic acid) [76]. The new synthesis strategies of resolvins and congeners will let further biological testing of this kind of anti-inflammatory lipid mediators.

3. Cardiovascular disease

Cardiovascular disease (CVD) is a general expression that comprises many different conditions affecting the heart and/or blood vessels. Nowadays, CVD is the leading cause of mortality in the western countries and its prevalence continues increasing progressively [20,23]. In fact, the number of deaths associated with CVD worldwide was a 29% in 1990 and it has been increased by 41% in 2013 [60,78]. This trend of increase is now increasing more commonly in lower-income countries derived from economic development and industrialization [96]. Obesity, and specifically the excess of visceral fat, favours the appearance of hypertension, dyslipidemia and resistance to glucose, which in turn can lead to the development of CVD [47,65]. Most of risk factors associated with CVD are preventable, since they are mainly due to non-healthy lifestyle, including excessive caloric intake and unbalanced diet, sedentary lifestyle, insufficient or poor quality sleep, chronic stress, and smoking [79]. Changes in dietary pattern, practicing physical activity and quit smoking are lifestyle interventions that successfully reduce the risk of CVD at risk subjects [69].

The inflammatory process plays a central role in the appearance and progression of CVD. Specifically, inflammation may underlie the physiopathology of the insulin resistance, endothelial dysfunction and, potentially, may contribute to cardiovascular risk [7]. Epidemiologic studies have evidenced the relationship between the mediators of inflammation and the prediction of cardiovascular risk [40,41]. Emerging evidence supports the idea that acting on some of the pro-inflammatory proteins or pathways leading to their synthesis may be an effective target in reducing the risk of cardiovascular events [29]. In this way, it is well established that obesity together with diabetes are two conditions characterized by a state of chronic, low-grade inflammation [17]. Adipose tissue synthesises and releases a range of bioactive molecules, known as adipokines, which can contribute to inflammation and to promote the development of obesity-derived cardiovascular complications [62]. These pro-inflammatory mediators include TNF-α and IL-1, IL-6 and C-reactive protein [68]. In addition, several *in vivo* studies using hypertensive models reported the existence of an association of vascular inflammation with endothelial dysfunction and hypertension. In general, these studies evidenced an increase in endothelial adhesion of molecules, impaired vasodilation and

decreased expression of the endothelial isoforms of nitric oxide synthase (eNO) [32,61,64]. In addition, atherosclerosis -characterized by accumulation of lipids and immune cells in the arterial wall- is directly related to inflammation. Oxidised LDL (ox-LDL) binds to endothelial cells by the lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) which exerts notable pro-inflammatory effects in atherogenesis [10,70].

4. Cardiovascular disease and resolvins

Resolvins are lipid mediators -derived from enzymatic oxidation via lipoxygenases action of DHA (D-resolvins) and EPA (E-resolvins) [86,89,101]- that play an important role in inflammation resolution. It was clearly evidenced in a previous study that a diet enriched with docosahexaenoic acid and monoglycerides significantly reduced the increase in proinflammatory marker levels (C reactive protein (CRP), IL-6, TNFα, and IL-1β) associated to high-fat/high-carbohydrate diet in rats, and also increased the levels of RvD2 and RvD3 [58]. The main function of resolvins is to block neutrophils and monocytes migration and infiltration protecting tissues against immune cell-mediated injury [89]. In addition, resolvins downregulate the expression of neutrophil adhesion molecules, reduce neutrophil oxidative burst, favour neutrophils apoptosis and clearance by macrophages, and participate in chemokine signalling termination by upregulating decoy receptors (C—C chemokine receptor type 5 (CCR5)) [2,81,85,98]. During inflammation resolution, E-resolvins could interact with leukotriene B4 neutrophils receptor and are able to reduce pro-inflammatory cytokine production enhancing the elimination of pro-inflammatory stimulus [87,89]. It is believed that E and D-resolvins present a similar function; for instance, both can inhibit nuclear factor-κB (NFκB) by a mechanism peroxisome proliferator-activated receptor γ (PPARγ) dependent [3,50].

Cardiovascular diseases are directly related to inflammatory state [39,54,74]; in this sense, resolvins could improve the prognosis of CVD. In fact, it has been shown that the lack of LOX-12 and LOX-15 -the two enzymes responsible of resolvins synthesis- accelerates the atherosclerosis development in murine models [55]. In this sense, atherosclerosis is a chronic inflammatory disease caused by an abnormal lipid metabolism, which induced an inflammatory response [99]. Atherosclerotic plaque is characterized by a high presence of oxidised lipids and lipoproteins (mainly ox-LDL, and oxidised VLDL) and by the presence of monocytes and neutrophil; these two cellular types are able to produce many pro-inflammatory mediators such as leukotrienes, and many chemokines as monocyte chemoattractant protein-1 (MCP-1) or IL-8, cytokines and grow factors which could increase the inflammatory status [99]. Resolvins could reduce this inflammatory state, since it has been observed that resolvins can inhibit neutrophil tissue infiltration decreasing the IL-8 production and reducing the surface expression of adhesion receptors, such as CD11b or CD18 [44]. In a mice model of atheroprogression, the administration of RvD2 and MaR1 prevented atheroprogression favouring macrophage profile toward a reparative phenotype and contributing to plaque stability [100]. Another study performed in murine model of atherosclerosis has shown that RvE1 administration significantly reduced the atherosclerotic lesions. RvE1 treatment also reduced TNF-α and Interferon-γ (IFN-γ) gene expression in aorta, albeit it did not reduce the macrophages on atherosclerotic plaque [80]. Similar results were obtained in a rabbit model of periodontitis and atherogenesis, in which RvE1 administration reduced the atherosclerotic plaque formation at the same time that decreased CRP levels and reduced macrophage infiltration into intima [31,51]. Consumption of 3.36 g of EPA and DHA by patients suffering from stable coronary artery disease restored the levels of proresolving lipid mediators and promoted macrophage phagocytosis of blood clots *in vitro* [16]. However, in a recent study the treatment with a high dose of n-3 PUFAs in patients with atherosclerotic vascular disease and type 2 diabetes receiving optimal medical therapy, resulted in increased EPA and DHA concentrations but without any change in RvD1 levels and in inflammatory markers [71].

As a consequence of atherosclerosis, vascular smooth muscle cells (VSMCs) are more proliferative and chemotactic, and are characterized by a reduced expression of contractile proteins and enhance production of pro-inflammatory cytokines [6]. Diverse studies evidenced that resolvins are capable to reduce VSMCs responses and ameliorate vascular injury, mediated by local activation of resolution mechanisms. In a rabbit model of arterial angioplasty, RvD2 significantly reduced cell proliferation, leukocyte recruitment and neointimal hyperplasia [56]. In the same study, RvD1 and RvD2 treatment in human VSMCs reduced proliferation, migration, monocyte adhesion, superoxide anion production and pro-inflammatory genes expression [1,56,103]. In a mouse model of arterial neointima formation (carotid ligation), RvD2 and MaR1 also reduced aortic smooth muscle cell proliferation and neointimal hyperplasia as well as neutrophil and macrophage recruitment [1]. Similar results were found in a rat model of arterial injury in which RvD1 attenuated rat arterial vascular smooth muscle cell inflammatory pathways. Oxidative stress levels and NFκB activation were significantly lower in the RvD1-treated samples [103]. Intact human arteries incubated with DHA *ex vivo* produced precursors of resolvins and D-series resolvins, and also inhibited monocyte adhesion to TNFα—stimulated endothelial cells [9]. This effect was partially reversed by antibodies against the RvD1 receptors. The authors concluded that the locally production of proresolving mediators of vascular origin are necessary in promoting resolution of the inflammatory response.

Platelet aggregation and thrombosis are two factors directly linked to atherosclerosis and inflammation, and could favour the apparition of myocardial infarction or stroke [19]. It was reported that resolvins could prevent platelet aggregation and induce vasodilatation reducing the risk to suffer atherosclerosis [13]. In this sense, it has been reported that RvE1 could reduce the platelet aggregation induced by thromboxane [21]. RvE1 incubation of human platelet-rich plasma reduced platelet ADP-stimulated P-selectin mobilization and polymerized actin content. Similarly, RvE1 reduced both ADP-stimulated and thromboxane receptor agonist U46619-stimulated platelet aggregation whereas did not block collagen-stimulated aggregation [15]. Treatment with fish oil protected against thrombosis and injury induced by vascular remodelling in mice evidenced by a reduced platelet activity, reduced local inflammatory reactions and enhanced resolution at least in part derived from an increase in RvE1 levels [30].

In an *in vivo* and *in vitro* study, RvE1 exerted protective effects against myocardial infarction [42]. In a rat model of myocardial ischemia/reperfusion, intravenous RvE1significantly reduced infarct size in a dose dependent manner. In the same study, an *in vitro* assay with H9c2 cells exposed to hypoxia/reoxygenation RvE1 increased the cell viability and decreased apoptosis. The mechanism of action seems to be mediated by activation of phosphoinositide 3-kinase (PI3K)/Akt and Extracellular-Regulated Kinase 1/2 (ERK1/2) signalling pathways, activation of endothelial nitric oxide synthase (eNOS) by phosphorylation and reduction of caspase-3 activity. The inhibition of Epidermal growth factor (EGF) receptor -which has been reported to mediate the epithelial wound healing effects of RvE1-blocked the protective effects of RvE1 [42]. Similar results were reported by other authors, evidencing that the protective effects of RvD1 were mediated by the PI3K/Akt pathway [28]. In this sense, the treatment with a PI3K/Akt inhibitor was capable to block the cardioprotection by RvD1 [26]. The same group

also evidenced that RvD1 diminished infarct size and neutrophil accumulation in the infarcted myocardium, but also attenuated depression-like symptoms increasing performance in the forced swim and social interaction tests in a rat model of myocardial infarction [27].

Moreover, the role of RvD1 in resolving post-myocardial infarction inflammation was investigated in C57BL/6J-mice subjected to coronary artery ligation. RvD1 limited neutrophil recruitment in the spleen and left ventricle, reduced the macrophage density preventing their accumulation in the infarcted area, and increased resolving lipid mediators (RvD1, RvD2, Maresin 1 and Lipoxin A4) in the spleen. RvD1 also reduced the expression of pro-fibrotic genes and reduced collagen deposition, ameliorating post-myocardial infarction fibrosis and stabilizing the extracellular matrix [38]. In a mice model of hind limb ischemia, RvD2 enhanced perfusion recovery stimulating arteriogenic revascularization [106]. The treatment with RvD2 induced a higher number of regenerated myocytes, a reduction of the neutrophil accumulation, and TNF-α and granulocyte macrophage colony-stimulating factor levels. Another study investigated the effects of RvD1 on hepatic ischemia/reperfusion injury [107]. Treatment with RvD1 prior to the ischemic insult inhibited inflammatory pathways, as it was evidenced by reductions in IL-6, TNF-α and myeloperoxidase levels and apoptosis, as well as increased phosphorylation of Akt. These protective effects of RvD1 on hepatic ischemia injury were blocking when a PI3K inhibitor (LY294002) was administered.

An interesting report by Thul et al. [97] showed that human subjects with a higher salivary RvD1/LTB4 ratio had a significantly lesser intima media thickness than those with lower ratio. The authors suggested that the lipid mediators RvD1 and LTB4 could serve as a biomarker of non-resolving inflammation. Finally, a study investigating sex differences in humans between the inflammatory response of males and females concluded that female sex protects against systemic inflammation-induced endothelial dysfunction [73]. The underlying mechanism seems to be related with an accelerated resolution of inflammation in females, specifically via neutrophils, mediated by an elevation of the lipid mediators, mainly D-resolvins.

5. Conclusion

Inflammation is a physiological process to protect tissues and cells against a wide range of aggressions. However, if the inflammatory response is excessive or prolonged over time it can lead to tissue damage and disease. In this sense, inflammation is associated with numerous cardiovascular diseases. The existence of lipid mediators that orchestrate the correct resolution of the disease opens new possibilities of study for the development of anti-inflammatory drugs. Resolvins could be a suitable treatment against cardiovascular diseases, mainly due to their anti-inflammatory properties which can reduce inflammatory state associated to atherosclerosis, inhibit neutrophil tissue infiltration and platelet aggregation, induce neutrophil apoptosis and decrease pro-inflammatory cytokines production. Resolvins can also prevent platelet aggregation and favouring vasodilatation which in turn, can reduce atherosclerosis risk. RvE1 can reduce myocardial infarct size in rat models. RvD1 limited neutrophil recruitment in left ventricle, and reduce the macrophage density preventing their accumulation in the infarcted area. Although the obtained results are promising, the actual knowledge is still in very preliminary research to determine their effectiveness. Many additional studies are needed to evaluate its therapeutic efficacy in clinical trials, pharmacological doses and possible toxicity.

Acknowledgements

This work has been granted by the Programme of Promotion of Biomedical Research and Health Sciences CIBEROBN CB12/03/30038, and supported by the Vicerrectoría de Investigación y Desarrollo from University of Concepción, Chile (216.073.031–1.0IN and 217.073.033–1.0). The authors hereby acknowledge the PhD grant provided by the University of the Balearic Island and the FPI/1648/2014 grant provided by Conselleria d'Educació, Cultura i Universitats, Direcció General d'Universitats i Recerca, Govern de les Illes Balears, within the program framework cofinanced by Fondo Social Europeo.

References

[1] D. Akagi, M. Chen, R. Toy, A. Chatterjee, M.S. Conte, Systemic delivery of proresolving lipid mediators resolvin D2 and maresin 1 attenuates intimal hyperplasia in mice, FASEB J. 29 (2015) 2504–2513.
[2] A. Ariel, G. Fredman, Y.P. Sun, A. Kantarci, T.E. Van Dyke, A.D. Luster, C.N. Serhan, Apoptotic neutrophils and T cells sequester chemokines during immune response resolution through modulation of CCR5 expression, Nat. Immunol. 7 (2006) 1209–1216.
[3] M. Arita, F. Bianchini, J. Aliberti, A. Sher, N. Chiang, S. Hong, R. Yang, N.A. Petasis, C.N. Serhan, Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1, J. Exp. Med. 201 (2005) 713–722.
[4] G.L. Bannenberg, N. Chiang, A. Ariel, M. Arita, E. Tjonahen, K.H. Gotlinger, S. Hong, C.N. Serhan, Molecular circuits of resolution: formation and actions of resolvins and protectins, J. Immunol. 174 (2005) 4345–4355.
[5] M. Bennett, D.W. Gilroy, Lipid mediators in inflammation, Microbiol. Spectr. 4 (2016).
[6] M.R. Bennett, S. Sinha, G.K. Owens, Vascular smooth muscle cells in atherosclerosis, Circ. Res. 118 (2016) 692–702.
[7] P. Calabro, E. Golia, V. Maddaloni, M. Malvezzi, B. Casillo, C. Marotta, R. Calabro, P. Golino, Adipose tissue-mediated inflammation: the missing link between obesity and cardiovascular disease? Intern. Emerg. Med. 4 (2009) 25–34.
[8] P.C. Calder, P. Yaqoob, Understanding omega-3 polyunsaturated fatty acids, Postgrad. Med. 121 (2009) 148–157.
[9] A. Chatterjee, S. Komshian, B.E. Sansbury, B. Wu, G. Mottola, M. Chen, M. Spite, M.S. Conte, Biosynthesis of proresolving lipid mediators by vascular cells and tissues, FASEB J. (2017), http://dx.doi.org/10.1096/fj.201700082Rfj.201700082R.
[10] X. Chen, H. Zhang, S. McAfee, C. Zhang, The reciprocal relationship between adiponectin and LOX-1 in the regulation of endothelial dysfunction in ApoE knockout mice, Am. J. Physiol. Heart Circ. Physiol. 299 (2010) H605–H612.
[11] N. Chiang, G. Fredman, F. Backhed, S.F. Oh, T. Vickery, B.A. Schmidt, C.N. Serhan, Infection regulates pro-resolving mediators that lower antibiotic requirements, Nature 484 (2012) 524–528.
[12] J. Dalli, B.D. Kraft, R.A. Colas, M. Shinohara, L.E. Fredenburgh, D.R. Hess, N. Chiang, K. Welty-Wolf, A.M. Choi, C.A. Piantadosi, C.N. Serhan, The regulation of proresolving lipid mediator profiles in baboon pneumonia by inhaled carbon monoxide, Am. J. Respir. Cell Mol. Biol. 53 (2015) 314–325.
[13] U.N. Das, Essential fatty acids and their metabolites could function as endogenous HMG-CoA reductase and ACE enzyme inhibitors, anti-arrhythmic, anti-hypertensive, anti-atherosclerotic, anti-inflammatory, cytoprotective, and cardioprotective molecules, Lipids Health Dis. 7 (2008) 37.
[14] U.N. Das, Lipoxins, resolvins, protectins, maresins, and nitrolipids: connecting lipids, inflammation, and cardiovascular disease risk, Curr. Cardiovasc. Risk Rep. 4 (2010) 24–31.
[15] M. Dona, G. Fredman, J.M. Schwab, N. Chiang, M. Arita, A. Goodarzi, G. Cheng, U.H. von Andrian, C.N. Serhan, Resolvin E1, an EPA-derived mediator in whole blood, selectively counterregulates leukocytes and platelets, Blood 112 (2008) 848–855.
[16] T.K. Elajami, R.A. Colas, J. Dalli, N. Chiang, C.N. Serhan, F.K. Welty, Specialized proresolving lipid mediators in patients with coronary artery disease and their potential for clot remodeling, FASEB J. 30 (2016) 2792–2801.
[17] A. Festa, R. D'Agostino Jr., K. Williams, A.J. Karter, E.J. Mayer-Davis, R.P. Tracy, S.M. Haffner, The relation of body fat mass and distribution to markers of chronic inflammation, Int. J. Obes. Relat. Metab. Disord. 25 (2001) 1407–1415.
[18] S. Fichtlscherer, C. Heeschen, A.M. Zeiher, Inflammatory markers and coronary artery disease, Curr. Opin. Pharmacol. 4 (2004) 124–131.
[19] J.H. Foley, E.M. Conway, Cross talk pathways between coagulation and inflammation, Circ. Res. 118 (2016) 1392–1408.
[20] G.C. Fonarow, The global burden of atherosclerotic vascular disease, Nat. Clin. Pract. Cardiovasc. Med. 4 (2007) 530–531.
[21] G. Fredman, T.E. Van Dyke, C.N. Serhan, Resolvin E1 regulates adenosine diphosphate activation of human platelets, Arterioscler. Thromb. Vasc. Biol.

30 (2010) 2005–2013.
[22] H. Fukuda, R. Muromoto, Y. Takakura, K. Ishimura, R. Kanada, D. Fushihara, M. Tanabe, K. Matsubara, T. Hirao, K. Hirashima, H. Abe, M. Arisawa, T. Matsuda, S. Shuto, Design and synthesis of cyclopropane congeners of resolvin E2, an endogenous proresolving lipid mediator, as its stable equivalents, Org. Lett. 18 (2016) 6224–6227.
[23] GBD, Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the global burden of disease study 2015, Lancet 388 (2016) 1459–1544.
[24] B. Geering, C. Stoeckle, S. Conus, H.U. Simon, Living and dying for inflammation: neutrophils, eosinophils, basophils, Trends Immunol. 34 (2013) 398–409.
[25] M. Giera, A. Ioan-Facsinay, R. Toes, F. Gao, J. Dalli, A.M. Deelder, C.N. Serhan, O.A. Mayboroda, Lipid and lipid mediator profiling of human synovial fluid in rheumatoid arthritis patients by means of LC-MS/MS, Biochim. Biophys. Acta 1821 (2012) 1415–1424.
[26] K. Gilbert, J. Bernier, V. Bourque-Riel, M. Malick, G. Rousseau, Resolvin D1 reduces infarct size through a phosphoinositide 3-kinase/protein kinase B mechanism, J. Cardiovasc. Pharmacol. 66 (2015a) 72–79.
[27] K. Gilbert, J. Bernier, R. Godbout, G. Rousseau, Resolvin D1, a metabolite of omega-3 polyunsaturated fatty acid, decreases post-myocardial infarct depression, Mar. Drugs 12 (2014) 5396–5407.
[28] K. Gilbert, M. Malick, N. Madingou, C. Touchette, V. Bourque-Riel, L. Tomaro, G. Rousseau, Metabolites derived from omega-3 polyunsaturated fatty acids are important for cardioprotection, Eur. J. Pharmacol. 769 (2015b) 147–153.
[29] E. Golia, G. Limongelli, F. Natale, F. Fimiani, V. Maddaloni, I. Pariggiano, R. Bianchi, M. Crisci, L. D'Acierno, R. Giordano, G. Di Palma, M. Conte, P. Golino, M.G. Russo, R. Calabro, P. Calabro, Inflammation and cardiovascular disease: from pathogenesis to therapeutic target, Curr. Atheroscler. Rep. 16 (2014) 435.
[30] Y. Gong, M. Lin, L. Piao, X. Li, F. Yang, J. Zhang, B. Xiao, Q. Zhang, W.L. Song, H. Yin, L. Zhu, C.D. Funk, Y. Yu, Aspirin enhances protective effect of fish oil against thrombosis and injury-induced vascular remodelling, Br. J. Pharmacol. 172 (2015) 5647–5660.
[31] H. Hasturk, R. Abdallah, A. Kantarci, D. Nguyen, N. Giordano, J. Hamilton, T.E. Van Dyke, Resolvin E1 (RvE1) attenuates atherosclerotic plaque formation in diet and inflammation-induced atherogenesis, Arterioscler. Thromb. Vasc. Biol. 35 (2015) 1123–1133.
[32] H.N. Hayenga, H. Aranda-Espinoza, Stiffness increases mononuclear cell transendothelial migration, Cell Mol. Bioeng. 6 (2013) 253–256.
[33] S. Hong, K. Gronert, P.R. Devchand, R.L. Moussignac, C.N. Serhan, Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation, J. Biol. Chem. 278 (2003) 14677–14687.
[34] S. Hong, E. Tjonahen, E.L. Morgan, Y. Lu, C.N. Serhan, A.F. Rowley, Rainbow trout (Oncorhynchus mykiss) brain cells biosynthesize novel docosahexaenoic acid-derived resolvins and protectins-mediator lipidomic analysis, Prostagl. Other Lipid Mediat 78 (2005) 107–116.
[35] G.S. Hotamisligil, Inflammation and metabolic disorders, Nature 444 (2006) 860–867.
[36] Y. Isobe, M. Arita, S. Matsueda, R. Iwamoto, T. Fujihara, H. Nakanishi, R. Taguchi, K. Masuda, K. Sasaki, D. Urabe, M. Inoue, H. Arai, Identification and structure determination of novel anti-inflammatory mediator resolvin E3, 17,18-dihydroxyeicosapentaenoic acid, J. Biol. Chem. 287 (2012) 10525–10534.
[37] R.R. Ji, Z.Z. Xu, G. Strichartz, C.N. Serhan, Emerging roles of resolvins in the resolution of inflammation and pain, Trends Neurosci. 34 (2011) 599–609.
[38] V. Kain, K.A. Ingle, R.A. Colas, J. Dalli, S.D. Prabhu, C.N. Serhan, M. Joshi, G.V. Halade, Resolvin D1 activates the inflammation resolving response at splenic and ventricular site following myocardial infarction leading to improved ventricular function, J. Mol. Cell Cardiol. 84 (2015) 24–35.
[39] S. Kaptoge, E. Di Angelantonio, G. Lowe, M.B. Pepys, S.G. Thompson, R. Collins, J. Danesh, C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis, Lancet 375 (2010) 132–140.
[40] S. Kaptoge, E. Di Angelantonio, L. Pennells, A.M. Wood, I.R. White, P. Gao, M. Walker, A. Thompson, N. Sarwar, M. Caslake, A.S. Butterworth, P. Amouyel, G. Assmann, S.J. Bakker, E.L. Barr, E. Barrett-Connor, E.J. Benjamin, C. Bjorkelund, H. Brenner, E. Brunner, R. Clarke, J.A. Cooper, P. Cremer, M. Cushman, G.R. Dagenais, R.B. D'Agostino Sr., R. Dankner, G. Davey-Smith, D. Deeg, J.M. Dekker, G. Engstrom, A.R. Folsom, F.G. Fowkes, J. Gallacher, J.M. Gaziano, S. Giampaoli, R.F. Gillum, A. Hofman, B.V. Howard, E. Ingelsson, H. Iso, T. Jorgensen, S. Kiechl, A. Kitamura, Y. Kiyohara, W. Koenig, D. Kromhout, L.H. Kuller, D.A. Lawlor, T.W. Meade, A. Nissinen, B.G. Nordestgaard, A. Onat, D.B. Panagiotakos, B.M. Psaty, B. Rodriguez, A. Rosengren, V. Salomaa, J. Kauhanen, J.T. Salonen, J.A. Shaffer, S. Shea, I. Ford, C.D. Stehouwer, T.E. Strandberg, R.W. Tipping, A. Tosetto, S. Wassertheil-Smoller, P. Wennberg, R.G. Westendorp, P.H. Whincup, L. Wilhelmsen, M. Woodward, G.D. Lowe, N.J. Wareham, K.T. Khaw, N. Sattar, C.J. Packard, V. Gudnason, P.M. Ridker, M.B. Pepys, S.G. Thompson, J. Danesh, C-reactive protein, fibrinogen, and cardiovascular disease prediction, N. Engl. J. Med. 367 (2012) 1310–1320.
[41] S. Kaptoge, S.R. Seshasai, P. Gao, D.F. Freitag, A.S. Butterworth, A. Borglykke, E. Di Angelantonio, V. Gudnason, A. Rumley, G.D. Lowe, T. Jorgensen, J. Danesh, Inflammatory cytokines and risk of coronary heart disease: new prospective study and updated meta-analysis, Eur. Heart J. 35 (2014) 578–589.
[42] K.T. Keyes, Y. Ye, Y. Lin, C. Zhang, J.R. Perez-Polo, P. Gjorstrup, Y. Birnbaum, Resolvin E1 protects the rat heart against reperfusion injury, Am. J. Physiol. Heart Circ. Physiol. 299 (2010) H153–H164.
[43] P.M. Kidd, Omega-3 DHA and EPA for cognition, behavior, and mood: clinical findings and structural-functional synergies with cell membrane phospholipids, Altern. Med. Rev. 12 (2007) 207–227.
[44] S. Krishnamoorthy, A. Recchiuti, N. Chiang, S. Yacoubian, C.H. Lee, R. Yang, N.A. Petasis, C.N. Serhan, Resolvin D1 binds human phagocytes with evidence for proresolving receptors, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 1660–1665.
[45] A. Kumar, S.S. Mastana, M.R. Lindley, n-3 Fatty acids and asthma, Nutr. Res. Rev. 29 (2016) 1–16.
[46] V.A. Kumar, A.K. Abbas, J.C. Aster, Robbins y Cotran. Patología Estructural y Funcional, Elsevier, 2015.
[47] L.J. Laslett, P. Alagona Jr., B.A. Clark 3rd, J.P. Drozda Jr., F. Saldivar, S.R. Wilson, C. Poe, M. Hart, The worldwide environment of cardiovascular disease: prevalence, diagnosis, therapy, and policy issues: a report from the American college of cardiology, J. Am. Coll. Cardiol. 60 (2012) S1–S49.
[48] C.H. Lee, Resolvins as new fascinating drug candidates for inflammatory diseases, Arch. Pharm. Res. 35 (2012) 3–7.
[49] C.C. Leslie, Cytosolic phospholipase A(2): physiological function and role in disease, J. Lipid Res. 56 (2015) 1386–1402.
[50] Z. Liao, J. Dong, W. Wu, T. Yang, T. Wang, L. Guo, L. Chen, D. Xu, F. Wen, Resolvin D1 attenuates inflammation in lipopolysaccharide-induced acute lung injury through a process involving the PPARgamma/NF-kappaB pathway, Respir. Res. 13 (2012) 110.
[51] P. Libby, P.M. Ridker, Inflammation and atherosclerosis: role of C-reactive protein in risk assessment, Am. J. Med. 116 (Suppl 6A) (2004) 9S–16S.
[52] H.L. Liu, Z.J. Yin, L. Xiao, Y.N. Xu, Q. Qu le, Identification and evaluation of omega-3 fatty acid desaturase genes for hyperfortifying alpha-linolenic acid in transgenic rice seed, J. Exp. Bot. 63 (2012) 3279–3287.
[53] M. Martorell, X. Capo, M.M. Bibiloni, A. Sureda, A. Mestre-Alfaro, J.M. Batle, I. Llompart, J.A. Tur, A. Pons, Docosahexaenoic acid supplementation promotes erythrocyte antioxidant defense and reduces protein nitrosative damage in male athletes, Lipids 50 (2015) 131–148.
[54] M.M. McDermott, K. Liu, L. Ferrucci, L. Tian, J.M. Guralnik, H. Tao, P.M. Ridker, M.H. Criqui, Relation of interleukin-6 and vascular cellular adhesion molecule-1 levels to functional decline in patients with lower extremity peripheral arterial disease, Am. J. Cardiol. 107 (2011) 1392–1398.
[55] A.J. Merched, K. Ko, K.H. Gotlinger, C.N. Serhan, L. Chan, Atherosclerosis: evidence for impairment of resolution of vascular inflammation governed by specific lipid mediators, FASEB J. 22 (2008) 3595–3606.
[56] T. Miyahara, S. Runge, A. Chatterjee, M. Chen, G. Mottola, J.M. Fitzgerald, C.N. Serhan, M.S. Conte, D-series resolvin attenuates vascular smooth muscle cell activation and neointimal hyperplasia following vascular injury, FASEB J. 27 (2013) 2220–2232.
[57] A. Mohebi-Nejad, B. Bikdeli, Omega-3 supplements and cardiovascular diseases, Tanaffos 13 (2014) 6–14.
[58] C. Morin, E. Rousseau, P.U. Blier, S. Fortin, Effect of docosahexaenoic acid monoacylglyceride on systemic hypertension and cardiovascular dysfunction, Am. J. Physiol. Heart Circ. Physiol. 309 (2015) H93–H102.
[59] D. Mozaffarian, J.H. Wu, Omega-3 fatty acids and cardiovascular disease: effects on risk factors, molecular pathways, and clinical events, J. Am. Coll. Cardiol. 58 (2011) 2047–2067.
[60] C.J.L. Murray, A.D. Lopez, The Global Burden of Disease: a Comprehensive Assessment of Mortality and Disability from Diseases, Injuries, and Risk Factors in 1990 and Projected to 2020, Harvard University Press, Cambridge, Mass, 1996.
[61] K. Nagano, J. Ishida, M. Unno, T. Matsukura, A. Fukamizu, Apelin elevates blood pressure in ICR mice with LNAME induced endothelial dysfunction, Mol. Med. Rep. 7 (2013) 1371–1375.
[62] K. Nakamura, J.J. Fuster, K. Walsh, Adipokines: a link between obesity and cardiovascular disease, J. Cardiol. 63 (2014) 250–259.
[63] C. Nathan, A. Ding, Nonresolving inflammation, Cell 140 (2010) 871–882.
[64] C.Y. Ng, Y. Kamisah, O. Faizah, K. Jaarin, The role of repeatedly heated soybean oil in the development of hypertension in rats: association with vascular inflammation, Int. J. Exp. Pathol. 93 (2012) 377–387.
[65] T.J. Niiranen, R.S. Vasan, Epidemiology of cardiovascular disease: recent novel outlooks on risk factors and clinical approaches, Expert Rev. Cardiovasc. Ther. 14 (2016) 855–869.
[66] N. Ogawa, T. Sugiyama, M. Morita, Y. Suganuma, Y. Kobayashi, Total synthesis of resolvin D5, J. Org. Chem. 82 (2017) 2032–2039.
[67] S.F. Oh, P.S. Pillai, A. Recchiuti, R. Yang, C.N. Serhan, Pro-resolving actions and stereoselective biosynthesis of 18S E-series resolvins in human leukocytes and murine inflammation, J. Clin. Invest. 121 (2011) 569–581.
[68] N. Ouchi, J.L. Parker, J.J. Lugus, K. Walsh, Adipokines in inflammation and metabolic disease, Nat. Rev. Immunol. 11 (2011) 85–97.
[69] T.A. Pearson, S.N. Blair, S.R. Daniels, R.H. Eckel, J.M. Fair, S.P. Fortmann, B.A. Franklin, L.B. Goldstein, P. Greenland, S.M. Grundy, Y. Hong, N.H. Miller, R.M. Lauer, I.S. Ockene, R.L. Sacco, J.F. Sallis Jr., S.C. Smith Jr., N.J. Stone, K.A. Taubert, AHA guidelines for primary prevention of cardiovascular disease and stroke: 2002 update: consensus panel guide to comprehensive risk

reduction for adult patients without coronary or other atherosclerotic vascular diseases, Am. Heart Assoc. Sci. Advis. Coord. Comm. Circ. 106 (2002) 388–391.
[70] A. Pirillo, G.D. Norata, A.L. Catapano, LOX-1, OxLDL, and atherosclerosis, Mediat. Inflamm. 2013 (2013) 152786.
[71] M. Poreba, M. Mostowik, A. Siniarski, R. Golebiowska-Wiatrak, K.P. Malinowski, M. Haberka, E. Konduracka, J. Nessler, A. Undas, G. Gajos, Treatment with high-dose n-3 PUFAs has no effect on platelet function, coagulation, metabolic status or inflammation in patients with atherosclerosis and type 2 diabetes, Cardiovasc. Diabetol. 16 (2017) 50.
[72] D.A. Pratt, K.A. Tallman, N.A. Porter, Free radical oxidation of polyunsaturated lipids: new mechanistic insights and the development of peroxyl radical clocks, Acc. Chem. Res. 44 (2011) 458–467.
[73] K.S. Rathod, V. Kapil, S. Velmurugan, R.S. Khambata, U. Siddique, S. Khan, S. Van Eijl, L.C. Gee, J. Bansal, K. Pitrola, C. Shaw, F. D'Acquisto, R.A. Colas, F. Marelli-Berg, J. Dalli, A. Ahluwalia, Accelerated resolution of inflammation underlies sex differences in inflammatory responses in humans, J. Clin. Invest. 127 (2017) 169–182.
[74] P.M. Ridker, C.H. Hennekens, J.E. Buring, N. Rifai, C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women, N. Engl. J. Med. 342 (2000) 836–843.
[75] A.R. Rodríguez, B.W. Spur, First total synthesis of 7(S),17(S)-resolvin D5, a potent anti-inflammatory docosanoid, Tetrahedron Lett. 46 (2005) 3623–3627.
[76] A.R. Rodríguez, B.W. Spur, First total synthesis of the anti-inflammatory lipid mediator Resolvin D6, Tetrahedron Lett. 53 (2012) 86–89.
[77] I. Roifman, P.L. Beck, T.J. Anderson, M.J. Eisenberg, J. Genest, Chronic inflammatory diseases and cardiovascular risk: a systematic review, Can. J. Cardiol. 27 (2011) 174–182.
[78] G.A. Roth, M.H. Forouzanfar, A.E. Moran, R. Barber, G. Nguyen, V.L. Feigin, M. Naghavi, G.A. Mensah, C.J. Murray, Demographic and epidemiologic drivers of global cardiovascular mortality, N. Engl. J. Med. 372 (2015) 1333–1341.
[79] B. Ruiz-Nunez, L. Pruimboom, D.A. Dijck-Brouwer, F.A. Muskiet, Lifestyle and nutritional imbalances associated with Western diseases: causes and consequences of chronic systemic low-grade inflammation in an evolutionary context, J. Nutr. Biochem. 24 (2013) 1183–1201.
[80] K. Salic, M.C. Morrison, L. Verschuren, P.Y. Wielinga, L. Wu, R. Kleemann, P. Gjorstrup, T. Kooistra, Resolvin E1 attenuates atherosclerosis in absence of cholesterol-lowering effects and on top of atorvastatin, Atherosclerosis 250 (2016) 158–165.
[81] J.M. Schwab, N. Chiang, M. Arita, C.N. Serhan, Resolvin E1 and protectin D1 activate inflammation-resolution programmes, Nature 447 (2007) 869–874.
[82] R.C. Schwanke, R. Marcon, A.F. Bento, J.B. Calixto, EPA- and DHA-derived resolvins' actions in inflammatory bowel disease, Eur. J. Pharmacol. 785 (2016) 156–164.
[83] C.N. Serhan, Systems approach to inflammation resolution: identification of novel anti-inflammatory and pro-resolving mediators, J. Thromb. Haemost. 7 (Suppl 1) (2009) 44–48.
[84] C.N. Serhan, Pro-resolving lipid mediators are leads for resolution physiology, Nature 510 (2014) 92–101.
[85] C.N. Serhan, N. Chiang, Novel endogenous small molecules as the checkpoint controllers in inflammation and resolution: entree for resoleomics, Rheum. Dis. Clin. North Am. 30 (2004) 69–95.
[86] C.N. Serhan, N. Chiang, J. Dalli, The resolution code of acute inflammation: novel pro-resolving lipid mediators in resolution, Semin. Immunol. 27 (2015a) 200–215.
[87] C.N. Serhan, N. Chiang, T.E. Van Dyke, Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators, Nat. Rev. Immunol. 8 (2008) 349–361.
[88] C.N. Serhan, J. Dalli, R.A. Colas, J.W. Winkler, N. Chiang, Protectins and maresins: new pro-resolving families of mediators in acute inflammation and resolution bioactive metabolome, Biochim. Biophys. Acta 1851 (2015b) 397–413.
[89] C.N. Serhan, S. Hong, K. Gronert, S.P. Colgan, P.R. Devchand, G. Mirick, R.L. Moussignac, Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals, J. Exp. Med. 196 (2002) 1025–1037.
[90] C.N. Serhan, N.A. Petasis, Resolvins and protectins in inflammation resolution, Chem. Rev. 111 (2011) 5922–5943.
[91] C.N. Serhan, T. Takano, N. Chiang, K. Gronert, C.B. Clish, Formation of endogenous "anti-inflammatory" lipid mediators by transcellular biosynthesis. Lipoxins and aspirin-triggered lipoxins inhibit neutrophil recruitment and vascular permeability, Am. J. Respir. Crit. Care Med. 161 (2000) S95–S101.
[92] M. Singh, Essential fatty acids, DHA and human brain, Indian J. Pediatr. 72 (2005) 239–242.
[93] Y.P. Sun, S.F. Oh, J. Uddin, R. Yang, K. Gotlinger, E. Campbell, S.P. Colgan, N.A. Petasis, C.N. Serhan, Resolvin D1 and its aspirin-triggered 17R epimer. Stereochemical assignments, anti-inflammatory properties, and enzymatic inactivation, J. Biol. Chem. 282 (2007) 9323–9334.
[94] S. Suram, L.J. Silveira, S. Mahaffey, G.D. Brown, J.V. Bonventre, D.L. Williams, N.A. Gow, D.L. Bratton, R.C. Murphy, C.C. Leslie, Cytosolic phospholipase A(2) alpha and eicosanoids regulate expression of genes in macrophages involved in host defense and inflammation, PLoS One 8 (2013) e69002.
[95] S. Tejada, M. Martorell, X. Capo, J.A. Tur, A. Pons, A. Sureda, Omega-3 fatty acids in the management of epilepsy, Curr. Top. Med. Chem. 16 (2016) 1897–1905.
[96] K.K. Teo, H. Dokainish, The emerging epidemic of cardiovascular risk factors and atherosclerotic disease in developing countries, Can. J. Cardiol. 33 (2017) 358–365.
[97] S. Thul, C. Labat, M. Temmar, A. Benetos, M. Back, Low salivary resolvin D1 to leukotriene B4 ratio predicts carotid intima media thickness: a novel biomarker of non-resolving vascular inflammation, Eur. J. Prev. Cardiol. 24 (2017) 903–906.
[98] M. Uddin, B.D. Levy, Resolvins: natural agonists for resolution of pulmonary inflammation, Prog. Lipid Res. 50 (2011) 75–88.
[99] J. Viola, O. Soehnlein, Atherosclerosis - a matter of unresolved inflammation, Semin. Immunol. 27 (2015) 184–193.
[100] J.R. Viola, P. Lemnitzer, Y. Jansen, G. Csaba, C. Winter, C. Neideck, C. Silvestre-Roig, G. Dittmar, Y. Doring, M. Drechsler, C. Weber, R. Zimmer, N. Cenac, O. Soehnlein, Resolving lipid mediators maresin 1 and resolvin D2 prevent atheroprogression in mice, Circ. Res. 119 (2016) 1030–1038.
[101] K.H. Weylandt, C.Y. Chiu, B. Gomolka, S.F. Waechter, B. Wiedenmann, Omega-3 fatty acids and their lipid mediators: towards an understanding of resolvin and protectin formation, Prostagl. Other Lipid Mediat. 97 (2012) 73–82.
[102] J.T. Willerson, P.M. Ridker, Inflammation as a cardiovascular risk factor, Circulation 109 (2004) II2–10.
[103] B. Wu, G. Mottola, A. Chatterjee, K.D. Lance, M. Chen, I.O. Siguenza, T.A. Desai, M.S. Conte, Perivascular delivery of resolvin D1 inhibits neointimal hyperplasia in a rat model of arterial injury, J. Vasc. Surg. 65 (2017) 207–217 e203.
[104] N.S. Yadav, A. Wierzbicki, M. Aegerter, C.S. Caster, L. Perez-Grau, A.J. Kinney, W.D. Hitz, J.R. Booth Jr., B. Schweiger, K.L. Stecca, et al., Cloning of higher plant omega-3 fatty acid desaturases, Plant Physiol. 103 (1993) 467–476.
[105] E. Zakynthinos, N. Pappa, Inflammatory biomarkers in coronary artery disease, J. Cardiol. 53 (2009) 317–333.
[106] M.J. Zhang, B.E. Sansbury, J. Hellmann, J.F. Baker, L. Guo, C.M. Parmer, J.C. Prenner, D.J. Conklin, A. Bhatnagar, M.A. Creager, M. Spite, Resolvin D2 enhances postischemic revascularization while resolving inflammation, Circulation 134 (2016) 666–680.
[107] T. Zhang, H.H. Shu, L. Chang, F. Ye, K.Q. Xu, W.Q. Huang, Resolvin D1 protects against hepatic ischemia/reperfusion injury in rats, Int. Immunopharmacol. 28 (2015) 322–327.

Manuscript VIII

Cyclooxygenase-2 inhibitors as a therapeutic target in inflammatory diseases

Miguel D. Ferrer, Carla Busquests-Cortés, Xavier Capó, Silvia Tejada, Josep A. Tur, Antoni Pons, Antoni Sureda.

Curr Med Chem. 2018 May 13.

doi: 10.2174/0929867325666180514112124. [Epub ahead of print]

Send Orders for Reprints to reprints@benthamscience.ae

Current Medicinal Chemistry, 2018, *25*, 1-15

REVIEW ARTICLE

Cyclooxygenase-2 Inhibitors as a Therapeutic Target in Inflammatory Diseases

Miguel D. Ferrer[1,2], Carla Busquests-Cortés[1], Xavier Capó[1], Silvia Tejada[3], Josep A. Tur[1,2], Antoni Pons[1,2] and Antoni Sureda[1,2,*]

[1]*Laboratory for Physical Activity Sciences and Research Group in Community Nutrition and Oxidative Stress. Department of Basic Biology and Health Sciences. IUNICS, University of Balearic Islands, Palma, Spain;* [2]*CIBEROBN (Fisiopatología de la Obesidad y la Nutrición CB12/03/30038) Instituto de Salud Carlos III, Madrid, Spain;* [3]*Experimental Laboratory, Research Unit, Son Llàtzer Hospital, IUNICS, Ctra. Manacor km 4, E-07198, Palma de Mallorca, Balearic Islands, Spain*

ARTICLEHISTORY

Received: October 31, 2016
Revised: April 12, 2017
Accepted: April 22, 2017

DOI:
10.2174/0929867325666180514112124

Abstract: Inflammation plays a crucial role in the development of many complex diseases and disorders including autoimmune diseases, metabolic syndrome, neurodegenerative diseases, and cardiovascular pathologies. Prostaglandins play a regulatory role in inflammation. Cyclooxygenases are the main mediators of inflammation by catalyzing the initial step of arachidonic acid metabolism and prostaglandin synthesis. The differential expression of the constitutive isoform COX-1 and the inducible isoform COX-2, and the finding that COX-1 is the major form expressed in the gastrointestinal tract, lead to the search for COX-2-selective inhibitors as anti-inflammatory agents that might diminish the gastrointestinal side effects of traditional non-steroidal anti-inflammatory drugs (NSAIDs). COX-2 isoform is expressed predominantly in inflammatory cells and decidedly upregulated in chronic and acute inflammations, becoming a critical target for many pharmacological inhibitors. COX- 2 selective inhibitors happen to show equivalent efficacy with that of conventional NSAIDs, but they have reduced gastrointestinal side effects. This review would elucidate the most recent findings on selective COX-2 inhibition and their relevance to human pathology, concretely in inflammatory pathologies characterized by a prolonged pro-inflammatory status, including autoimmune diseases, metabolic syndrome, obesity, atherosclerosis, neurodegenerative diseases, chronic obstructive pulmonary disease, arthritis, chronic inflammatory bowel disease and cardiovascular pathologies.

Keywords: Cyclooxygenase, COX inhibitors, inflammation, interleukin, natural compound. prostaglandin.

1. INTRODUCTION

Inflammatory diseases are a kind of illnesses characterized by a prolonged pro-inflammatory state mainly marked by a new connective tissue formation [1]. A large number of diseases are included in this category, such as autoimmune diseases, metabolic syndrome, neurodegenerative diseases, chronic obstructive pulmonary diseases, chronic inflammatory bowel disease and cardiovascular diseases.

*Address correspondence to this author at the Research Group on Community Nutrition and Oxidative Stress, University of Balearic Islands, Ctra. Valldemossa, km 7.5, E-07122 - Palma de Mallorca, Balearic Islands, Spain; Tel: +34-971173142; Fax: +34-971173184; E-mail: tosugo@hotmail.com

Nowadays, it is considered that inflammation is a tissue process which consists of a set of molecular, cellular and vascular defensive phenomena against physical, chemical or biological attacks. It is also established that inflammation is the initial response to restore homeostasis and tissue function [2]. The acute inflammatory response is characterized by an increase in blood flow, changes in vascular permeability, accumulation of leukocytes and inflammatory mediators production, such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), IL-6, IL-1, IL-8, γ interferon (IFNγ) and other chemokines [3, 4]. Some of these cytokines can activate nuclear factor κβ (NFκβ) expression, a transcription factor that can mediate the inflammatory response

0929-8673/18 $58.00+.00

[5] by regulating the transcription of many acute phase proteins and a large variety of stress response genes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [5]. In fact, COX-2 plays an important role during inflammatory response [6].

Cyclooxygenases (COXs) are a family of oxidorreductase enzymes that catalyse eicosanoids biosynthesis through arachidonic acid (AA) oxidation. Three isoforms of COXs have been described to date, COX-1, COX-2 and COX-3 [7, 8]. COXs are considered bifunctional enzymes due to their both cyclooxygenase and peroxidase activities [9]. COXs are conserved enzymes and can be found in all vertebrates and even in some invertebrates as Porifera or cnidaria [10]. However, in unicellular organisms and plants, a kind of oxygenases called pathogen inducible oxygenases has been found. They are able to oxidize polyunsaturated fatty acids and they have 30% of homology with COXs [11].

COX-1, the first discovered COX isoform, is constitutively expressed in almost all tissues, although it is mainly located in quiescent cells of platelets, kidneys and the gastrointestinal (GI) tract, and its metabolites play an important role in maintaining the physiological conditions of the organism [12]. COX-2, the inducible isoform, is only expressed in some stages of cell differentiation or during replication. Besides, COX-2 expression has been observed in pathological processes such as inflammation and angiogenesis, among others [6]. COX-1 and COX-2 catalyse AA conversion in prostaglandins trough a two-steps reaction [13]. Firstly, two molecules of oxygen are added to AA generating prostaglandin G_2 (PGG_2); after this reaction, PGG_2 is reduced generating PGH_2, which is the origin of the rest of prostaglandins, tromboxan A_2 and prostacyclins [14]. COX-3 has similar structural and catalytic features to COX-1 and COX-2, but it exhibits 20% of the activity of COX-1 and COX-2. Anyhow, this isoform has not been isolated in humans yet [7]. COX-2 is a suitable drug target against inflammatory diseases and tumorigenesis, because it is the major contributor enzyme to prostanoid synthesis in inflammatory processes and its expression is upregulated by inflammatory mediators, and also under hypoxic conditions and in many cancers.

2. CYCLOOXYGENASES CHARACTERISTICS

COXs (EC 1.14.99.1) are also known as prostaglandin-endoperoxide synthase, fatty acid cyclooxygenases, prostaglandin synthetases, prostaglandin G/H synthases, prostaglandin synthases, and prostaglandin synthetase. COXs are hemoproteins acting as both dioxygenases and peroxidases that are able to catalyze the synthesis of prostaglandin H_2 (PGH_2) from AA. Two COX isoforms (COX-1 and COX-2), with 60% amino acid sequence homology encoded by distinct genes but expressing different profiles, have been described [15, 16]. COX-3 is a third isoform derived from the COX-1 gene that maintains the intron 1 in its mRNA [7, 17]. Prostaglandins produced by the COX enzymes are ubiquitous in human physiology and regulate numerous processes [18, 19] mediating basic housekeeping functions in the body [20, 21] (Fig. **1**). COX-1 and COX-2 isoforms are bifunctional, they are membrane-bound located on the lumenal surfaces of the endoplasmic reticulum, and on the inner and outer membranes of the nuclear envelope [22]. COX-1 is expressed in most tissues whereas COX-2 is also expressed in both brain and kidney but it is primarily an inducible enzyme, whose expression is activated in response to cytokines, mitogens, endotoxin, and tumor promoters in a variety of cell types [9]. The structure and mechanism of COX isoenzymes and the structure-function relationships of COX inhibitors have been excellently reviewed [9, 23-26]. In addition, the structural details and the interaction between the substrate, inhibitors and the catalytic and regulatory sites of COXs are published in several reviews [25-28] and also in the protein data bank structure [29].

AA oxygenation occurs in two sequential reactions (Fig. **1**); the first cyclooxygenase reaction is a dioxygenase reaction in the cyclooxygenase active site generating PGG_2, and the second one is the posterior PGG_2 reduction until PGH_2 in the peroxidase active site. Dioxygenase reaction of COXs begins with abstraction of the 13-pro-S-hydrogen from AA by a tyrosyl radical centred on Tyr-385 of the enzyme in the rate-determining step to generate an arachidonoyl radical [30, 31]. Then, two oxygens are sequentially added to the arachidonyl chain with concomitant rearrangements to form the bicyclic hydroperoxide PGG_2 that diffuses to the peroxidase active site. The peroxidase activity of COXs reduces the 15-hydroperoxyl of group of PGG_2 to generate PGH_2, the final product of COXs from AA. It has been described that both COX-1 and COX-2 isoforms have similar efficiency catalyzing the conversion of AA to PGH_2 [30, 32]. In fact, both isoforms are quite similar structurally [33] and mechanistically [9, 26], with only subtle kinetic differences in substrate [34] and inhibitor specificities [24, 35], and hydroperoxide activator requirements [36, 37].

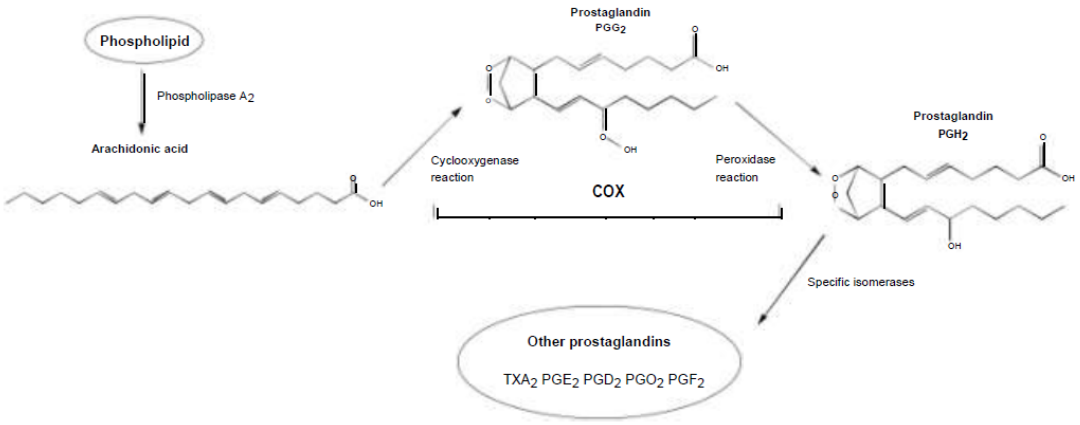


Fig. (1). Main biosynthesis steps of the arachidonic acid to prostaglandin H_2 (PGH_2) via the prostaglandin-endoperoxide synthase or COX pathway. PGH_2 is an important precursor of eicosanoids such as prostaglandins, thromboxanes, leukotrienes and resolvins, among others.

COX-1 and COX-2 are homodimers composed of strong association of two 70 kDa monomers with identical sequences that can be dissociated into their monomers only under denaturation [38]; dimerization is required for structural integrity and catalytic activity [38]. Briefly, each COX monomer consists of three structural domains [23]: a short N-terminal epidermal growth factor domain, a membrane binding domain, and a large, globular C-terminal catalytic domain [39]. The cyclooxygenase and peroxidase active sites are located on opposite sides of the catalytic domain; this domain constitutes the main catalytic monomer with the heme prosthetic group located at the base of the peroxidase site. The epidermal growth factor domain and the catalytic domain create the dimer interface, being the two membrane binding domains on the same side of the homodimer. However, although COX enzymes are homodimers, they act as conformational heterodimers during both catalysis and inhibition [39-42].

The maximal COX activity is attained with one heme per dimer [43] and, similarly, the stoichiometry of some inhibitors (such as flurbiprofen and other non-specific nonsteroidal anti-inflammatory drugs) is one inhibitory molecule per dimer [44]. COX enzymes operate like an allosteric/catalytic couple, in which one COX active site has a catalytic activity with heme bound (modulated by the ligand occupying the COX site of the partner monomer), and an allosteric monomer without heme [39, 42]. In this sense, COXs acquire a stable, asymmetric allosteric and catalytic form during folding and processing, working as a conformational heterodimer [45]. The only heme is bound to the peroxidase active site of the catalytic subunit, which binds to the substrate fatty acids and also a subset of COX inhibitors. Moreover, both substrate and non-substrate fatty acids and a second subset of COX inhibitors can also bind to the COX site of the allosteric monomer [46]. To sum up, the COXs are allosteric enzymes that may bind substrates, activators, or inhibitors to the allosteric subunit, and this can influence the binding in the catalytic subunit via the dimer interface communication [47]. Kinetically, COX-2 inhibition can occur in rapid-reversible or slow-tight ways. Compounds that are rapid-reversible COX-2 inhibitors are named as "substrate-selective". They bind the allosteric subunit at very low concentrations and induce a conformational change that blocks the catalytic activity of the other subunit. Binding of a second inhibitor molecule in the catalytic subunit blocks AA oxygenation, although this typically needs high inhibitor concentrations [40]. In fact, the oxygenation of all substrates can be blocked by slow, tight-binding inhibitors bind in the catalytic monomer at equal concentrations [44, 48].

The substrates of COXs can be n-3 and n-6 18-22 carbon polyunsaturated fatty acids, although with varying efficiencies and, generally, with higher Km values than AA [34, 49-52]. COXs also can be an endocannabinoid metabolizing enzyme due their role oxygenating arachidonate-containing lipids [27]. The fatty acid binding COXs is interpreted as a mode of production, leading to the inhibition of prostaglandins from the AA oxidation [53-55]. In fact, different fatty acids could compete with AA for binding to the allosteric or catalytic sites of COXs and regulating eicosanoid synthesis [56]. Although saturated and monounsaturated fatty acids are not COX substrates, they can bind to the al-

losteric subunit regulating COX activities; palmitic acid binds allosteric subunit stimulating COX-2 but inhibiting COX-1 [56]. C-22 n-3 polyunsaturated fatty acids, such as docosahexaenoic (DHA), have higher affinities for catalytic than for allosteric COX subunits; whereas C-20 n-3 polyunsaturated fatty acids, such as eicosapentaenoate (EPA), preferentially bind the COX-1catalytic subunit and the COX-2 allosteric one [56]. Fish oil containing both EPA and DHA reduces about 50% prostaglandin, suggesting that fatty acids alter the rate of prostaglandin production [56]. However, the products of n-3 polyunsaturated fatty acid oxidation by COXs are relevant for the inflammation resolution [57]. Synthetic inhibitors, such as aspirin, produce a highly conserved Ser-530 COX form by acetylation of the catalytic subunit [30, 41, 58]. The Acetylated-COXs transform AA into 15R-hydroxyeicosapentaenoic acid (15R-HETE) [59, 60], which is a precursor of potent anti-inflammatory molecules [61-63]; and transforms EPA and DHA in bioactive trihydroxylated compounds named resolvin E1 (RvE1) and 17R-resolvin D1 (17R-RvD1) [57, 64]. Resolvins play an active role in the resolution of inflammation processes [64].

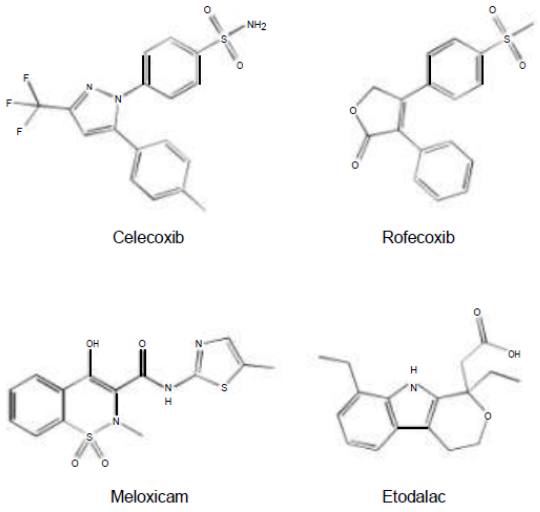


Fig. (2). Chemical structure of cyclooxygenase 2 inhibitors celecoxib, rofecoxib, meloxicam and etodalac.

COX-1 is mainly expressed in the gastrointestinal tract, and its activity inhibition displays side effects. For that reason, research is centered on COX-2-selective inhibitors as potential anti-inflammatory agents [65].

3. CLASSICAL CYCLOOXYGENASE INHIBITORS

Non-steroidal anti-inflammatory drugs (NSAIDs) are a type of drugs with large range of applications in clinic, although their indications are limited due to various adverse effects and interactions which may be potentially severe, so they should be prescribed in selected patients [66]. Concretely, traditional NSAIDs can exert nonselective inhibition effects on COX-1/COX-2, and thereby they inhibit the synthesis of prostaglandins and thromboxanes. Nonetheless, the tissue location of COX-1 underlines GI side effects such as stomach irritation, GI bleeding and even ulcers provoked by traditional NSAIDs that inhibit both COX-1 and COX-2, particularly the aspirin. These drugs exert their therapeutic effects by inhibiting COX-2-dependent prostanoid biosynthesis and COX-1 dependent gastroprotective prostaglandins production, which in turn cause gastrointestinal damage [65, 66]. Therefore, this fact led to the search for a new generation COX-2-selective inhibitors (known as Coxibs) as anti-inflammatory agents that might attenuate the GI side effects and diminish tissue toxicity of traditional non-steroidal anti-inflammatory drugs (NSAIDs). The use of Coxibs in humans not only provided the opportunity for obtaining anti-inflammatory, analgesic and antipyretic effects with equivalent efficacy as the rest of NSAIDs and a better adverse effect profile, but also unraveled a cardiovascular protective role of COX-2 by generation of prostacyclin [67, 68].

COX-1 and COX-2 isoforms present a high degree of homology, but a substitution at position 523 (isoleucine in COX-1 and valine in COX-2) makes the difference in terms of selectivity. The single methyl group provides extra space in the active site, creating a COX-2 side pocket with larger solvent accessible surface, suitable for bulky drugs as coxibs [69]. Another substitution of phenylalanine in COX-1 for leucine in COX-2 leads to a more flexible active site that let chemically dissimilar drugs as meloxicam to inhibit COX-2. Another remarkable dissimilarity between COX-1 and COX-2 is the type of inhibition they experiment. While NSAIDs bind to COX-1 through reversible hydrogen-bonding and the inhibition is carried out by stearic hindrance, COX-2 traps the inhibitors owing to an active closure of the lower enzyme site. This fact results in a meta-stable transitional state with the ligand irreversibly bind [70].

Traditionally, NSAIDs have been classified by chemical structure, although most recent drugs can be bibliographically found according to its mechanism of

action. Then, NSAIDS can be separated into two groups: non-selective cyclooxygenase inhibitors and selective COX-2 inhibitors. Most NSAIDs act inhibiting non-selectively both COX-1 and COX-2 isoenzymes in a competitively reversible way (except aspirin, in which the inhibition is irreversible). COX-2 selective inhibitors represent a noteworthy therapeutic development because of their ability to circumvent side effects associated with COX-1 inhibition. The first group of non-selective inhibitors is generally organized as follows: salicylates, where aspirin stands out; propionic acid derivatives, with ibuprofen as an archetypal drug; acetic acid derivatives, such as diclofenac; enolic acid derivatives, such as piroxicam, and sulfonanilides. The second group embraces selective COX-2 inhibitors, also called Coxibs [71]. Essentially two groups of drugs have been shown to inhibit COX-2 selectively. The first group is called Coxibs, and include celecoxib, rofecoxib, valdecoxib, parecoxib, etoricoxib, lumiracoxib and etoricoxib, tri-cyclic drugs that access the COX-2 side-pocket [69, 70]. Coxibs were discovered to be COX-2 selective since their finding and were developed and exposed as improved GI safety compounds compared to traditional NSAIDs. The second group includes previously described NSAIDS that were retrospectively found to show COX-2 selective inhibitory activity, such as meloxicam, nimesulide or etodolac.

4. CYCLOOXYGENASE-2 INHIBITORS AND INFLAMMATORY DISEASES

There is a lot of available data about the therapeutic use of COX-2 inhibitors in diverse inflammatory diseases including inflammatory bowel diseases which include ulcerative colitis and Crohn's disease, osteoarthritis and neurological diseases especially epilepsy. It is well established that COX-2 plays an important role in the development of ulcerative colitis because prostaglandins are involved in the inflammatory process, enhancing the activity of 5-lipooxygenase and the release of inflammatory leukotrienes [72]. Celecoxib treatment has been reported to reduce the damage and neutrophil infiltration in the mucous membrane of the large intestine, and also reduce the levels of IL-1β in a mouse model of experimentally-induced colonic lesions [72]. Similar effects of rofecoxib were observed in a mouse model of induced colitis. Rofecoxib increased the amount of intestinal mucus that acts as a protective barrier. It is also observed that rofecoxib decreased myeloperoxidase levels in the intestinal mucosa and reduced colonic inflammation due to suppression in neutrophils infiltration [73]. However, it is not clear if Coxibs have more beneficial effects than side effects since diverse studies have reported that Coxibs can exacerbate inflammatory bowel diseases and Crohn's disease signs [74-76].

Osteoarthritis is the most common joint disorder in western countries, affecting over 70% of adults aged 55 to 70 years [77]. COX-2 inhibitors are often used in arthritis treatment. It has been observed that celecoxib was able to reduce cartilage damage; furthermore, a reduced loss of chondrocytes and an increase in proteoglycan synthesis were also observed [78]. However, the underlying mechanisms of celecoxib treatment on the disease progression remain still unclear [79-81]. Pro-inflammatory cytokines play an important role in osteoarthritis pathogenesis due to an inhibitory activity on proteoglycan synthesis and on chondrocyte apoptosis induction. It has been observed that treatment with celecoxib reduced IL-6 levels in synovial fluid, and TNF-α and IL-1β expression in the synovial membrane [78, 81, 82]. *In vitro*, it was also demonstrated that chondrocytes' pro-inflammatory cytokine secretion was reduced after celecoxib treatment, which could slow down the progression of the disease [83]. In addition, celecoxib treatment also influences bone structure. Some studies have demonstrated that celecoxib administration can reduce the mineral bone loss and enhance trabecular bone volume but without affecting bone formation [84-86]. Finally, it has been observed that rofecoxib is able to reduce joint pain and improve life quality in arthritic patients after three weeks of treatment [87-89].

Epilepsy is a condition of the brain characterized by recurrent epileptic seizures. It has been observed that prostaglandin brain levels together with a high COX-2 expression increase dramatically in seizures produced by an epileptic status suggesting that COX-2 plays an important role in epilepsy [90]. It has been observed that oral administration of celecoxib has an anticonvulsant action, but these effects were reversed by PGE_2 intracerebroventricular administration in animal murine models [91]. It has been demonstrated that intraperitoneal injection of rofecoxib also reduced convulsions induced by pentylenetetrazol (PTZ) [92]; however, other studies using oral administration of rofecoxib for 5 days previous PTZ induction did not show any effect on the severity of seizures [93]. COX-2 inhibitors could be great strategies to fight against a migraine as it has been observed that doses of 25 and 50 mg of rofecoxib significantly reduced headaches two hours after administration [94]. Coxibs are also used in Alzheimer's disease treatment although their

beneficial effects are not clear [95]. Several studies have demonstrated that rofecoxib slows down the disease progression and could reduce the reactive oxygen species' production in rat's brain [96-99]. However, other studies have shown no effects of rofecoxib administration on disease progression [95, 100]. The administration of Celecoxib as a treatment against Alzheimer's disease was studied, but no significant effects on the disease were observed [101, 102].

5. SIDE EFFECTS OF CYCLOOXYGENASE-2 INHIBITORS

Not long after the development and introduction of the first NSAIDs in the late seventies, the first reports on side effects in the cardiovascular and renal systems started to appear. Relationships between the inhibition of COX and platelet thrombus formation [103] and occasional decreases in glomerular filtration rate and renal function [104] were reported as early as 1980s. Although some contradictory results have been obtained throughout the years, the evidence point out to a certain increase of the risk of cardiovascular incidences, especially when high doses are administered on a long-time basis. The mechanisms by which the Coxibs exert their effects on the vasculature seem to be multifactorial. On one hand, these inhibitors can activate platelet production and function, thus promptingan increase in blood viscosity and the formation of thrombi, which can further lead to the development of a cardiovascular event. As prostaglandins are involved in the inhibition of platelet aggregation [105] and thromboxane A induces pro-aggregative processes [106], the selective inhibition of COX-2 without inhibiting COX-1 disposes to the activation of platelet aggregation. On the other hand, these compounds can increase the blood pressure, especially in hypertensive subjects [107, 108] through a decrease in renal blood flow and subsequent sodium and water retention [67, 109, 110].

Several clinical trials carried out in the recent years have evidenced this relationship between NSAIDs use and increased risk of cardiovascular events. The VIGOR study revealed an increase of the risk of myocardial infarction associated to the use of rofecoxib, but only in a subgroup of patients with high risk of infarction and without affecting overall mortality [111]. Similar results with the same rofecoxib were obtained in subsequent clinical trials such as the APPROVE study, in which the use of rofecoxib was related to an increased incidence of thrombotic and cerebrovascular events and myocardial infarctions [112] but without affecting overall cardiovascular mortality. The CLASS study performed on 8059 patients with osteoarthritis or rheumatoid arthritis revealed no differences in the incidence of cardiovascular events between celecoxib and ibuprofen or diclofenac [113]. This lack of effect was supported by the results of the ADAPT study in patients over 70 years with a family history of Alzheimer's disease in which the rate of cardiovascular events in patients receiving celecoxib did not differ from receiving placebo [114]. However, another study published in the same year on the effect of celecoxib in the prevention of adenomas showed a nearly 2-fold-increased cardiovascular risk and a trend for a dose-related increase in cardiovascular events and blood pressure [115].

The results of different meta-analyses point altogether to a moderate increase in the risk of vascular events associated to the use of these NSAIDs [116] but this risk is highly dependent on the concrete inhibitor used and some inhibitors such as celecoxib and meloxicam seem to be safer than others such as rofecoxib [117-119].

These results evidence that the use of certain selective COX-2 inhibitors can increase the risk of suffering cardiovascular events, especially in predisposed high-risk patients. This is why the American Heart Association guidelines published in 2007 stated that in these patients, the use of COX-2 inhibitors should be limited to patients, for whom there are no appropriate alternatives, and then, only in the lowest dose and for the shortest duration necessary [120] and the FDA raised warnings in 2005 and 2015 on the increase in myocardial infarction and cerebrovascular accidents associated to NSAIDs. This fact and the recent market removal of some coxibs such due to their undesirable cardiovascular side effects clearly encourage focusing on future research to investigate and assess alternative templates with COX-2 inhibitory activity such as natural compounds.

6. NATURAL COMPOUNDS AS CYCLOOXYGENASE-2 INHIBITORS

Taking into account that most COX inhibitors exhibit deleterious effects which can lead to gastrointestinal, renal and cardiovascular toxicity, much attention has been focused on natural compounds. Although these natural compounds are not specific COX inhibitors, they are able to reduce inflammation by inhibition of the expression of pro-inflammatory mediators including COX-2. In this way, diverse authors reported that plant extracts or plant secondary metabolites such as phenolic compounds exert anti-inflammatory activ-

ity by ameliorating the levels and/or expression of various inflammatory mediators.

A number of studies performed *in vitro* investigated the anti-inflammatory effects of specific compounds mainly in LPS-stimulated murine macrophage cell lines (RAW 264.7 and THP-1). In these studies, the inhibitory effects of phenolic compounds such as naringenin, quercetin, procyanidin C1, monotropein, 3,4-dihydroxytoluene, gamma-irradiated genistein and resveratrol and chromone isoeugenol against COX-2 expression were clearly evidenced [121-127]. Resveratrol and its related compounds, orcinol and 4-allylphenol were also effective in COX-2 expression when RAW264.7 cells were stimulated with *Porphyromonas gingivalis* fimbriae [128]. Phenolic compounds also exerted COX-2 inhibitory activity in other cell types including HaCaT keratinocytes and Caco-2 intestinal cells [129, 130]. However, it is important to note that other compounds than polyphenols have the capability to inhibit COX-2. In a research done by Cam and de Mejia [131], lunasin, a 43–amino-acid bioactive peptide derived from soybean, inhibited proinflammatory markers by downregulating the activation of Akt-mediated NFκβ pathways through interaction with αVβ3 integrins.

Isolated compounds were also reported to be effective in inhibiting COX-2 and inflammatory processes in diverse animal models. Berberine hydrochloride, a natural extract from *Rhizoma coptidis* (Ranunculaceae), improved intestinal mucosa inflammation and reduced COX-2 expression in a rat model of acute endotoxemia induced by LPS administration [132]. In another study, chelidonic acid, a constituent of *Chelidonium majus* (Papaveraceae), was also effective against dextran sulfate sodium (DSS)-induced ulcerative colitis in the mouse. Chelidonic acid administration attenuated PGE_2 production levels and COX-2 and hypoxia induced factor-1α (HIF-1α) expression in colonic tissues [133]. 4-vinyl-2,6-dimethoxyphenol (canolol) were also investigated in DSS-induced colitis in the mouse. Inflammatory mediators, such as COX-2 and cytokines, and oxidative injury of DNA were ameliorated by canolol treatment [134]. In the same animal model, the treatment with fraxinellone, a natural occurring lactone, significantly alleviated the main signs of colitis in mice [135]. Moreover, the expression of macrophage-related molecules in the colon, including adhesion molecules, iNOS and COX-2 were markedly inhibited. Rhododendron, isolated from *Rhododendron brachycarpum* (Ericaceae) leaves, was also found to be effective as an anti-inflammatory agent in trinitrochlorobenzene (TNCB)-treated mouse ear skins [136]. Isofraxidin, a coumarin compound, significantly lowered LPS-induced mortality and the levels of inflammatory mediators in serum and bronchoalveolar lavage fluid and COX-2 protein expression in lung tissues [137].

Several studies have investigated the anti-inflammatory effects of plant extracts or diverse compositions mainly from traditional Asian medicine. In a study, primary human chondrocytes were isolated from patients with osteoarthritis, SW1353 chondrocytes and THP-1 macrophages pretreated with an extract from the heartwood of *Caesalpinia sappan* (Leguminosae) prior to stimulation with interleukin-1beta (IL-1β) or LPS evidenced a significant inhibition of COX-2 transcription [138]. Another study using LPS-stimulated RAW 264.7 macrophages evidenced that *Schizonepeta tenuifolia* (Lamiaceae) ethanolic extract significantly decreased COX-2 and prostaglandin PGE_2 levels, and NO production [139]. Korean Red Ginseng (*Panax ginseng*) water extract was also reported to suppress acrolein-induced COX-2 expression and to reduce apoptosis in HUVECs [140]. *Oldenlandia diffusa* (Rubiaceae) extract, a traditional oriental medicine for inflammation, protected mice against DSS-induced colitis by suppressing the plasma levels of IL-6, IL-1β and expression of COX-2 in colon tissues [141]. In the same animal model, inhibitory effects on COX-2 and iNOS expression were also reported after the treatment with a fungus *Phellinus linteus* extract germinated on brown rice [142]. Similar results were obtained when the extract was used in LPS-stimulated RAW 264.7 macrophages. Using trinitrobenzene sulfonic acid (TNBS)-induced rat model of inflammatory bowel disease, diverse authors investigated the protective effects of several compounds. In this way, *Pogostemon cablin* (Lamiaceae), a traditional Korean medicine, suppressed clinical signs of colitis and reduced COX-2 expression in a dose-dependent manner [143]. Similarly, *Gegen-qinlian decoction*, an oral Chinese medicine compound, assayed in rats with TNBS-induced colitis resulted in reduced colonic injury, inflammatory mediators levels, and iNOS, COX-2, macrophage inflammatory protein-2 (MIP-2), intercellular adhesion molecule-1 (ICAM-1) and toll-like receptor (TLR)-2 and -4 expressions [144]. The anti-inflammatory effects of *Caragana tangutica* (Fabaceae: Papilionoideae) were studied in diverse mouse models of inflammation including ear and paw oedema and lung inflammation. The *C. tangutica* ethyl acetate extract significantly reduced the release of PGE_2 and COX-2 expression [145]. In addition, the treatment with a composition based on the extracts from the leaf of *Uncaria gambir* (Rubiaceae) and

the root bark of *Morus alba* (Moraceae) suppressed paw edema and ear thickness in animal models by inhibiting COX-2 and lipoxygenase (5-LOX) enzyme activities [146].

In addition, synthesized phenolic compounds were newly designed and tested as potential COX-2 inhibitors. For example, the novel 2-{6-fluoro-2-[(4-methyl-2-pridinyl)carbonyl]-1H-indol-3-yl}acetic acid compound was an effective and selective COX-2 inhibitor *in vitro* using human umbilical vein endothelial cells (HUVECs) and with anti-inflammatory capability against carrageenan-induced foot in Sprague-Dawley rats [147]. The synthetic pyranochalcone-derived compound, (E)-3-(3,4-Dimethoxyphenyl)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)prop-2-en-1-one was also effective in ameliorating inflammation in a mouse model of collagen-induced arthritis [148]. In another assay, the synthetic flavonoid, (E)-1-(4-ethoxyphenyl)-3-(4-nitrophenyl)-prop-2-en-1-one (ETH) inhibited LPS-induced inflammation in LPS-stimulated RAW 264.7 macrophages via suppressing NF κβsignalling pathway [149]. Finally, an interesting study by Srinivas *et al.,* [150] reported that new synthesized 1,2-oxazine-based derivatives exerted a high degree of selectivity in inhibition towards COX-2 over COX-1. Molecular docking analyses evidenced that the presence of an isoleucine residue in the active site of COX-1 was responsible for the lower affinity to COX-1.

Neurodegenerative diseases seem to be linked to inflammation in microglia and other neuronal cells, representing a target for studying new treatments. Unfortunately, current COX inhibitors used as possible treatments have failed to obtain positive results in this kind of illnesses, despite the fact that some studies have revealed hopeful findings [151]. However, other stud-ies related to neuroinflammation and natural com-pounds as COX inhibitors have already been performed on the cells. The sesquiterpene torilin, isolated from *Ulmus davidiana* var. Japonica, has been used in tradi-tional medicine for inflammation. Torilin was able to reduce the extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, the cyclic AMP-responsive element (CRE)-binding protein (CREB) and NFκβ in lipopolysaccharide (LPS)-stimulated microglial BV2 cells. This resulted in a decrease of the inducible nitric oxide synthase (iNOS), COX-2 and IL-1β expressions, and also a decrease in the following release of nitric oxide, prostaglandin E_2 and IL-1β [152]. Similar results were found in other study using the same cell model, observing the suppression of NFκβ and TLR2 or TLR4 signalling pathways, and without affecting cell viability by *Ganoderma lucidum* extract [153], and by a multi-herb mixture (PMC-12) [154]. In a model rat of cerebral ischemia, *Eleutherococcus senticosus* (eleutheroside E, eleutheroside B and chlorogenic acid) extract was used as a treatment. The authors reported a task memory improvement and a reduction of the death of hippocampal neurons, and also an inhibition of the expression of COX-2, glial fibrillary acid protein (GFAP, astrocytes marker) and CD11b antibody (OX-42, microglia marker) in that region in a dose-dependent manner [155]. In the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydro pyridine (MPTP)-induced mouse model of Parkinson's disease, inflammatory and oxidative responses were observed in astrocyte and microglia, this was reflected by a rise of the expressions of the NFκβ, iNOS and COX-2, and an upregulation of the tumor necrosis factor alpha (TNF-α) and IL-1β expression in the strata. The flavonoid procyanidin (pycnogenol®), an extract of the maritime pine bark, reversed these effects; however, the used compound was a mixture of procyanidin and polyphenols [156]. More recently, the flavonoid hesperidin given to mice with cognitive impairment induced by streptozotocin showed similar results, as it improved the memory performance and inhibited the overexpression of several markers, including COX-2, iNOS, NFκβ and GFAP [157] The use of ethanol extracts of *Ophiocordyceps sinensis* displayed similar results in a rat model of focal cerebral ischemia/reperfusion [158]. Although some clinical assays have been performed to study the effects of natural compounds in neurological diseases [159-161], the results are not conclusive and do not aim to research the role of COXs. More research is needed to deepen the knowledge of the effects of natural products as COX inhibitors in order to ameliorate the neurological diseases driven by inflammation processes.

7. MECHANISM OF ACTION OF NATURAL COMPOUNDS

The inflammatory response is a complex network that involves multiple signalling cascades. However, when analysing the mechanism of action of these compounds or extracts, the underlying pathways are mostly the same. The final target of the different assayed compounds is the inhibition of the NFκβ signalling pathway which is an inflammatory situation that up-regulates the expression of diverse pro-inflammatory genes including COX-2 [162]. Cytokines, such as TNF-α and IL-1β, and pathogen-associated molecular patterns, such as LPS via TLR4, mediate inflammation and immune responses by activating NFκβ, MAPKs and PI3K/Akt signalling pathways [130, 136, 138]. In addi-

tion, NFκβ signalling is also closely associated to MAPK, and PI3K/Akt signalling. Most of the compounds have been reported to suppress the activation of MAPKs and PI3K/Akt pathways via reducing the phosphorylation of ERK1/2, p38 and/or Akt [121, 132, 163]. Moreover, the treatment with natural compounds is associated to a reduced degradation and phosphorylation of IκBα and/or NFκβ subunit p65, and reduced phosphorylation of the upstream signalling protein IKKα/β [127, 136]. These compounds also prevented the nuclear translocation of NFκβ and can directly interfere with the DNA binding activity of NFκβ subunits [136, 138].

CONCLUSION

COXs, and specifically COX-2, are considered the main regulators of inflammatory processes since they catalyze the initial step of arachidonic acid metabolism and prostaglandin biosynthesis, central messenger molecules in the process of inflammation.

Therapeutic interventions aiming to reduce the degree of inflammation in chronic inflammatory diseases may need to be focused on the inhibition of some of the enzymes directly involved in the synthesis of inflammatory mediators such as COX-2. The use of NSAIDs that inhibit non-selectively both COX-1 and COX-2 isoenzymes or COX-2 selective inhibitors have been reported as a promising approach to counteracting the higher inflammatory mediators production induced by over-expressed COX-2 in inflammatory diseases. However, the classical COX inhibitors have been reported to exert some degree of undesirable side effects including cardiovascular or gastrointestinal effects. Altogether, it clearly encourages developing new therapeutic drugs for the treatment of mental diseases that overcome the side effects of currently known COX inhibitors. Natural compounds, mainly polyphenols and derivates, are a new and interesting approach as they have been reported to act as potent anti-inflammatory molecules with a promising future in the therapeutic management of inflammatory diseases. Although the underlying mechanisms of action of the natural compounds are becoming well known, the absence of clinical trials makes strongly necessary additional studies to determine adequate dosages, combinations, safety and efficacy in order to establish the therapeutic uses of these compounds for the pharmacological treatment of inflammatory diseases.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGMENTS

This work has been granted by the Programme of Promotion of Biomedical Research and Health Sciences CIBEROBN CB12/03/30038. The authors hereby acknowledge the PhD grant provided by the University of the Balearic Island and the FPI/1648/2014 grant provided by Conselleria d'Educació, Cultura i Universitats, Direcció General d'Universitats i Recerca, Govern de les Illes Balears, within the program framework cofinanced by Fondo Social Europeo.

REFERENCES

[1] Cicchitti, L.; Martelli, M.; Cerritelli, F. Chronic inflammatory disease and osteopathy: a systematic review. *PLoS One,* **2015**, 10, e0121327.

[2] Sun, S.; Ji, Y.; Kersten, S.; Qi, L. Mechanisms of inflammatory responses in obese adipose tissue. *Annu Rev Nutr,* **2012**, 32, 261-286.

[3] Feghali, C.A.; Wright, T.M. Cytokines in acute and chronic inflammation. *Front Biosci,* **1997**, 2, d12-26.

[4] Koj, A. Initiation of acute phase response and synthesis of cytokines. *Biochim Biophys Acta,* **1996**, 1317, 84-94.

[5] Pahl, H.L. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene,* **1999**, 18, 6853-6866.

[6] Katori, M.; Majima, M. Cyclooxygenase-2: its rich diversity of roles and possible application of its selective inhibitors. *Inflamm Res,* **2000**, 49, 367-392.

[7] Chandrasekharan, N.V.; Dai, H.; Roos, K.L.; Evanson, N.K.; Tomsik, J.; Elton, T.S.; Simmons, D.L. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc Natl Acad Sci U S A,* **2002**, 99, 13926-13931.

[8] Ricciotti, E.; FitzGerald, G.A. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol,* **2011**, 31, 986-1000.

[9] Smith, W.L.; DeWitt, D.L.; Garavito, R.M. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem,* **2000**, 69, 145-182.

[10] Jarving, R.; Jarving, I.; Kurg, R.; Brash, A.R.; Samel, N. On the evolutionary origin of cyclooxygenase (COX) isozymes: characterization of marine invertebrate COX genes points to independent duplication events in vertebrate and invertebrate lineages. *J Biol Chem,* **2004**, 279, 13624-13633.

[11] Sanz, A.; Moreno, J.I.; Castresana, C. PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. *Plant Cell,* **1998**, 10, 1523-1537.

[12] Dubois, R.N.; Abramson, S.B.; Crofford, L.; Gupta, R.A.; Simon, L.S.; Van De Putte, L.B.; Lipsky, P.E. Cyclooxygenase in biology and disease. *FASEB J,* **1998**, 12, 1063-1073.

[13] Kulmacz, R.J.; van der Donk, W.A.; Tsai, A.L. Comparison of the properties of prostaglandin H synthase-1 and -2. *Prog Lipid Res,* **2003**, 42, 377-404.

[14] Vane, J.R.; Bakhle, Y.S.; Botting, R.M. Cyclooxygenases 1 and 2. *Annu Rev Pharmacol Toxicol,* **1998**, 38, 97-120.

[15] Kujubu, D.A.; Fletcher, B.S.; Varnum, B.C.; Lim, R.W.; Herschman, H.R. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J Biol Chem,* **1991**, 266, 12866-12872.

[16] Xie, W.L.; Chipman, J.G.; Robertson, D.L.; Erikson, R.L.; Simmons, D.L. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci U S A,* **1991**, 88, 2692-2696.

[17] Qin, N.; Zhang, S.P.; Reitz, T.L.; Mei, J.M.; Flores, C.M. Cloning, expression, and functional characterization of human cyclooxygenase-1 splicing variants: evidence for intron 1 retention. *J Pharmacol Exp Ther,* **2005**, 315, 1298-1305.

[18] Simmons, D.L.; Botting, R.M.; Hla, T. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol Rev,* **2004**, 56, 387-437.

[19] Smyth, E.M.; Grosser, T.; Wang, M.; Yu, Y.; FitzGerald, G.A. Prostanoids in health and disease. *J Lipid Res,* **2009**, 50 Suppl, S423-428.

[20] Trappe, T.A.; Liu, S.Z. Effects of prostaglandins and COX-inhibiting drugs on skeletal muscle adaptations to exercise. *J Appl Physiol (1985),* **2013**, 115, 909-919.

[21] Weinheimer, E.M.; Jemiolo, B.; Carroll, C.C.; Harber, M.P.; Haus, J.M.; Burd, N.A.; LeMoine, J.K.; Trappe, S.W.; Trappe, T.A. Resistance exercise and cyclooxygenase (COX) expression in human skeletal muscle: implications for COX-inhibiting drugs and protein synthesis. *Am J Physiol Regul Integr Comp Physiol,* **2007**, 292, R2241-2248.

[22] Morita, I.; Schindler, M.; Regier, M.K.; Otto, J.C.; Hori, T.; DeWitt, D.L.; Smith, W.L. Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. *J Biol Chem,* **1995**, 270, 10902-10908.

[23] Blobaum, A.L.; Marnett, L.J. Structural and functional basis of cyclooxygenase inhibition. *J Med Chem,* **2007**, 50, 1425-1441.

[24] DeWitt, D.L. Cox-2-selective inhibitors: the new super aspirins. *Mol Pharmacol,* **1999**, 55, 625-631.

[25] Garavito, R.M.; Mulichak, A.M. The structure of mammalian cyclooxygenases. *Annu Rev Biophys Biomol Struct,* **2003**, 32, 183-206.

[26] Marnett, L.J.; Rowlinson, S.W.; Goodwin, D.C.; Kalgutkar, A.S.; Lanzo, C.A. Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. *J Biol Chem,* **1999**, 274, 22903-22906.

[27] Hermanson, D.J.; Gamble-George, J.C.; Marnett, L.J.; Patel, S. Substrate-selective COX-2 inhibition as a novel strategy for therapeutic endocannabinoid augmentation. *Trends Pharmacol Sci,* **2014**, 35, 358-367.

[28] Xu, S.; Rouzer, C.A.; Marnett, L.J. Oxicams, a class of nonsteroidal anti-inflammatory drugs and beyond. *IUBMB Life,* **2014**, 66, 803-811.

[29] Europe, P.D.B.i. Available: http://www.ebi.ac.uk/pdbe/entry/search/index?ec_number:1 .14.99.1

[30] Schneider, C.; Pratt, D.A.; Porter, N.A.; Brash, A.R. Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. *Chem Biol,* **2007**, 14, 473-488.

[31] Tsai, A.L.; Palmer, G.; Wu, G.; Peng, S.; Okeley, N.M.; van der Donk, W.A.; Kulmacz, R.J. Structural characterization of arachidonyl radicals formed by aspirin-treated prostaglandin H synthase-2. *J Biol Chem,* **2002**, 277, 38311-38321.

[32] Tsai, A.L.; Kulmacz, R.J. Prostaglandin H synthase: resolved and unresolved mechanistic issues. *Arch Biochem Biophys,* **2010**, 493, 103-124.

[33] Malkowski, M.G.; Ginell, S.L.; Smith, W.L.; Garavito, R.M. The productive conformation of arachidonic acid bound to prostaglandin synthase. *Science,* **2000**, 289, 1933-1937.

[34] Laneuville, O.; Breuer, D.K.; Xu, N.; Huang, Z.H.; Gage, D.A.; Watson, J.T.; Lagarde, M.; DeWitt, D.L.; Smith, W.L. Fatty acid substrate specificities of human prostaglandin-endoperoxide H synthase-1 and -2. Formation of 12-hydroxy-(9Z, 13E/Z, 15Z)-octadecatrienoic acids from alpha-linolenic acid. *J Biol Chem,* **1995**, 270, 19330-19336.

[35] Marnett, L.J.; Kalgutkar, A.S. Cyclooxygenase 2 inhibitors: discovery, selectivity and the future. *Trends Pharmacol Sci,* **1999**, 20, 465-469.

[36] Kulmacz, R.J.; Wang, L.H. Comparison of hydroperoxide initiator requirements for the cyclooxygenase activities of prostaglandin H synthase-1 and -2. *J Biol Chem,* **1995**, 270, 24019-24023.

[37] Tsai, A.; Wu, G.; Palmer, G.; Bambai, B.; Koehn, J.A.; Marshall, P.J.; Kulmacz, R.J. Rapid kinetics of tyrosyl radical formation and heme redox state changes in prostaglandin H synthase-1 and -2. *J Biol Chem,* **1999**, 274, 21695-21700.

[38] Xiao, G.; Chen, W.; Kulmacz, R.J. Comparison of structural stabilities of prostaglandin H synthase-1 and -2. *J Biol Chem,* **1998**, 273, 6801-6811.

[39] Yuan, C.; Rieke, C.J.; Rimon, G.; Wingerd, B.A.; Smith, W.L. Partnering between monomers of cyclooxygenase-2 homodimers. *Proc Natl Acad Sci U S A,* **2006**, 103, 6142-6147.

[40] Prusakiewicz, J.J.; Duggan, K.C.; Rouzer, C.A.; Marnett, L.J. Differential sensitivity and mechanism of inhibition of COX-2 oxygenation of arachidonic acid and 2-arachidonoylglycerol by ibuprofen and mefenamic acid. *Biochemistry,* **2009**, 48, 7353-7355.

[41] Sharma, N.P.; Dong, L.; Yuan, C.; Noon, K.R.; Smith, W.L. Asymmetric acetylation of the cyclooxygenase-2 homodimer by aspirin and its effects on the oxygenation of arachidonic, eicosapentaenoic, and docosahexaenoic acids. *Mol Pharmacol,* **2010**, 77, 979-986.

[42] Sidhu, R.S.; Lee, J.Y.; Yuan, C.; Smith, W.L. Comparison of cyclooxygenase-1 crystal structures: cross-talk between monomers comprising cyclooxygenase-1 homodimers. *Biochemistry,* **2010**, 49, 7069-7079.

[43] Kulmacz, R.J.; Lands, W.E. Prostaglandin H synthase. Stoichiometry of heme cofactor. *J Biol Chem,* **1984**, 259, 6358-6363.

[44] Kulmacz, R.J.; Lands, W.E. Stoichiometry and kinetics of the interaction of prostaglandin H synthase with anti-inflammatory agents. *J Biol Chem,* **1985**, 260, 12572-12578.

[45] Dong, L.; Sharma, N.P.; Jurban, B.J.; Smith, W.L. Pre-existent asymmetry in the human cyclooxygenase-2 sequence homodimer. *J Biol Chem,* **2013**, 288, 28641-28655.

[46] Dong, L.; Vecchio, A.J.; Sharma, N.P.; Jurban, B.J.; Malkowski, M.G.; Smith, W.L. Human cyclooxygenase-2 is a sequence homodimer that functions as a conformational heterodimer. *J Biol Chem,* **2011**, 286, 19035-19046.

[47] Yuan, C.; Sidhu, R.S.; Kuklev, D.V.; Kado, Y.; Wada, M.; Song, I.; Smith, W.L. Cyclooxygenase Allosterism, Fatty Acid-mediated Cross-talk between Monomers of

Cyclooxygenase Homodimers. *J Biol Chem,* **2009**, 284, 10046-10055.

[48] Duggan, K.C.; Walters, M.J.; Musee, J.; Harp, J.M.; Kiefer, J.R.; Oates, J.A.; Marnett, L.J. Molecular basis for cyclooxygenase inhibition by the non-steroidal anti-inflammatory drug naproxen. *J Biol Chem,* **2010**, 285, 34950-34959.

[49] Larsen, L.N.; Dahl, E.; Bremer, J. Peroxidative oxidation of leuco-dichlorofluorescein by prostaglandin H synthase in prostaglandin biosynthesis from polyunsaturated fatty acids. *Biochim Biophys Acta,* **1996**, 1299, 47-53.

[50] Malkowski, M.G.; Thuresson, E.D.; Lakkides, K.M.; Rieke, C.J.; Micielli, R.; Smith, W.L.; Garavito, R.M. Structure of eicosapentaenoic and linoleic acids in the cyclooxygenase site of prostaglandin endoperoxide H synthase-1. *J Biol Chem,* **2001**, 276, 37547-37555.

[51] Rieke, C.J.; Mulichak, A.M.; Garavito, R.M.; Smith, W.L. The role of arginine 120 of human prostaglandin endoperoxide H synthase-2 in the interaction with fatty acid substrates and inhibitors. *J Biol Chem,* **1999**, 274, 17109-17114.

[52] Rowlinson, S.W.; Crews, B.C.; Lanzo, C.A.; Marnett, L.J. The binding of arachidonic acid in the cyclooxygenase active site of mouse prostaglandin endoperoxide synthase-2 (COX-2). A putative L-shaped binding conformation utilizing the top channel region. *J Biol Chem,* **1999**, 274, 23305-23310.

[53] Needleman, P.; Whitaker, M.O.; Wyche, A.; Watters, K.; Sprecher, H.; Raz, A. Manipulation of platelet aggregation by prostaglandins and their fatty acid precursors: pharmacological basis for a therapeutic approach. *Prostaglandins,* **1980**, 19, 165-181.

[54] Lagarde, M.; Drouot, B.; Guichardant, M.; Dechavanne, M. Uptake and effect on arachidonic acid oxygenation of some icosaenoic acids in human platelets. *Biomed Biochim Acta,* **1984**, 43, S319-322.

[55] Spector, A.A.; Kaduce, T.L.; Figard, P.H.; Norton, K.C.; Hoak, J.C.; Czervionke, R.L. Eicosapentaenoic acid and prostacyclin production by cultured human endothelial cells. *J Lipid Res,* **1983**, 24, 1595-1604.

[56] Dong, L.; Zou, H.; Yuan, C.; Hong, Y.H.; Kuklev, D.V.; Smith, W.L. Different fatty acids compete with arachidonic acid for binding to the allosteric or catalytic subunits of cyclooxygenases to regulate prostanoid synthesis. *J Biol Chem,* **2016**, 291, 4069-4078.

[57] Serhan, C.N.; Clish, C.B.; Brannon, J.; Colgan, S.P.; Chiang, N.; Gronert, K. Novel functional sets of lipid-derived mediators with antiinflammatory actions generated from omega-3 fatty acids via cyclooxygenase 2-nonsteroidal antiinflammatory drugs and transcellular processing. *J Exp Med,* **2000**, 192, 1197-1204.

[58] Smith, W.L. Nutritionally essential fatty acids and biologically indispensable cyclooxygenases. *Trends Biochem Sci,* **2008**, 33, 27-37.

[59] Holtzman, M.J.; Turk, J.; Shornick, L.P. Identification of a pharmacologically distinct prostaglandin H synthase in cultured epithelial cells. *J Biol Chem,* **1992**, 267, 21438-21445.

[60] Lecomte, M.; Laneuville, O.; Ji, C.; DeWitt, D.L.; Smith, W.L. Acetylation of human prostaglandin endoperoxide synthase-2 (cyclooxygenase-2) by aspirin. *J Biol Chem,* **1994**, 269, 13207-13215.

[61] Chiang, N.; Gronert, K.; Clish, C.B.; O'Brien, J.A.; Freeman, M.W.; Serhan, C.N. Leukotriene B4 receptor transgenic mice reveal novel protective roles for lipoxins and aspirin-triggered lipoxins in reperfusion. *J Clin Invest,* **1999**, 104, 309-316.

[62] Chiang, N.; Takano, T.; Clish, C.B.; Petasis, N.A.; Tai, H.H.; Serhan, C.N. Aspirin-triggered 15-epi-lipoxin A4 (ATL) generation by human leukocytes and murine peritonitis exudates: development of a specific 15-epi-LXA4 ELISA. *J Pharmacol Exp Ther,* **1998**, 287, 779-790.

[63] Xiao, G.; Tsai, A.L.; Palmer, G.; Boyar, W.C.; Marshall, P.J.; Kulmacz, R.J. Analysis of hydroperoxide-induced tyrosyl radicals and lipoxygenase activity in aspirin-treated human prostaglandin H synthase-2. *Biochemistry,* **1997**, 36, 1836-1845.

[64] Serhan, C.N.; Chiang, N.; Van Dyke, T.E. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol,* **2008**, 8, 349-361.

[65] Zarghi, A.; Arfaei, S. Selective COX-2 Inhibitors: a review of their structure-activity relationships. *Iran J Pharm Res,* **2011**, 10, 655-683.

[66] Rao, P.; Knaus, E.E. Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. *J Pharm Pharm Sci,* **2008**, 11, 81s-110s.

[67] Patrono, C. Cardiovascular effects of cyclooxygenase-2 inhibitors: a mechanistic and clinical perspective. *Br J Clin Pharmacol,* **2016**, 82, 957-964.

[68] Perrone, M.G.; Scilimati, A.; Simone, L.; Vitale, P. Selective COX-1 inhibition: A therapeutic target to be reconsidered. *Curr Med Chem,* **2010**, 17, 3769-3805.

[69] Hawkey, C.J. COX-2 inhibitors. *Lancet,* **1999**, 353, 307-314.

[70] Hawkey, C.J. COX-1 and COX-2 inhibitors. *Best Pract Res Clin Gastroenterol,* **2001**, 15, 801-820.

[71] White, W.B. Cardiovascular effects of the cyclooxygenase inhibitors. *Hypertension,* **2007**, 49, 408-418.

[72] Sklyarov, A.Y.; Panasyuk, N.B.; Fomenko, I.S. Role of nitric oxide-synthase and cyclooxygenase/lipooxygenase systems in development of experimental ulcerative colitis. *J Physiol Pharmacol,* **2011**, 62, 65-73.

[73] Martin, A.R.; Villegas, I.; Alarcon de la Lastra, C. The COX-2 inhibitor, rofecoxib, ameliorates dextran sulphate sodium induced colitis in mice. *Inflamm Res,* **2005**, 54, 145-151.

[74] Gornet, J.M.; Hassani, Z.; Modiglian, R.; Lemann, M. Exacerbation of Crohn's colitis with severe colonic hemorrhage in a patient on rofecoxib. *Am J Gastroenterol,* **2002**, 97, 3209-3210.

[75] Matuk, R.; Crawford, J.; Abreu, M.T.; Targan, S.R.; Vasiliauskas, E.A.; Papadakis, K.A. The spectrum of gastrointestinal toxicity and effect on disease activity of selective cyclooxygenase-2 inhibitors in patients with inflammatory bowel disease. *Inflamm Bowel Dis,* **2004**, 10, 352-356.

[76] Miao, X.P.; Ouyang, Q.; Li, H.Y.; Wen, Z.H.; Zhang, D.K.; Cui, X.Y. Role of selective cyclooxygenase-2 inhibitors in exacerbation of inflammatory bowel disease: A systematic review and meta-analysis. *Curr Ther Res Clin Exp,* **2008**, 69, 181-191.

[77] Bijlsma, J.W.; Knahr, K. Strategies for the prevention and management of osteoarthritis of the hip and knee. *Best Pract Res Clin Rheumatol,* **2007**, 21, 59-76.

[78] de Boer, T.N.; Huisman, A.M.; Polak, A.A.; Niehoff, A.G.; van Rinsum, A.C.; Saris, D.; Bijlsma, J.W.; Lafeber, F.J.; Mastbergen, S.C. The chondroprotective effect of selective COX-2 inhibition in osteoarthritis: ex vivo evaluation of human cartilage tissue after *in vivo* treatment. *Osteoarthritis Cartilage,* **2009**, 17, 482-488.

[79] Ding, C.; Cicuttini, F.; Jones, G. Do NSAIDs affect longitudinal changes in knee cartilage volume and knee cartilage defects in older adults? *Am J Med,* **2009**, 122, 836-842.

[80] Raynauld, J.P.; Martel-Pelletier, J.; Beaulieu, A.; Bessette, L.; Morin, F.; Choquette, D.; Haraoui, B.; Abram, F.; Pelletier, J.P. An open-label pilot study evaluating by magnetic resonance imaging the potential for a disease-modifying effect of celecoxib compared to a modelized historical control cohort in the treatment of knee osteoarthritis. *Semin Arthritis Rheum,* **2010**, 40, 185-192.

[81] Sawitzke, A.D.; Shi, H.; Finco, M.F.; Dunlop, D.D.; Bingham, C.O., 3rd; Harris, C.L.; Singer, N.G.; Bradley, J.D.; Silver, D.; Jackson, C.G.; Lane, N.E.; Oddis, C.V.; Wolfe, F.; Lisse, J.; Furst, D.E.; Reda, D.J.; Moskowitz, R.W.; Williams, H.J.; Clegg, D.O. The effect of glucosamine and/or chondroitin sulfate on the progression of knee osteoarthritis: a report from the glucosamine/chondroitin arthritis intervention trial. *Arthritis Rheum,* **2008**, 58, 3183-3191.

[82] Alvarez-Soria, M.A.; Largo, R.; Santillana, J.; Sanchez-Pernaute, O.; Calvo, E.; Hernandez, M.; Egido, J.; Herrero-Beaumont, G. Long term NSAID treatment inhibits COX-2 synthesis in the knee synovial membrane of patients with osteoarthritis: differential proinflammatory cytokine profile between celecoxib and aceclofenac. *Ann Rheum Dis,* **2006**, 65, 998-1005.

[83] Sanchez, C.; Mateus, M.M.; Defresne, M.P.; Crielaard, J.M.; Reginster, J.Y.; Henrotin, Y.E. Metabolism of human articular chondrocytes cultured in alginate beads. Longterm effects of interleukin 1beta and nonsteroidal antiinflammatory drugs. *J Rheumatol,* **2002**, 29, 772-782.

[84] Katagiri, M.; Ogasawara, T.; Hoshi, K.; Chikazu, D.; Kimoto, A.; Noguchi, M.; Sasamata, M.; Harada, S.; Akama, H.; Tazaki, H.; Chung, U.I.; Takato, T.; Nakamura, K.; Kawaguchi, H. Suppression of adjuvant-induced arthritic bone destruction by cyclooxygenase-2 selective agents with and without inhibitory potency against carbonic anhydrase II. *J Bone Miner Res,* **2006**, 21, 219-227.

[85] Noguchi, M.; Kimoto, A.; Kobayashi, S.; Yoshino, T.; Miyata, K.; Sasamata, M. Effect of celecoxib, a cyclooxygenase-2 inhibitor, on the pathophysiology of adjuvant arthritis in rat. *Eur J Pharmacol,* **2005**, 513, 229-235.

[86] Tsuboi, H.; Nampei, A.; Matsui, Y.; Hashimoto, J.; Kawai, S.; Ochi, T.; Yoshikawa, H. Celecoxib prevents juxta-articular osteopenia and growth plate destruction adjacent to inflamed joints in rats with collagen-induced arthritis. *Mod Rheumatol,* **2007**, 17, 115-122.

[87] Hochberg, M.C. Treatment of rheumatoid arthritis and osteoarthritis with COX-2-selective inhibitors: a managed care perspective. *Am J Manag Care,* **2002**, 8, S502-517.

[88] Shen, H.; Sprott, H.; Aeschlimann, A.; Gay, R.E.; Michel, B.A.; Gay, S. Analgesic action of acetaminophen in symptomatic osteoarthritis of the knee. *Rheumatology (Oxford),* **2006**, 45, 765-770.

[89] Theiler, R.; Bischoff, H.A.; Good, M.; Uebelhart, D. Rofecoxib improves quality of life in patients with hip or knee osteoarthritis. *Swiss Med Wkly,* **2002**, 132, 566-573.

[90] Yamagata, K.; Andreasson, K.I.; Kaufmann, W.E.; Barnes, C.A.; Worley, P.F. Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids. *Neuron,* **1993**, 11, 371-386.

[91] Oliveira, M.S.; Furian, A.F.; Royes, L.F.; Fighera, M.R.; Fiorenza, N.G.; Castelli, M.; Machado, P.; Bohrer, D.; Veiga, M.; Ferreira, J.; Cavalheiro, E.A.; Mello, C.F. Cyclooxygenase-2/PGE2 pathway facilitates pentylenetetrazol-induced seizures. *Epilepsy Res,* **2008**, 79, 14-21.

[92] Akula, K.K.; Dhir, A.; Kulkarni, S.K. Rofecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor increases pentylenetetrazol seizure threshold in mice: possible involvement of adenosinergic mechanism. *Epilepsy Res,* **2008**, 78, 60-70.

[93] Claycomb, R.J.; Hewett, S.J.; Hewett, J.A. Prophylactic, prandial rofecoxib treatment lacks efficacy against acute PTZ-induced seizure generation and kindling acquisition. *Epilepsia,* **2011**, 52, 273-283.

[94] Krymchantowski, A.V.; Bigal, M.E. Rofecoxib in migraine. *Expert Rev Neurother,* **2005**, 5, 55-61.

[95] Aisen, P.S.; Thal, L.J.; Ferris, S.H.; Assaid, C.; Nessly, M.L.; Giuliani, M.J.; Lines, C.R.; Norman, B.A.; Potter, W.Z. Rofecoxib in patients with mild cognitive impairment: further analyses of data from a randomized, double-blind, trial. *Curr Alzheimer Res,* **2008**, 5, 73-82.

[96] Aisen, P.S. Evaluation of selective COX-2 inhibitors for the treatment of Alzheimer's disease. *J Pain Symptom Manage,* **2002**, 23, S35-40.

[97] Cakala, M.; Malik, A.R.; Strosznajder, J.B. Inhibitor of cyclooxygenase-2 protects against amyloid beta peptide-evoked memory impairment in mice. *Pharmacol Rep,* **2007**, 59, 164-172.

[98] Minghetti, L. Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. *J Neuropathol Exp Neurol,* **2004**, 63, 901-910.

[99] Nivsarkar, M.; Banerjee, A.; Padh, H. Cyclooxygenase inhibitors: a novel direction for Alzheimer's management. *Pharmacol Rep,* **2008**, 60, 692-698.

[100] Reines, S.A.; Block, G.A.; Morris, J.C.; Liu, G.; Nessly, M.L.; Lines, C.R.; Norman, B.A.; Baranak, C.C. Rofecoxib: no effect on Alzheimer's disease in a 1-year, randomized, blinded, controlled study. *Neurology,* **2004**, 62, 66-71.

[101] Breitner, J.C.; Baker, L.D.; Montine, T.J.; Meinert, C.L.; Lyketsos, C.G.; Ashe, K.H.; Brandt, J.; Craft, S.; Evans, D.E.; Green, R.C.; Ismail, M.S.; Martin, B.K.; Mullan, M.J.; Sabbagh, M.; Tariot, P.N. Extended results of the Alzheimer's disease anti-inflammatory prevention trial. *Alzheimers Dement,* **2011**, 7, 402-411.

[102] Soininen, H.; West, C.; Robbins, J.; Niculescu, L. Long-term efficacy and safety of celecoxib in Alzheimer's disease. *Dement Geriatr Cogn Disord,* **2007**, 23, 8-21.

[103] Bourgain, R.H.; Six, F.; Andries, R. The action of cyclooxygenase and prostacyclin-synthetase inhibitors on platelet-vessel wall interaction. *Artery,* **1980**, 8, 96-100.

[104] Lifschitz, M.D. Renal effects of nonsteroidal anti-inflammatory agents. *J Lab Clin Med,* **1983**, 102, 313-323.

[105] Tang, S.Y.; Monslow, J.; Todd, L.; Lawson, J.; Pure, E.; FitzGerald, G.A. Cyclooxygenase-2 in endothelial and vascular smooth muscle cells restrains atherogenesis in hyperlipidemic mice. *Circulation,* **2014**, 129, 1761-1769.

[106] Moran, A.E.; Roth, G.A.; Narula, J.; Mensah, G.A. 1990-2010 global cardiovascular disease atlas. *Glob Heart,* **2014**, 9, 3-16.

[107] Aw, T.J.; Haas, S.J.; Liew, D.; Krum, H. Meta-analysis of cyclooxygenase-2 inhibitors and their effects on blood pressure. *Arch Intern Med,* **2005**, 165, 490-496.

[108] Pope, J.E.; Anderson, J.J.; Felson, D.T. A meta-analysis of the effects of nonsteroidal anti-inflammatory drugs on blood pressure. *Arch Intern Med,* **1993**, 153, 477-484.

[109] Harirforoosh, S.; Aghazadeh-Habashi, A.; Jamali, F. Extent of renal effect of cyclo-oxygenase-2-selective inhibitors is

pharmacokinetic dependent. *Clin Exp Pharmacol Physiol,* **2006**, 33, 917-924.

[110] Kohli, P.; Steg, P.G.; Cannon, C.P.; Smith, S.C., Jr.; Eagle, K.A.; Ohman, E.M.; Alberts, M.J.; Hoffman, E.; Guo, J.; Simon, T.; Sorbets, E.; Goto, S.; Bhatt, D.L. NSAID use and association with cardiovascular outcomes in outpatients with stable atherothrombotic disease. *Am J Med,* **2014**, 127, 53-60 e51.

[111] Bombardier, C.; Laine, L.; Reicin, A.; Shapiro, D.; Burgos-Vargas, R.; Davis, B.; Day, R.; Ferraz, M.B.; Hawkey, C.J.; Hochberg, M.C.; Kvien, T.K.; Schnitzer, T.J. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N Engl J Med,* **2000**, 343, 1520-1528, 1522 p following 1528.

[112] Bresalier, R.S.; Sandler, R.S.; Quan, H.; Bolognese, J.A.; Oxenius, B.; Horgan, K.; Lines, C.; Riddell, R.; Morton, D.; Lanas, A.; Konstam, M.A.; Baron, J.A. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N Engl J Med,* **2005**, 352, 1092-1102.

[113] Silverstein, F.E.; Faich, G.; Goldstein, J.L.; Simon, L.S.; Pincus, T.; Whelton, A.; Makuch, R.; Eisen, G.; Agrawal, N.M.; Stenson, W.F.; Burr, A.M.; Zhao, W.W.; Kent, J.D.; Lefkowith, J.B.; Verburg, K.M.; Geis, G.S. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: A randomized controlled trial. Celecoxib Long-term Arthritis Safety Study. *JAMA,* **2000**, 284, 1247-1255.

[114] Cardiovascular and cerebrovascular events in the randomized, controlled Alzheimer's Disease Anti-Inflammatory Prevention Trial (ADAPT). *PLoS Clin Trials,* **2006**, 1, e33.

[115] Solomon, S.D.; Pfeffer, M.A.; McMurray, J.J.; Fowler, R.; Finn, P.; Levin, B.; Eagle, C.; Hawk, E.; Lechuga, M.; Zauber, A.G.; Bertagnolli, M.M.; Arber, N.; Wittes, J. Effect of celecoxib on cardiovascular events and blood pressure in two trials for the prevention of colorectal adenomas. *Circulation,* **2006**, 114, 1028-1035.

[116] Kearney, P.M.; Baigent, C.; Godwin, J.; Halls, H.; Emberson, J.R.; Patrono, C. Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. *BMJ,* **2006**, 332, 1302-1308.

[117] White, W.B.; West, C.R.; Borer, J.S.; Gorelick, P.B.; Lavange, L.; Pan, S.X.; Weiner, E.; Verburg, K.M. Risk of cardiovascular events in patients receiving celecoxib: a meta-analysis of randomized clinical trials. *Am J Cardiol,* **2007**, 99, 91-98.

[118] Asghar, W.; Jamali, F. The effect of COX-2-selective meloxicam on the myocardial, vascular and renal risks: a systematic review. *Inflammopharmacology,* **2015**, 23, 1-16.

[119] Bhala, N.; Emberson, J.; Merhi, A.; Abramson, S.; Arber, N.; Baron, J.A.; Bombardier, C.; Cannon, C.; Farkouh, M.E.; FitzGerald, G.A.; Goss, P.; Halls, H.; Hawk, E.; Hawkey, C.; Hennekens, C.; Hochberg, M.; Holland, L.E.; Kearney, P.M.; Laine, L.; Lanas, A.; Lance, P.; Laupacis, A.; Oates, J.; Patrono, C.; Schnitzer, T.J.; Solomon, S.; Tugwell, P.; Wilson, K.; Wittes, J.; Baigent, C. Vascular and upper gastrointestinal effects of non-steroidal anti-inflammatory drugs: meta-analyses of individual participant data from randomised trials. *Lancet,* **2013**, 382, 769-779.

[120] Antman, E.M.; Bennett, J.S.; Daugherty, A.; Furberg, C.; Roberts, H.; Taubert, K.A. Use of nonsteroidal antiinflammatory drugs: an update for clinicians: a scientific statement from the American Heart Association. *Circulation,* **2007**, 115, 1634-1642.

[121] Byun, E.B.; Sung, N.Y.; Byun, E.H.; Song, D.S.; Kim, J.K.; Park, J.H.; Song, B.S.; Park, S.H.; Lee, J.W.; Byun, M.W.; Kim, J.H. The procyanidin trimer C1 inhibits LPS-induced MAPK and NF-kappaB signaling through TLR4 in macrophages. *Int Immunopharmacol,* **2013**, 15, 450-456.

[122] Byun, E.B.; Sung, N.Y.; Park, J.N.; Yang, M.S.; Park, S.H.; Byun, E.H. Gamma-irradiated resveratrol negatively regulates LPS-induced MAPK and NF-kappaB signaling through TLR4 in macrophages. *Int Immunopharmacol,* **2015**, 25, 249-259.

[123] Byun, E.B.; Sung, N.Y.; Yang, M.S.; Lee, B.S.; Song, D.S.; Park, J.N.; Kim, J.H.; Jang, B.S.; Choi, D.S.; Park, S.H.; Yu, Y.B.; Byun, E.H. Anti-inflammatory effect of gamma-irradiated genistein through inhibition of NF-kappaB and MAPK signaling pathway in lipopolysaccharide-induced macrophages. *Food Chem Toxicol,* **2014**, 74, 255-264.

[124] Chao, C.L.; Weng, C.S.; Chang, N.C.; Lin, J.S.; Kao, S.T.; Ho, F.M. Naringenin more effectively inhibits inducible nitric oxide synthase and cyclooxygenase-2 expression in macrophages than in microglia. *Nutr Res,* **2010**, 30, 858-864.

[125] Endale, M.; Park, S.C.; Kim, S.; Kim, S.H.; Yang, Y.; Cho, J.Y.; Rhee, M.H. Quercetin disrupts tyrosine-phosphorylated phosphatidylinositol 3-kinase and myeloid differentiation factor-88 association, and inhibits MAPK/AP-1 and IKK/NF-kappaB-induced inflammatory mediators production in RAW 264.7 cells. *Immunobiology,* **2013**, 218, 1452-1467.

[126] Shin, J.S.; Yun, K.J.; Chung, K.S.; Seo, K.H.; Park, H.J.; Cho, Y.W.; Baek, N.I.; Jang, D.; Lee, K.T. Monotropein isolated from the roots of *Morinda officinalis* ameliorates proinflammatory mediators in RAW 264.7 macrophages and dextran sulfate sodium (DSS)-induced colitis via NF-kappaB inactivation. *Food Chem Toxicol,* **2013**, 53, 263-271.

[127] Su, K.Y.; Yu, C.Y.; Chen, Y.P.; Hua, K.F.; Chen, Y.L. 3,4-Dihydroxytoluene, a metabolite of rutin, inhibits inflammatory responses in lipopolysaccharide-activated macrophages by reducing the activation of NF-kappaB signaling. *BMC Complement Altern Med,* **2014**, 14, 21.

[128] Murakami, Y.; Kawata, A.; Ito, S.; Katayama, T.; Fujisawa, S. The Radical Scavenging activity and cytotoxicity of resveratrol, orcinol and 4-allylphenol and their inhibitory effects on cox-2 gene expression and Nf-kappab activation in RAW264.7 cells stimulated with *Porphyromonas gingivalis*-fimbriae. *In Vivo,* **2015**, 29, 341-349.

[129] Lim, K.M.; Bae, S.; Koo, J.E.; Kim, E.S.; Bae, O.N.; Lee, J.Y. Suppression of skin inflammation in keratinocytes and acute/chronic disease models by caffeic acid phenethyl ester. *Arch Dermatol Res,* **2015**, 307, 219-227.

[130] Xiong, Y.; Chen, D.; Yu, C.; Lv, B.; Peng, J.; Wang, J.; Lin, Y. Citrus nobiletin ameliorates experimental colitis by reducing inflammation and restoring impaired intestinal barrier function. *Mol Nutr Food Res,* **2015**, 59, 829-842.

[131] Cam, A.; de Mejia, E.G. RGD-peptide lunasin inhibits Akt-mediated NF-kappaB activation in human macrophages through interaction with the alphaVbeta3 integrin. *Mol Nutr Food Res,* **2012**, 56, 1569-1581.

[132] Feng, A.W.; Yu, C.; Mao, Q.; Li, N.; Li, Q.R.; Li, J.S. Berberine hydrochloride attenuates cyclooxygenase-2 expression in rat small intestinal mucosa during acute endotoxemia. *Fitoterapia,* **2011**, 82, 976-982.

[133] Kim, D.S.; Kim, S.J.; Kim, M.C.; Jeon, Y.D.; Um, J.Y.; Hong, S.H. The therapeutic effect of chelidonic acid on ulcerative colitis. *Biol Pharm Bull,* **2012**, 35, 666-671.

[134] Fang, J.; Seki, T.; Tsukamoto, T.; Qin, H.; Yin, H.; Liao, L.; Nakamura, H.; Maeda, H. Protection from inflammatory bowel disease and colitis-associated carcinogenesis with 4-vinyl-2,6-dimethoxyphenol (canolol) involves suppression of oxidative stress and inflammasome activation. *Carcinogenesis,* **2013**, 34, 2833-2841.

[135] Wu, X.F.; Ouyang, Z.J.; Feng, L.L.; Chen, G.; Guo, W.J.; Shen, Y.; Wu, X.D.; Sun, Y.; Xu, Q. Suppression of NF-kappaB signaling and NLRP3 inflammasome activation in macrophages is responsible for the amelioration of experimental murine colitis by the natural compound fraxinellone. *Toxicol Appl Pharmacol,* **2014**, 281, 146-156.

[136] Jeon, Y.J.; Kim, B.H.; Kim, S.; Oh, I.; Lee, S.; Shin, J.; Kim, T.Y. Rhododendrin ameliorates skin inflammation through inhibition of NF-kappaB, MAPK, and PI3K/Akt signaling. *Eur J Pharmacol,* **2013**, 714, 7-14.

[137] Niu, X.; Wang, Y.; Li, W.; Mu, Q.; Li, H.; Yao, H.; Zhang, H. Protective effects of Isofraxidin against lipopolysaccharide-induced acute lung injury in mice. *Int Immunopharmacol,* **2015**, 24, 432-439.

[138] Wu, S.Q.; Otero, M.; Unger, F.M.; Goldring, M.B.; Phrutivorapongkul, A.; Chiari, C.; Kolb, A.; Viernstein, H.; Toegel, S. Anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract in human chondrocytes and macrophages. *J Ethnopharmacol,* **2011**, 138, 364-372.

[139] Byun, M.W. Schizonepeta tenuifolia ethanol extract exerts anti-inflammatory activity through the inhibition of TLR4 signaling in lipopolysaccharide-stimulated macrophage cells. *J Med Food,* **2014**, 17, 350-356.

[140] Lee, S.E.; Park, Y.S. Korean Red Ginseng water extract inhibits COX-2 expression by suppressing p38 in acrolein-treated human endothelial cells. *J Ginseng Res,* **2014**, 38, 34-39.

[141] Kim, S.J.; Kim, Y.G.; Kim, D.S.; Jeon, Y.D.; Kim, M.C.; Kim, H.L.; Kim, S.Y.; Jang, H.J.; Lee, B.C.; Hong, S.H.; Um, J.Y. *Oldenlandia diffusa* ameliorates dextran sulphate sodium-induced colitis through inhibition of NF-kappaB activation. *Am J Chin Med,* **2011**, 39, 957-969.

[142] Song, M.; Park, H.J. Anti-inflammatory effect of *Phellinus linteus* grown on germinated brown rice on dextran sodium sulfate-induced acute colitis in mice and LPS-activated macrophages. *J Ethnopharmacol,* **2014**, 154, 311-318.

[143] Park, S.Y.; Neupane, G.P.; Lee, S.O.; Lee, J.S.; Kim, M.Y.; Kim, S.Y.; Park, B.C.; Park, Y.J.; Kim, J.A. Protective effects of *Pogostemon cablin* Bentham water extract on inflammatory cytokine expression in TNBS-induced colitis in rats. *Arch Pharm Res,* **2014**, 37, 253-262.

[144] Xu, B.L.; Zhang, G.J.; Ji, Y.B. Active components alignment of *Gegenqinlian* decoction protects ulcerative colitis by attenuating inflammatory and oxidative stress. *J Ethnopharmacol,* **2015**, 162, 253-260.

[145] Niu, X.; Li, Y.; Li, W.; Hu, H.; Yao, H.; Li, H.; Mu, Q. The anti-inflammatory effects of *Caragana tangutica* ethyl acetate extract. *J Ethnopharmacol,* **2014**, 152, 99-105.

[146] Yimam, M.; Lee, Y.C.; Kim, T.W.; Moore, B.; Jiao, P.; Hong, M.; Kim, H.J.; Nam, J.B.; Kim, M.R.; Oh, J.S.; Cleveland, S.; Hyun, E.J.; Chu, M.; Jia, Q. Analgesic and anti-inflammatory effect of UP3005, a botanical composition containing two standardized extracts of *Uncaria gambir* and *Morus alba*. *Pharmacognosy Res,* **2015**, 7, S39-46.

[147] Hayashi, S.; Ueno, N.; Murase, A.; Nakagawa, Y.; Takada, J. Novel acid-type cyclooxygenase-2 inhibitors: Design, synthesis, and structure-activity relationship for anti-inflammatory drug. *Eur J Med Chem,* **2012**, 50, 179-195.

[148] Li, X.; Peng, F.; Xie, C.; Wu, W.; Han, X.; Chen, L. (E)-3-(3,4-Dimethoxyphenyl)-1-(5-hydroxy-2,2-dimethyl-2H-chromen-6-yl)prop-2-en-1 -one ameliorates the collagen-arthritis via blocking ERK/JNK and NF-kappaB signaling pathway. *Int Immunopharmacol,* **2013**, 17, 1125-1133.

[149] Xu, J.; Jia, Y.Y.; Chen, S.R.; Ye, J.T.; Bu, X.Z.; Hu, Y.; Ma, Y.Z.; Guo, J.L.; Liu, P.Q. (E)-1-(4-ethoxyphenyl)-3-(4-nitrophenyl)-prop-2-en-1-one suppresses LPS-induced inflammatory response through inhibition of NF-kappaB signaling pathway. *Int Immunopharmacol,* **2013**, 15, 743-751.

[150] Srinivas, V.; Mohan, C.D.; Baburajeev, C.P.; Rangappa, S.; Jagadish, S.; Fuchs, J.E.; Sukhorukov, A.Y.; Chandra; Mason, D.J.; Sharath Kumar, K.S.; Madegowda, M.; Bender, A.; Basappa; Rangappa, K.S. Synthesis and characterization of novel oxazines and demonstration that they specifically target cyclooxygenase 2. *Bioorg Med Chem Lett,* **2015**, 25, 2931-2936.

[151] Carlson, N.G.; Rojas, M.A.; Redd, J.W.; Tang, P.; Wood, B.; Hill, K.E.; Rose, J.W. Cyclooxygenase-2 expression in oligodendrocytes increases sensitivity to excitotoxic death. *J Neuroinflammation,* **2010**, 7, 25.

[152] Choi, Y.; Lee, M.K.; Lim, S.Y.; Sung, S.H.; Kim, Y.C. Inhibition of inducible NO synthase, cyclooxygenase-2 and interleukin-1beta by torilin is mediated by mitogen-activated protein kinases in microglial BV2 cells. *Br J Pharmacol,* **2009**, 156, 933-940.

[153] Yoon, H.M.; Jang, K.J.; Han, M.S.; Jeong, J.W.; Kim, G.Y.; Lee, J.H.; Choi, Y.H. *Ganoderma lucidum* ethanol extract inhibits the inflammatory response by suppressing the NF-kappaB and toll-like receptor pathways in lipopolysaccharide-stimulated BV2 microglial cells. *Exp Ther Med,* **2013**, 5, 957-963.

[154] Park, M.Y.; Jung, Y.S.; Park, J.H.; Choi, Y.W.; Lee, J.; Kim, C.M.; Baek, J.U.; Choi, B.T.; Shin, H.K. PMC-12, a Prescription of traditional Korean medicine, improves amyloid beta-induced cognitive deficits through modulation of neuroinflammation. *Evid Based Complement Alternat Med,* **2015**, 2015, 768049.

[155] Lee, D.; Park, J.; Yoon, J.; Kim, M.Y.; Choi, H.Y.; Kim, H. Neuroprotective effects of *Eleutherococcus senticosus* bark on transient global cerebral ischemia in rats. *J Ethnopharmacol,* **2012**, 139, 6-11.

[156] Khan, M.M.; Kempuraj, D.; Thangavel, R.; Zaheer, A. Protection of MPTP-induced neuroinflammation and neurodegeneration by Pycnogenol. *Neurochem Int,* **2013**, 62, 379-388.

[157] Javed, H.; Vaibhav, K.; Ahmed, M.E.; Khan, A.; Tabassum, R.; Islam, F.; Safhi, M.M. Effect of hesperidin on neurobehavioral, neuroinflammation, oxidative stress and lipid alteration in intracerebroventricular streptozotocin induced cognitive impairment in mice. *J Neurol Sci,* **2015**, 348, 51-59.

[158] Kong, R.; Zhang, Y.; Zhang, S.; Liu, M.; Sun, W.; Xing, Y.; Guan, Y.; Han, C.; Liu, Z. Protective effect of ethanol extracts of the Chinese caterpillar Mushroom, *Ophiocordyceps sinensis* (Ascomycetes), on the experimental middle cerebral artery occlusion/reperfusion (MCAO/R) Model. *Int J Med Mushrooms,* **2015**, 17, 997-1003.

[159] Engelhart, M.J.; Geerlings, M.I.; Ruitenberg, A.; van Swieten, J.C.; Hofman, A.; Witteman, J.C.; Breteler, M.M. Dietary intake of antioxidants and risk of Alzheimer disease. *JAMA,* **2002**, 287, 3223-3229.

[160] Laurin, D.; Masaki, K.H.; Foley, D.J.; White, L.R.; Launer, L.J. Midlife dietary intake of antioxidants and risk of late-life incident dementia: the Honolulu-Asia Aging Study. *Am J Epidemiol,* **2004**, 159, 959-967.

[161] White, L.R.; Petrovitch, H.; Ross, G.W.; Masaki, K.; Hardman, J.; Nelson, J.; Davis, D.; Markesbery, W. Brain aging and midlife tofu consumption. *J Am Coll Nutr,* **2000**, 19, 242-255.

[162] Zhang, Q.; Piao, X.L.; Piao, X.S.; Lu, T.; Wang, D.; Kim, S.W. Preventive effect of *Coptis chinensis* and berberine on intestinal injury in rats challenged with lipopolysaccharides. *Food Chem Toxicol,* **2011**, 49, 61-69.

[163] Wu, J.; Chen, C.; Hu, X.; Cai, X.; Guan, Y.; Hu, H.; Wang, Q.; Chen, X.; Cai, B.; Jing, X. Suppressing cyclooxygenase-2 prevents nonalcoholic and inhibits apoptosis of hepatocytes that are involved in the Akt/p53 signal pathway. *Biochem Biophys Res Commun,* **2016**, 469, 1034-1040.

PMID: 29756563

La commedia è finita

Ópera Pagliacci, Ruggero Leoncavallo, 1892