Universidad de Navarra

Facultad de Farmacia y Nutrición

Effects of DHA supplementation and physical exercise on adipose tissue and metabolic health: studies in aged obese female mice and postmenopausal women

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Dña. Elisa Félix Soriano

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Quienes no se mueven no notan sus cadenas

Rosa Luxemburgo

Cuidado con lo que deseas, porque vas a conseguirlo

Ángela Díaz Villalba

A mis padres

A mi abuela

A mi bisa

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Ahora sí, *vámonos que nos vamos*

LIST OF ABBREVIATIONS

A

AA: Arachidonic Acid

ACC: Acetyl Carboxylase

ACOX1: Acyl-CoA oxidase 1

ACK: Ammonium-Chloride-Potassium

ACSM: American College of Sports Medicine

ALOX5AP: Arachidonate 5-Lipoxygenase-Activating Protein

ALX/FPR2: Lipoxin Receptor/N-Formyl Peptide Receptor-2

AMPK: Adenosine Monophosphate (AMP)-activated protein kinase

ATGL: Adipose Triglyceride Lipase

AUC: Area Under the Curve

B

BAT: Brown Adipose Tissue

β-Klotho: Beta-klotho

β3AR: Beta 3-Adrenergic Receptor

BMC: Bone Mineral Content

BMD: Bone Mineral Density

BMI: Body Mass Index

BSA: Bovine Serum Albumin

C

CD11b: Integrin Alpha M

CD206: Mannose Receptor, C 1(Cluster of Differentiation 206)

CD: Cluster of Differentiation

CEIMD: *Centro de Estudios e Investigación en Medicina del Deporte*

ChemR23: Chemerin Receptor 23

COX-2: Cyclooxygenase-2

CRP: C-Reactive Protein

CT: Control

cysLT: cysLeukotriene

D

DAPI: 4',6-Diamidine-2-Phenilindol

DBP: Diastolic Blood Pressure

DGAT: Diglyceride acyltransferase

DHA: Docosahexaenoic Acid

DIO: Diet-Induced Obese

DIOMEG: Diet-Induced obese + Omega-3

DIOEX: Diet-Induced obese + Exercise

DMEM: Dulbecco's Modified Eagle's Medium

DPA: Docosapentaenoic Acid

DXA: Dual X-ray Absorptiometry

E

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetraacetic Acid

EGR1: Early Growth Response 1

EFSA: European Food Safety Agency

ELISA: Enzyme-Linked ImmunoSorbent Assay

EPA: Eicosapentaenoic Acid

F

FFAs: Free Fatty Acids

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

FGF21: Fibroblast Growth Factor 21

FGFR1: Fibroblast Growth Factor Receptor 1

FNDC5: Fibronectin type III Domain-Containing protein 5,

F4/80: EGF-like Module-containing Mucin-like Hormone receptor-like 1

G

GLUT: Glucose Transporter

GPB: Green Fluorescent Protein

GPR120: G-Protein Coupled Receptor 120

GPR32: G-Protein Coupled Receptor 32

GTT: Glucose Tolerance Test

H

HDL-Chol: HDL-Cholesterol

HFD: High-Fat Diet

HIIT: High Intensity Interval Training

hMADS: Human Adipose Tissue-Derived Mesenchymal Stem Cells

HOMA-IR: Homeostatic Model Assessment for Insulin Resistance

HRP: Horseradish Peroxidase

HSL: Hormone-Sensitive Lipase

I

iBAT: interscapular Brown Adipose Tissue

IL: Interleukin

IR: Insulin Resistance

ISAK: International Society for the Advancement of Kinanthropometry

K

KO: Knockout

L

LDL-Chol: LDL-Cholesterol

LOX: Lipoxygenase

LPA: Light Physical Activity

LPL: Lipoprotein Lipase

LT: Leukotriene

LX: Lipoxin

Ly6G: Lymphocyte antigen 6 complex locus G6D

M

MaR: Maresin

MCP-1: Monocyte Chemoattractant Protein-1

MedDiet: Mediterranean Diet

MET: Metabolic Equivalent of the Task

MicroPET: Micro Positron Emission Tomography

MGL: Monoglyceride Lipase

MICT: Mild Intensity Continuous Training

MPA: Moderate Physical Activity

MU: Metabolic Unit

MUFA: Monounsaturated Fatty Acids

N

NE: Norepinephrine

NF-κB: Nuclear Factor-kappa B

NLR: Neutrophil-to-Lymphocyte Ratio

n-3: DPA: Omega-3 Docosapentaenoic Acid

n-3 + P: Omega-3 + Placebo

n-3 PUFA: Omega-3 Polyunsaturated Fatty Acids

n-3 + RT: Omega-3 + Resistance Training

O

OGTT: Oral glucose tolerance test

P

P: Placebo

PA: Physical Activity

PBS: Phosphate Buffered Saline

PD: Protectin

PET/CT: Positron Emission Tomography-Computed Tomography

PG: Prostaglandin

PGC1α: Peroxisome Proliferator-Activated Receptor γ Co-activator 1α

PLR: Platelet-to-Lymphocyte Ratio

PLSDA: Partial Least Squares-Discriminant Analysis

PMSF: Phenylmethylsulphonylfluoride

PPARs: Peroxisome Proliferator-Activated Receptors

PRDM16: PR-Domain containing 16

Pro-LM: Proinflammatory Lipid Mediator

Q

qRT-PCR: Quantitative Real Time Polymerase Chain Reaction

R

RT: Resistance Training

Rv: Resolvin

RvD: D-series Resolvin

RvE: E-series Resolvin

RvT: T-series Resolvin

S

SAT: Subcutaneous Adipose Tissue

scWAT: Subcutaneous Adipose Tissue

SBP: Systolic Blood Pressure

SCD1: Stearoyl-CoA desaturase-1

SDS: Sodium Dodecyl Sulfate

SENC: Spanish Society for Communitarian Nutrition

SII index: Systemic Immune-Inflammation index

SIRT-1: Sirtuin 1

SPA: Sedentary Physical Activity

SPM: Specialized proresolving lipid mediator

SUN: *Seguimiento Universidad de Navarra*

SUVmax: Maximum Standardized Uptake Value

SVF: Stroma Vascular Fraction

T

TBS: Tris-Buffered Saline

TLR: Toll like receptor

TNF-α: Tumor Necrosis Factor-α

TNFR: Tumor Necrosis Factor Receptor

Total-Chol: Total Cholesterol

Treg: Regulatory T Cells

TRPV1: Transient Receptor Potential Vanilloid 1

TSH: Thyroid Stimulating Hhormone

TyG index: Triglycerides to Glucose index

T2DM: Type 2 Diabetes Mellitus

U

UCP1: Uncoupling Protein 1

V

VAT: Visceral Adipose Ttissue

VPA: Vigorous Physical Activity

vWAT: Visceral White Adipose Tissue

W

WAT: White Adipose Tissue

WHO: World Health Organization

WT: Wild Type

#

1-RM: 1 Repetition Maximum

[^{18}F]FDG: [^{18}F]Fluoro-2-deoxy-2-D-glucose

GENE SYMBOLS

Mouse/HUMAN

Acox1: acyl-Coenzyme A oxidase 1, palmitoyl	https://www.ncbi.nlm.nih.gov/gene/11430
Adipoq/*ADIPONECTIN*: adiponectin, C1Q and collagen domain containing	https://www.ncbi.nlm.nih.gov/gene/11450
ADIPONECTIN RECEPTOR (ADIPOR1): adiponectin receptor 1	https://www.ncbi.nlm.nih.gov/gene/51094
Atgl (*Pnpla2*): patatin-like phospholipase domain containing 2	https://www.ncbi.nlm.nih.gov/gene/66853
β3AR (Adrb3): adrenergic receptor, beta 3	https://www.ncbi.nlm.nih.gov/gene/11556
Ccl2/*CCL2*: chemokine (C-C motif) ligand 2	https://www.ncbi.nlm.nih.gov/gene/20296
Cd11c/*CD11C* (*Itgax*): integrin alpha X	https://www.ncbi.nlm.nih.gov/gene/16411
Cd137 (*Tnfsrf9*)/CD137: tumor necrosis factor receptor superfamily, member 9	https://www.ncbi.nlm.nih.gov/gene/21942
Cd206 (Mrc1): mannose receptor, C type 1	https://www.ncbi.nlm.nih.gov/gene/17533
CHEMERIN (*RARRES2*): retinoic acid receptor responder 2	https://www.ncbi.nlm.nih.gov/gene/5919
ChemR23 (Cmklr1): chemokine-like receptor 1	https://www.ncbi.nlm.nih.gov/gene/14747
Cidea: cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	https://www.ncbi.nlm.nih.gov/gene/12683
Cpt1a: carnitine palmitoyltransferase 1a, liver	https://www.ncbi.nlm.nih.gov/gene/12894
Cpt1b: carnitine palmitoyltransferase 1b, muscle	https://www.ncbi.nlm.nih.gov/gene/12895
Dgat1: diacylglycerol O-acyltransferase 1	https://www.ncbi.nlm.nih.gov/gene/13350
Egr1: early growth response 1	https://www.ncbi.nlm.nih.gov/gene/13653
Fasn: fatty acid synthase	https://www.ncbi.nlm.nih.gov/gene/14104
Fgf21: fibroblast growth factor 21	https://www.ncbi.nlm.nih.gov/gene/56636
Fgfr1: fibroblast growth factor receptor 1	https://www.ncbi.nlm.nih.gov/gene/14182
FNDC5: fibronectin type III domain containing 5	https://www.ncbi.nlm.nih.gov/gene/252995
ALX/FPR2 (Fpr2): formyl peptide receptor 2	https://www.ncbi.nlm.nih.gov/gene/14289
FNDC5: fibronectin type III domain containing 5	https://www.ncbi.nlm.nih.gov/gene/252995
Glut4 (Scl2a4): solute carrier family 2 (facilitated glucose transporter), member 4	https://www.ncbi.nlm.nih.gov/gene/20528
Gpr120 (*Ffar4*): free fatty acid receptor 4	https://www.ncbi.nlm.nih.gov/gene/107221
Hsl (*Lipe*): lipase, hormone sensitive	https://www.ncbi.nlm.nih.gov/gene/16890
Icam (Icam1): intercellular adhesion molecule 1	https://www.ncbi.nlm.nih.gov/gene/15894
Il1b: interleukin 1 beta	https://www.ncbi.nlm.nih.gov/gene/16176
Il10: interleukin 10	https://www.ncbi.nlm.nih.gov/gene/16153
Il2: interleukin 2	https://www.ncbi.nlm.nih.gov/gene/16183
Il4: interleukin 4	https://www.ncbi.nlm.nih.gov/gene/16189
Il6/*IL6*: interleukin 6	https://www.ncbi.nlm.nih.gov/gene/16193
β-klotho (Klb): klotho beta	https://www.ncbi.nlm.nih.gov/gene/83379

Lep/*LEPTIN*: leptin	https://www.ncbi.nlm.nih.gov/gene/16846
Lpl: lipoprotein lipase	https://www.ncbi.nlm.nih.gov/gene/16956
MMP9: matrix metallopeptidase 9	https://www.ncbi.nlm.nih.gov/gene/4318
Nrf1: nuclear respiratory factor 1	https://www.ncbi.nlm.nih.gov/gene/18181
Pgc1a (Ppargc1a): peroxisome proliferative activated receptor, gamma, coactivator 1 α	https://www.ncbi.nlm.nih.gov/gene/19017
Ppara: peroxisome proliferator activated receptor alpha	https://www.ncbi.nlm.nih.gov/gene/19013
Pparg: peroxisome proliferator activated receptor gamma	https://www.ncbi.nlm.nih.gov/gene/19016
Prdm16/PRDM16: PR domain containing 16	https://www.ncbi.nlm.nih.gov/gene/70673
p53 (Trp53): transformation related protein 53	https://www.ncbi.nlm.nih.gov/gene/22059
p21 (Cdkn1a): cyclin dependent kinase inhibitor 1A	https://www.ncbi.nlm.nih.gov/gene/12575
Resistin (Rtn): resistin	https://www.ncbi.nlm.nih.gov/gene/57264
Scd1: stearoyl-Coenzyme A desaturase 1	https://www.ncbi.nlm.nih.gov/gene/20249
Tbx1/TBX1: T-box 1	https://www.ncbi.nlm.nih.gov/gene/21380
Tfam: transcription factor A, mitochondrial	https://www.ncbi.nlm.nih.gov/gene/21780
TLR2: toll like receptor 2	https://www.ncbi.nlm.nih.gov/gene/7097
Tlr4/TLR4: toll like receptor 4	https://www.ncbi.nlm.nih.gov/gene/21898
Tmem26/TMEM26: transmembrane protein 26	https://www.ncbi.nlm.nih.gov/gene/327766
Tnf: tumor necrosis factor	https://www.ncbi.nlm.nih.gov/gene/21926
Ucp1/UCP1: uncoupling protein 1 (mitochondrial, proton carrier)	https://www.ncbi.nlm.nih.gov/gene/22227
Ucp2: uncoupling protein 2 (mitochondrial, proton carrier)	https://www.ncbi.nlm.nih.gov/gene/22228
Ucp3: uncoupling protein 3 (mitochondrial, proton carrier)	https://www.ncbi.nlm.nih.gov/gene/22229

Abstract

Adipose tissue is a dynamic organ distributed in different fat depots. Among them, the energy storing subcutaneous white adipose tissue (scWAT) is a strong contributor to metabolic flexibility, while the brown adipose tissue (BAT) is related to healthier metabolic profiles due to its role in energy dissipation and glucose/triglyceride homeostasis. Hence, fat cells were classified as white or brown adipocytes. However, a third type of beige adipocytes can be found within several white fat depots, showing a highly inducible thermogenic capacity through a process termed *browning* of WAT. Therefore, this process entails a promising strategy for the induction of a metabolically healthier WAT due to its BAT-like phenotype. However, the three adipocytes become dysfunctional in obesity and in aging, with brown and beige cells also showing a declined activity. Importantly, obesity and aging are associated to a chronic, low-grade unresolved inflammation that underlies the dysfunction of adipose tissue and the development of the associated metabolic derangements, making the adipose organ an attractive focus for anti-inflammatory lifestyle and nutritional therapies. Among the target populations, aging obese women represent a crucial preventive focus, since they experience an acceleration of the pathophysiological processes associated to aging and obesity in the menopausal transition. In this background, docosahexaenoic acid (DHA), probably through its derived proresolving lipid mediators (SPMs), as well as exercise training, *via* its beneficial effects in several metabolic organs including the adipose tissue, could be therapeutic approaches for aging and obesity. Hence, we hypothesized that DHA supplementation and/or exercise training could ameliorate adipose tissue dysfunction in aged obese female mice and in postmenopausal women with overweight/obesity, exerting concomitant reductions in biomarkers of metabolic disturbances. With this purpose, the first experimental study was based on long-term DHA dietary supplementation or exercise training, conducted in diet-induced obese mice from adulthood up to 18 months of age. Firstly, the effects of obesity on the aged scWAT inflammatory and metabolic status were analyzed, as well as on markers of beige adipocytes, together with their modulation by the DHA-enriched diet (*Chapter 1*). Secondly, a lipidomic approach was used to characterize the changes in proresolving (SPMs) and in proinflammatory lipid mediators' signatures of the interscapular BAT (iBAT) in aging and in obesity, in addition to their associations with iBAT inflammatory status and thermogenic function, and their modulation by the DHA-enriched diet (*Chapter 2*). On the other hand, the effects of long-term exercise were analyzed on scWAT and iBAT metabolic, inflammatory, and thermogenic status, in aged obese female mice (*Chapter 3*). Then, the studies in animals were partially translated to a randomized clinical trial in postmenopausal women with overweight/obesity to explore the effects of DHA supplementation and resistance training (RT), alone or in combination, on body composition, muscle strength and quality, glucose metabolism and metabolic biomarkers (*Chapter 4*), as well as in the systemic-adipose inflammatory axis (*Chapter 5*). The studies in animals revealed beneficial actions for DHA on improving scWAT metabolic and inflammatory status (*Chapter 1*). These effects included a reduction in adipocytes size and downregulation of lipogenic and inflammatory genes, along with the upregulation of antiinflammatory M2 macrophages. Moreover, the DHA-enriched diet also induced gene markers of beige adipocyte in scWAT. Regarding the effects on iBAT, obesity and aging induced a significant reduction in SPMs content, which could contribute to the proinflammatory status and reduced thermogenic function of this fat depot (*Chapter 2*). The DHA-enriched diet increased UCP1 levels and *n*-3 PUFA derived SPMs in iBAT, without modifying those derived from arachidonic acid (lipoxins). However, it could not recover the impaired responsiveness of iBAT to cold

exposure observed in aged obese mice. Concerning the effects of long-term exercise on the obese-aged mice (*Chapter 3*), the scWAT of aged trained animals showed an increase in fatty acid oxidation and a reduction of inflammatory gene markers, with a decline in macrophage infiltration and a positive modulation of thermogenic and beige adipocytes gene markers. In contrast, iBAT of aged obese mice was less responsive to exercise, and only a moderate increase was observed on thermogenic genes/proteins, almost without changes on the inflammatory status nor in fatty acid metabolism genes (*Chapter 3*). Therefore, the changes induced by DHA supplementation and exercise on the and iBAT of obese aged mice could have contributed to their beneficial effect at the systemic levels, since DHA reduced total and LDL-cholesterol, while exercise improved insulin resistance and glucose tolerance. Finally, the studies in postmenopausal women with overweight/obesity (*Chapters 4, 5*) revealed that DHA supplementation decreased diastolic blood pressure, the inflammatory platelet-to-lymphocyte ratio, and circulating triglycerides. On the other hand, RT induced local beneficial actions on fat mass loss and muscle mass gain, maintaining whole-body bone mineral content and improving glucose tolerance. Both treatments induced favourable modulations on circulatory C-reactive protein and scWAT mRNA levels of adipocytokines (*ADIPONECTIN, LEPTIN, CHEMERIN, IL6*) and M1 macrophages markers (*CD11c*), without inducing beige-characteristic genes in scWAT. The combination of both treatments did not have any relevant synergistic effect. Instead, it resulted in some negative interactions at the inflammatory gene expression level in scWAT and in the circulating C-reactive protein. Overall, this research provides evidence that DHA supplementation and exercise training induce a beneficial remodeling of adipose tissue that can contribute to prevent the systemic inflammation and metabolic disturbances that accompany obesity and aging. The present work also suggests novel insights on the potential relevance of SPMs on iBAT function in aging and obesity that open the field to future investigations.

Resumen

El tejido adiposo es un órgano dinámico distribuido en varios depósitos grasos. Entre ellos, el tejido adiposo blanco subcutáneo almacena energía y contribuye al mantenimiento de la flexibilidad metabólica, mientras que el tejido adiposo pardo está relacionado con fenotipos metabólicos más saludables debido a su papel en la disipación de energía y la homeostasis de glucosa y triglicéridos. Las células grasas se han clasificado tradicionalmente en adipocitos blancos y pardos. Sin embargo, se ha identificado un tercer tipo de adipocito denominado *beige,* que se puede encontrar dentro de varios depósitos de grasa blanca, mostrando una capacidad termogénica altamente inducible mediante el proceso de pardeamiento del tejido adiposo blanco. Por lo tanto, este proceso supone una estrategia prometedora para obtener un tejido adiposo blanco metabólicamente más saludable, debido a su fenotipo similar al pardo. Sin embargo, los tres tipos de adipocitos se tornan disfuncionales en la obesidad y en el envejecimiento, en los que también se da una disminución de la actividad de las células pardas y beige. Es importante destacar que la obesidad y el envejecimiento están asociados a una inflamación crónica de bajo grado no resuelta, que subyace a la disfunción del tejido adiposo y al desarrollo de los desórdenes metabólicos asociados, lo que hace al órgano adiposo un foco atractivo para las terapias antiinflamatorias basadas en la nutrición y el estilo de vida. Como población diana, las mujeres obesas de mediana edad suponen un foco preventivo crucial, ya que experimentan un aceleramiento en los procesos patofisiológicos de la obesidad y el envejecimiento debido a la transición menopáusica. En este contexto, el ácido docosahexaenoico (DHA), probablemente mediante los mediadores lipídicos proresolutivos de inflamación (*specialized proresolving lipid mediators*, SPMs) que se generan del mismo, así como el ejercicio físico a través de sus acciones en los órganos metabólicos incluido el adiposo, podrían ser enfoques terapéuticos para ambos procesos. Por ello, hipotetizamos que la suplementación con DHA y/o el ejercicio físico podrían mejorar la disfunción del tejido adiposo en ratones hembra obesas envejecidas y en mujeres postmenopáusicas con sobrepeso/obesidad, ejerciendo reducciones concomitantes en biomarcadores de alteraciones metabólicas. Con este propósito, el primer estudio experimental se basó en la suplementación con DHA o en un programa de entrenamiento, ambos de forma crónica, en ratones hembra con obesidad inducida por la dieta desde la edad adulta hasta los 18 meses de edad. En primer lugar, se analizaron los efectos de la obesidad sobre el estado inflamatorio y metabólico de la grasa blanca subcutánea envejecida, así como sobre los marcadores de adipocitos beige, junto con su modulación mediante la suplementación dietética con DHA (*Capítulo 1*). En segundo lugar, se investigaron los cambios en el perfil de mediadores lipídicos proresolutivos (SPMs) y proinflamatorios de la grasa parda interescapular en el envejecimiento y en obesidad, además de su asociación con marcadores del estado inflamatorio y función termogénica de la grasa parda, y su modulación por la suplementación dietética con DHA (*Capítulo 2*). Por otro lado, se analizaron los efectos del ejercicio a largo plazo sobre el estado metabólico, inflamatorio y termogénico de la grasa blanca subcutánea y parda interescapular de los ratones hembra obesos envejecidos (*Capítulo 3*). A continuación, el estudio en animales se trasladó parcialmente a un ensayo clínico aleatorizado en mujeres posmenopáusicas con sobrepeso/obesidad para explorar los efectos de la suplementación con DHA y el entrenamiento de fuerza, por separado o combinados, sobre la composición corporal, la fuerza y calidad muscular, el metabolismo de la glucosa y diversos biomarcadores metabólicos (*Capítulo 4*), así como en el eje inflamatorio adiposo-sistémico (*Capítulo 5*). Los estudios en animales revelaron acciones beneficiosas del DHA en la mejora del estado metabólico e inflamatorio del tejido adiposo blanco subcutáneo (*Capítulo 1*). Estos efectos incluyeron una reducción del tamaño de los adipocitos y la disminución de genes lipogénicos e inflamatorios, junto con una

estimulación de macrófagos antiinflamatorios M2. Además, la dieta enriquecida en DHA también indujo marcadores de adipocitos beige en la grasa blanca subcutánea. Con respecto a los efectos en grasa parda interescapular, la obesidad y el envejecimiento indujeron una reducción significativa en el contenido de SPMs, lo que podría contribuir a su estado proinflamatorio y reducción de la función termogénica (*Capítulo 2*). La dieta enriquecida en DHA aumentó los niveles de UCP1 y de los SPMs derivados de los omega-3 en el tejido adiposo pardo, sin modificar los derivados del ácido araquidónico (lipoxinas). Sin embargo, no pudo recuperar la caída en la capacidad de respuesta al frío observada en la grasa parda de ratones obesos envejecidos. En cuanto a los efectos del ejercicio en los animales obesos envejecidos (*Capítulo 3*), la grasa blanca subcutánea de los animales entrenados mostró un incremento en genes de oxidación de ácidos grasos y una reducción de genes inflamatorios, junto con una disminución en la infiltración de macrófagos y una estimulación de genes termogénicos y marcadores de adipocitos beige. Por el contrario, la grasa parda de los ratones obesos envejecidos respondió menos al ejercicio, y sólo se observó un aumento moderado en genes/proteínas termogénicas, sin apenas cambios en genes marcadores del estado inflamatorio ni del metabolismo de los ácidos grasos. Por lo tanto, los cambios inducidos por la suplementación con DHA y el ejercicio en las grasas blanca y parda de ratones obesos envejecidos podrían haber contribuido a su efecto beneficioso a nivel sistémico, ya que el DHA redujo los niveles de colesterol total y LDL, mientras que el ejercicio mejoró la resistencia a la insulina y la tolerancia a la glucosa. Finalmente, los estudios en mujeres posmenopáusicas con sobrepeso/obesidad (*Capítulos 4, 5*) revelaron que la suplementación con DHA disminuyó la presión arterial diastólica, el índice inflamatorio ratio-plaquetas/linfocitos y los triglicéridos circulantes. Por otro lado, el entrenamiento de fuerza indujo acciones beneficiosas locales sobre la pérdida de masa grasa y el aumento de masa muscular, manteniendo la cantidad mineral ósea corporal y mejorando la tolerancia a la glucosa. Ambos tratamientos indujeron modulaciones favorables en los niveles circulantes de proteína C-reactiva y en la expresión génica de adipocitoquinas (*ADIPONECTINA*, *LEPTINA*, *QUEMERINA, IL6)* y marcadores de macrófagos M1 (*CD11c*) en biopsias de grasa subcutánea, sin inducir genes característicos de pardeamiento en la grasa blanca subcutánea. La combinación de ambos no tuvo ningún efecto sinérgico relevante, sino que resultó en algunas interacciones negativas a nivel de expresión de genes inflamatorios en la grasa subcutánea y en la proteína C-reactiva circulante. En general, esta investigación proporciona evidencia de que la suplementación con DHA y el entrenamiento físico inducen una remodelación beneficiosa del tejido adiposo que puede contribuir a prevenir la inflamación sistémica y las alteraciones metabólicas que acompañan a la obesidad y el envejecimiento. El presente trabajo también sugiere nuevas perspectivas sobre la potencial relevancia de los SPMs en la función de la grasa parda en el envejecimiento y la obesidad, que abren el campo a futuras investigaciones.

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Introduction

1. Obesity, aging and menopause: at the core of metabolic diseases in women

Obesity was characterized as a pandemic disease already in the 2000s by the World Health Organization (WHO)[1]. Indeed, in 2019, more than half a billion adults -650 million- were obese[2], and the prevalence is projected to rise up to 1.1 billion affected by 2030[3]. In some developed countries, obesity is even expected to affect 1 in 2 subjects by that date[4]. In this context, the fact that obesity leads to an outnumber of comorbidities, among which the most characterized are type 2 diabetes mellitus, cardiovascular disease, metabolic syndrome, musculoskeletal disorders, cognitive decline and even certain types of cancer, makes this disease a global health issue[2]. Because these non-communicable diseases are among the top 10 death causes worldwide[5], prevention and treatment of obesity and its comorbidities are a crucial target to improve the burden of these illnesses. Similar to the obesity prevalence trends, non-communicable diseases contributed to 7 out of 10 worldwide leading death-causes in 2019, which is an increase from 4 out of the 10 leading causes back in 2000[5].

In this background, the worldwide lifespan is also increasingly growing[6]. In 2019, 703 million people were above 65 years, and it is expected that 1 in 6 people will be over 65 by 2050, which is approximately 16% of the world's population[6]. Like obesity, chronic age-related diseases include metabolic disorders such as the metabolic syndrome, obesity, type 2 diabetes mellitus and cardiovascular diseases that, in the older age, contribute to the coexistence of several conditions in the same subject[7]. As a matter of fact, the older age is characterized by the emergence of several complex health states commonly called geriatric syndromes[8]. Importantly, leading death causes in this population group are also non-communicable diseases, specifically cardiovascular disease and several types of cancer, in developed regions like the United States of America and the European Union[9,10].

Noteworthy, aging and obesity converge in a systemic, chronic low-grade inflammation that underlays and favors the development of their associated comorbidities[11,12]. According to geroscience, inflammation is one of the seven evolutionarily conserved mechanistic pillars of ageing that are shared by age-related disorders, including metabolic diseases[7]. Such aging-associated inflammation led to the appearance of the term *inflammaging*, coined by Claudio Franceschi to describe the chronic low-grade inflammation driven by endogenous signals in the absence of infection that accompany aging[13].

Body composition changes are one of the main factors joining the process of age and obesity associated inflammation. In most individuals, the percentage of body fat gradually increases between 20–25 years of age until about 65[14], while body fat percentages are at least 25% of the body composition in men and 30% in women with obesity[15]. However, body composition changes during aging are also characterized by fat mass redistribution towards the visceral cavity and the ectopic fat infiltration in other metabolic tissues such as liver and muscle[14]. In this regard, menopause entails a major physiologic difference between men and women, since the redistribution from gluteofemoral to visceral fat depots occurs mainly during the menopausal transition[16]. These events assist the consequent rise in circulating LDL-cholesterol, the loss of insulin sensitivity and the appearance of hypertension[16,17]. Similarly, aging and obesity lead to a decline in muscle mass that is accelerated during menopause[18]. Due to muscle crucial contributions to whole-body resting energy expenditure and glucose homeostasis, this process favors the decrease in resting metabolic

rate and an the impairment in glucose metabolism, promoting the increase in fat deposition and the appearance of glucose intolerance[17,19,20]. Moreover, as estrogens play a role in inflammatory and immune processes[21], their loss also contributes to the development of inflammaging during the aging process.

In turn, the prevalence of obesity and metabolic comorbidities such as impaired glucose tolerance and metabolic syndrome is higher in women than men in all countries[22]. Conversely, women's longevity is typically higher than men's[23], partly related to lower exposure to cardiovascular risk factors during their reproductive lifespan, as most of such factors appear after menopause[24]. Indeed, obesity, diabetes mellitus and metabolic syndrome prevalence is higher in postmenopausal compared to premenopausal women, and in aging women compared to age-matched men[22,25,26]. In fact, age at menarche and menopause are directly related with higher odds ratios for mortality[27], and the final menstrual period has been postulated as a marker of aging and health[28].

Interrelationships between obesity and the final menstrual period remain to be elucidated, as obesity, central adiposity and higher body mass index (BMI) have been observed to have both positive and neutral associations with the classic menopausal vasomotor symptoms[29]. Also, aging has been postulated as the main cause for weight gain in women, regardless of the menopausal transition[29]. However, longitudinal studies have reported that the linear association that exists between body fat mass and aging is also characterized by a peak increase in visceral fat after menopause[16].

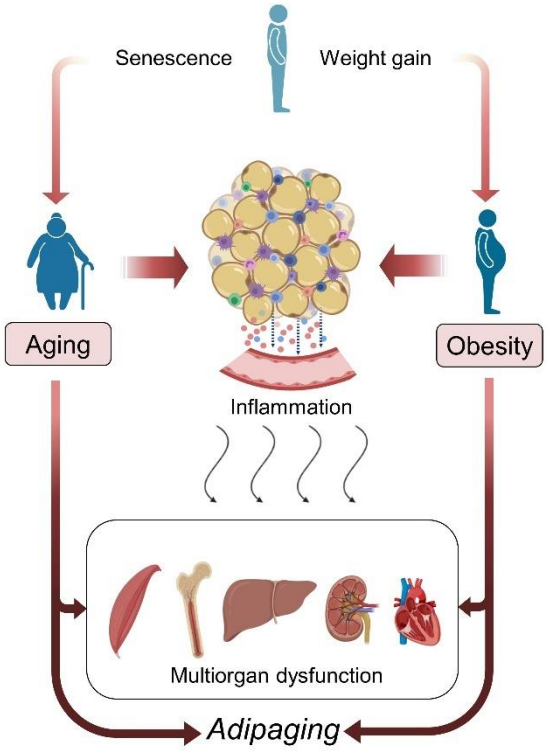

Figure 1. Adipaging, coined by Pérez *et al*.[30], joins the dysfunctional adipose tissue characterizing aging and obesity to their associated metabolic diseases, caused by lipotoxicity in several metabolic organs.

Therefore, because menopause, aging and obesity conform a triad that promotes the development of metabolic inflammation[29], the health status of aging women should be paid special attention. It is worth of mention that, from a mechanistic point of view, adipose tissue is at the basis of metabolic inflammation not only in obesity, but also in aging[7]. Hence, it is critical to understand the dysfunctional adipose-immune interactions that contribute to inflammation. In fact, *adipaging* has been proposed as a concept that illustrates the common pathways linking both conditions (**Figure 1**)[11]. As a consequence, focusing on adipose tissue as a target for antiinflammatory therapies represents an interesting approach for the treatment of obesity, aging, and their associated comorbidities[31–33].

2. Adipose tissue inflammation underlies obesity and aging-associated metabolic disorders

Adipose tissue is a dynamic organ that ranges from 4% to more than 40% of the body composition in adult humans[34]. The adipose organ is distributed in several depots with largely heterogeneous characteristics[35], but the existence of two types of adipose tissue is well established[36]. The two classic adipose tissues include the energy-storing white adipose tissue (WAT) and the energy-dissipating brown adipose tissue (BAT) , which are constituted by white and brown adipocytes, respectively[37]. However, a third new type of adipocytes (beige/brite) was found within some white fat depots but showing functional and morphological features of brown adipocytes. These include high multilocularity and the presence of cristae-rich mitochondria with inducible uncoupling protein 1 (UCP1), also producing heat instead of energy[38–40] (**Figure 2**). The discovery of active BAT in adult humans few years ago[40,41], together with the finding of beige adipocytes high inducibility by *browning* or *beiging* of WAT[42], led recent investigations to focus on strategies to increment or maintain these thermogenic adipocytes. This is of special relevance since the activity of brown and beige adipocytes is reduced by obesity and aging. Thus, these strategies could counteract the metabolic consequences of WAT dysfunction in obesity[43,44] and aging[45,46].

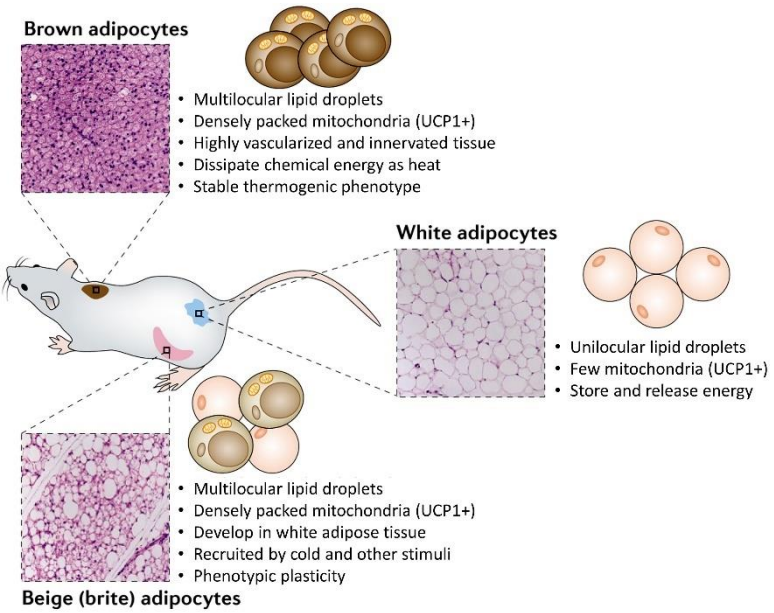

Figure 2. Three types of adipocytes (white, brown, and beige) can be found in discrete adipose depots in mice. Modified from Wang and Seale[47].

2.1. White adipose tissue in obesity and aging

WAT is a long-term storage organ which accumulates the excess of energy as triglycerides within lipid droplets, mobilizing them when needed, by pathways of lipogenesis and lipolysis, respectively[37]. Importantly, WAT is also an endocrine organ that secretes a myriad of pro and antiinflammatory cytokines, hormones, chemoattractant proteins and growth factors, collectively termed adipokines[48]. In obesity, WAT undergoes processes which comprise hyperplasia (increased adipocyte number) and hypertrophy (increased adipocyte size) in order to accumulate the excess of energy[49,50]. However, the increased cellularity that occurs thanks to tissue hyperplasia is limited, and finally leads to higher ratios of hypertrophy[50]. Indeed, WAT dysfunction is characterized by hypertrophic adipocytes which show an impaired adipokine secretion[51], with a reduction in the antiinflammatory adiponectin and an increment in the proinflammatory leptin, resistin, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein (MCP-1) and interleukins (IL-6, IL-8, IL-1β), that activate and attract pro-inflammatory immune cells[48,51]. Actually, obesity is associated with an increased macrophages infiltration and polarization towards the M1 proinflammatory phenotype instead of the M2 antiinflammatory[52]. Such M1 macrophages can constitute up to 50% of immune cells of the obese WAT and contribute to the proinflammatory secretion pattern with their derived cytokines, including IL-6 or MCP-1 among others[48]. M2 macrophages, however, secrete antiinflammatory cytokines like IL-10[48]. Furthermore, other types of immune cells (lymphocytes, eosinophils and natural killer, mast, dendritic and foam cells) can been found infiltrating the obese WAT, also contributing to local and systemic inflammation[48,53]. These events are accompanied by moderate local hypoxia due to the enlarged hypertrophic adipocytes, inducing adipocyte apoptosis or autophagy[48]. Necrotic adipocytes appear surrounded by M1 macrophages in a crown-like structure that, together with greater angiogenesis, is believed to represent a compensatory mechanism to facilitate vascularization[48]. However, both angiogenesis and hypoxia-induced fibrosis (an excess of deposition of extracellular matrix components) contribute to WAT inflammation[52] and dysfunction[49] (**Figure 3**).

The WAT was classically categorized into subcutaneous WAT (scWAT), which constitutes the largest site of fat storage (approximately 80% of whole body fat); and visceral WAT (vWAT), which accounts for a small fraction of body fatness (nearly 20% of total body fat in men, and 5-8% in women)[48]. The differences between both white fat depots are metabolically/clinically relevant, since scWAT represents a healthier WAT depot[35]. In aging, the dysfunctional adipose organ is characterized mainly by an increase in the ratio of vWAT to scWAT, due to differences in the activity of catecholamines activity and in levels of sex hormones and their receptors, which mediate fat deposition[54]. This shift contributes to increase the ratio of hypertrophic/hyperplasic adipocytes, as hyperplasia occurs mainly in the scWAT depots[55]. Hypertrophic adipocytes are directly associated to dyslipidemia, insulin resistance and type 2 diabetes[56,57], and accordingly, vWAT deposition predicts the development of metabolic and cardiovascular risk in obese subjects[58]. scWAT hyperplasic obesity, on the contrary, might have a protective role against such metabolic disturbances[59,60].

Importantly, the second characteristic of the aged adipose tissue is the appearance of senescent cells that lack the ability to divide in response to metabolic stress[61], in parallel to a decrease in the progenitor cells function and ability to incorporate lipids[62]. Consequently, the ability of WAT to undergo hyperplasia is significantly decreased[62]. Moreover, such senescent cells show a specific secretory phenotype that has been characterized across the adipocyte life stages[63]. In this context, a secretory pattern defined by

proinflammatory factors, including IL-6, MCP-1, and TNF-α, is maintained from senescent preadipocytes to senescent mature adipocytes[63]. Also, it is important to note that senescent cells drive senescence in their adjacent cells[64]. Hence, senescent adipose tissue contributes to local tissue inflammation, macrophage infiltration, immune response amplification, and in turn, consolidation of the inflammation at a whole adipose tissue level[64].

On the other hand, hypertrophic adipocytes also exhibit significant alterations in lipid metabolism, with an upregulation in basal lipolysis that results in higher circulating free fatty acids[65]. Importantly, free fatty acids interfere with local insulin actions and signaling in WAT and in main metabolic tissues, including skeletal muscle and liver, favoring not only ectopic fat deposition but also systemic inflammation and insulin resistance[66]. Insulin resistance further contributes to the upregulated basal lipolysis in hypertrophic adipocytes[67], and so do inflammatory signals[68,69], supporting a sustained release of free fatty acids. In turn, whole body metabolism assists the appearance of hyperinsulinemia, hyperglycemia and hyperlipidemia with the consequent development of obesity and aging associated comorbidities, including type 2 diabetes and cardiovascular disease[66].

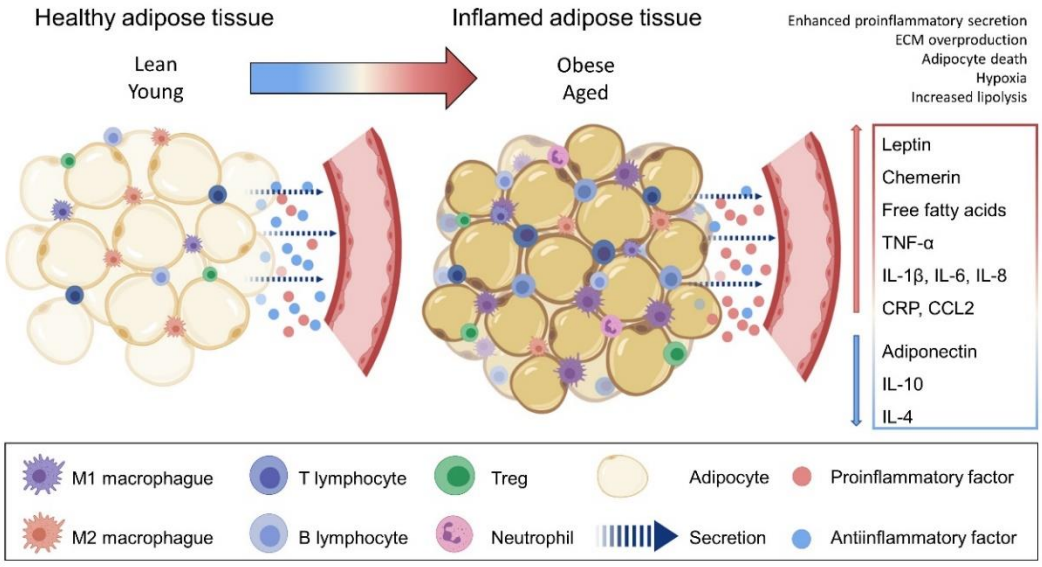

Figure 3. White adipose tissue inflammation, recruitment of immune cells and spoiled secretion of pro/antiinflammatory factors in aging and fat excess. Modified from Wang *et al.*[70]. ECM: Extracellular Matrix; TNF-α: Tumor Necrosis Factor-α; IL: Interleukins; MCP-1: Monocyte Chemoattractant Protein-1; Treg: Regulatory T Cells.

In summary, WAT secretes pro and antiinflammatory factors that act in an endocrine, paracrine and autocrine manner, driving local and systemic derangements when their secretion is impaired[71]. It is worth mentioning that adipose tissue can account for up to 50% of body mass in morbidly obese subjects, representing an important compartment of the immune system capable of influencing systemic inflammation[72]. Noteworthy, a population of metabolically healthy obese subjects has been described as insulin sensitive obese individuals, with normal plasma glucose, insulin, and lipid levels, in parallel to healthy inflammatory markers and Homeostatic Model Assessment for insulin resistance index (HOMA-IR). Among these subjects, adipose tissue appears with lower levels of infiltrated immune cells, and smaller adipocytes[73,74].

2.2. Brown adipose tissue in obesity and aging

BAT is a thermogenic tissue which main function is energy dissipation, mainly through UCP1. UCP1 is a protein located in the inner mitochondrial membrane that uncouples the respiratory chain and produces heat instead of energy. This process is known as adaptive thermogenesis, and it's mediated by beta (β)-adrenergic stimulation[36,39].

BAT differs from WAT not only in the presence of UCP1, as they arise from different developmental lineages (*Myf5*$^{+}$ and *Myf5*$^{-}$ progenitors, respectively)[49]. In fact, BAT is packed with a great number of dense mitochondria and smaller lipid droplets, which are more accessible for triglyceride hydrolysis and fatty acid oxidation[75]. Moreover, BAT metabolic fuels are glucose and free fatty acids[76], and investigations have proven BAT role in the physiological regulation of metabolism, including triglyceride[77] free fatty acids[78] and glucose clearance in rodents[79] and humans[42,78,80,81]. Although BAT endocrine function is still poorly characterized[82], it has been recently described that BAT produces batokines such as fibroblast growth factor 21 (FGF21), retinol binding protein, IL-6 and several lipid derived endocrine factors (lipokines) that modulate the function of BAT and other metabolic organs[78,83–85].

Hence, since BAT discovery in adult humans[40,41], observational studies have associated BAT activation with a leaner phenotype and with lower fasting insulin, as well as with improved insulin sensitivity and antiinflammatory adipokine secretion in clinical trials[86,87]. Upon activation, BAT has been related to weight loss[88] and decreased body fat mass[89], both due to an increase in energy expenditure, together with an increase in local and net glucose disposal[90]. Other human studies have also shown that BAT activity is also negatively associated with the development of obesity[36] and with BMI[91], fasting glycemia[92], and insulin resistance[80]. Thus, although BAT represents a small fraction of body mass (~0.1%), increasing BAT activity entails a therapeutic strategy to treat obesity, aging and their associated comorbidities[93,94].

However, it is now known that the presence and activity of BAT in humans declines as age and adiposity advance[40,41]. Therefore, this leads also to a loss of its potential preventive effects on the development of aging and obesity associated metabolic derangements[43,46] (**Figure 4**). Indeed, aging directly decreases BAT mass by decreasing its proliferative capacity[95], as well as reduces BAT activity due to lower sympathetic innervation, increased mitochondrial damage, and lower response of UCP1 to stimuli[95–97]. The aged BAT also appears with senescent cells in aging rodents[98]. Moreover, there are several indirect mechanisms by which aging can reduce BAT mass and activity[99]. Among them, *adipaging* and *inflammaging* may link the aging-induced decrease in BAT activity that appears with increased adiposity[100]. In fact, studies have shown a decreased mass/metabolic activity in BAT among obese subjects, in association with an increase in visceral adiposity, aging and hyperglycemia[40,101]. Also, a process of *whitening* of BAT has been described in animal models of obesity and in aging, with enlarged adipocytes showing a loss of response to β-adrenergic stimuli, together with mitochondrial dysfunction and loss[102–104].

In the background of obesity, evidence has suggested that compared to WAT, BAT is less susceptible to local inflammation in obesity due to immune cell infiltration[31]. However, BAT also contains different immune cells (macrophages, neutrophils, and lymphocytes)[105,106] that contribute to its inflammation and dysfunction[107]. Thus, the obese BAT presents an increased production of inflammatory cytokines, mainly TNF-α and MCP-1[31]. In this view, it has been hypothesized that inflammation with the production of these cytokines may indirectly impair the thermogenic activity of BAT by means of halting insulin sensitivity and glucose uptake in BAT[31]. In addition, inflammation induces catecholamine resistance and apoptosis in BAT

and inhibits the full differentiation of brown adipocytes. Therefore, these effects may contribute to impair the β-adrenergic-induced thermogenesis and to reduce BAT mass, respectively[108].

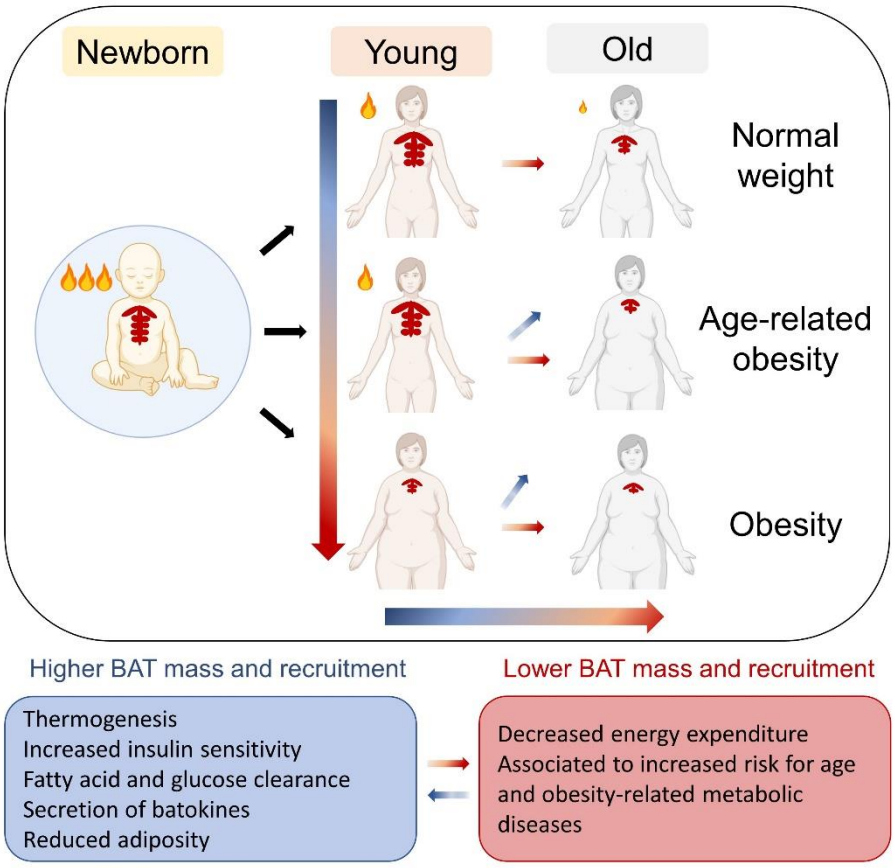

Figure 4. Brown adipose tissue presence in young and normal weight subjects, as well as its loss in aging and in obesity, and the respective consequences at local and systemic level.

Conversely, the appearance of active BAT in obese individuals is related to a metabolically healthier phenotype[101]. The therapeutic role of BAT in obesity is further supported by studies showing that BAT recruitment is positively associated with weight loss after bariatric surgery in obese individuals[109]. Moreover, in animal models of obesity, BAT transplantation results in an improved glucose homeostasis by increasing insulin sensitivity in cardiac muscle and in WAT[79]. The therapeutic potential of BAT in obesity is not limited to weight loss and glucose homeostasis, instead it also involves an improvement in lipid metabolism. Hence, there is evidence that BAT activation leads to an improvement in triglyceride and free fatty acids clearance in hyperlipidemic humans[110] and in rodent models of obesity[77].

Regarding sexual dimorphism, BAT activity is associated with the levels of female gonadotropic hormones in healthy and in aging adults[111,112].Evidence from observational studies have also shown that women are more likely to show active BAT.

2.3. Beige adipocytes in obesity and aging

During the last decade, a third type of adipose tissue consisting on beige or *brite* (brown-in-white) adipocytes was found within some WAT depots in mice, and in BAT depots in humans[38,42]. Beige/brite adipocytes are brown-like multilocular adipocytes that have thermogenic properties due to an increased mitochondrial function and to the expression of inducible UCP1[38]. Beige cells share *Myf5*$^{-}$ progenitors with white adipocytes[37] but express specific-beige genes *TBX1, TMEM26* and *CD137*[38].

However, the appearance of beige adipocytes within white fat depots has been studied mostly in rodents. Hence, beige adipocytes have been described to appear in WAT *via* transdifferentiation from white to brown-like adipocytes[113,114], or *de novo* from specific precursors that proliferate and differentiate into brown-like adipocytes[115]. Some investigations have suggested that both processes occur in WAT depots depending on their location[113]. Also, some studies have shown that the transdifferentiation from white to beige adipocytes is bidirectional, depending on the thermogenic and energy needs[115]. Therefore, more research is needed to clarify how the browning process takes place in WAT.

Moreover, there is few evidence of browning in human subcutaneous WAT[42,116]. However, it has been demonstrated that human adult BAT depots consist on a mixed population of brown and beige adipocytes[117,118], and gene-marker studies have suggested that supraclavicular and neck BAT actually consist on beige adipocytes[38,119]. In this context, evidence has revealed that progenitors within the neck BAT depot give rise to both brown, beige or white adipocytes depending on the depth of the location within the tissue[120].

Beige adipocytes have very low UCP1 protein levels compared to brown adipocytes, but its activity is highly inducible in response to stimulation[121]. These stimuli include, in addition to cold exposure and β-adrenergic stimulation, other endogenous, pharmacological and nutritional factors, such as natriuretic peptides, irisin, FGF21, cardiotrophin-1, retinoids and conjugated linoleic acid[39,122,123]. Noteworthy, a recent study has also supported that some immune-adipose interactions increase beige fat thermogenesis, reliant on eosinophils and macrophages pathways[124]. In mice, browning of WAT due to cold exposure or β-adrenergic stimuli is accompanied by a reduction in diet-induced obesity, dyslipidemia and hyperglycemia, along with a macrophage polarization towards M2 antiinflammatory phenotype in BAT and WAT[125,126]. Interestingly, cold upregulates the gene expression of *UCP1, TMEM26* and *TBX1* in human subcutaneous fat, and this process was inhibited by obesity and inflammation[127].

In the absence of beiging stimuli, UCP1 expression and mitochondrial content of beige adipocytes decrease, and beige adipocytes transition to a white adipocyte phenotype occurs[34]. This also takes place in aging and in obesity, in which the abundance of proinflammatory stimuli also decrease the presence of beige adipocytes[31,99]. In fact, the proposed mechanisms by which both conditions reduce the number of beige adipocytes include the reduction of beige precursors, their inability to differentiate under stimuli, and a sirtuin 1 (SIRT-1)-mediated pathway by which senescent markers increase in parallel to a decrease in beige adipogenesis[99]. Moreover, the same inflammatory mechanisms by which obesity and aging reduce thermogenesis in BAT act to reduce thermogenesis and browning in WAT[31] (summarized in **Figure 5**). In fact, it has been proposed that the inflammatory pathways that inhibit BAT proliferation, thermogenesis and glucose uptake act in a more severe manner to impair beige adipocytes induction and function[31].

However, in the last years an outnumber of stimuli that induce beiging have emerged. Among them, several lifestyle interventions including dietary and physical exercise could help to induce or maintain beige adipocytes, which in fact has been demonstrated already in animal models of obesity and of aging[128–133].

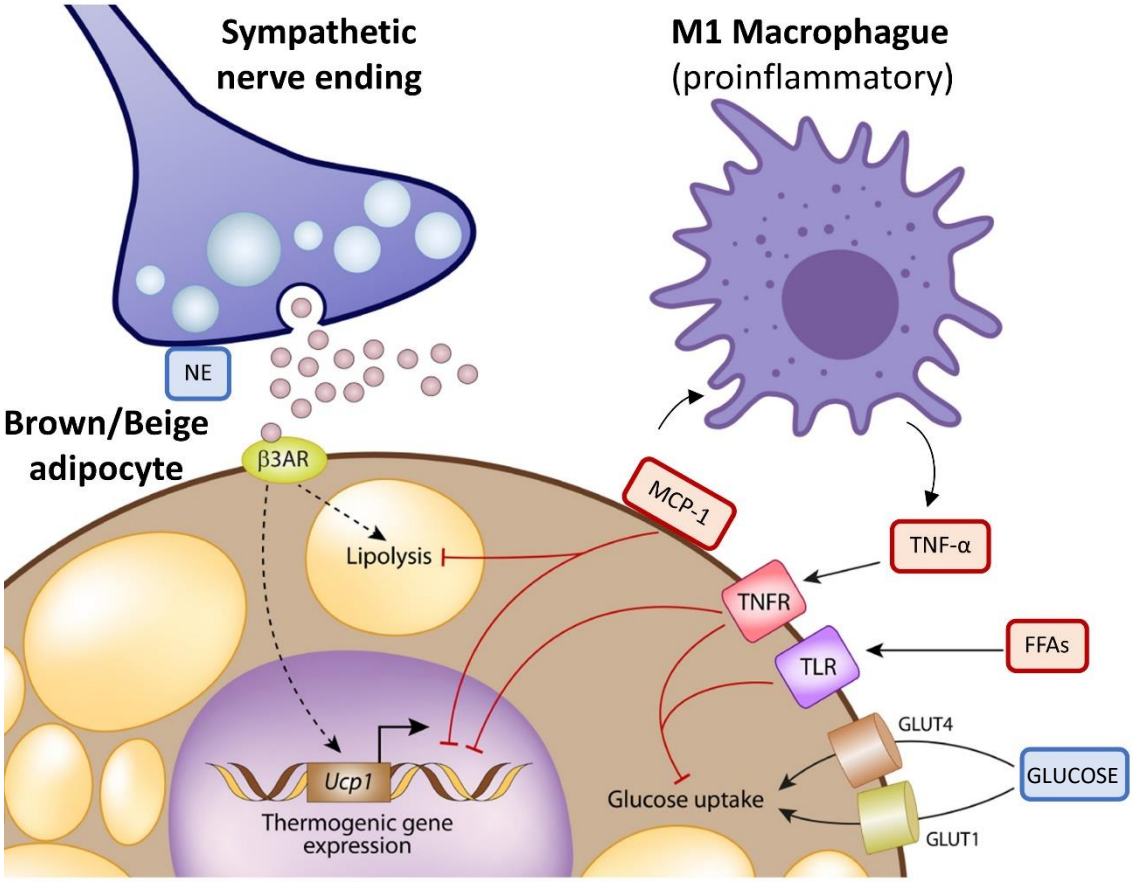

Figure 5. Proposed mechanisms for the effects of inflammation in brown and beige adipocytes, including catecholamine resistance and proinflammatory factors, proposed and modified from Villarroya *et al*.[31] NE: Norepinephrine; β3AR: Beta 3-Adrenergic Receptor; MCP-1: Monocyte Chemoattractant Protein-1; TNF-α: Tumor Necrosis Factor alpha; TNFR: Tumor Necrosis Factor Receptor; FFAs: Free Fatty Acids; TLR: Toll Like Receptor; GLUT4/1: Glucose Transporter 4/1; *Ucp1*: *Uncoupling Protein 1*.

2.4. Adipokines link adipose tissue dysfunction to metabolic inflammation in obesity and aging

Since leptin was discovered in the early nineties, the secretory role of adipose tissue in the maintenance of energy and metabolic homeostasis was soon recognized[134]. In fact, adipose tissue is now established as the largest endocrine and immune organ of the body[135]. However, as explained above, adipokine secretion is spoiled in obesity and in aging[136,137]. Importantly, adipokines exert several actions in metabolic organs such as the skeletal muscle, liver, adipose tissue, and the vascular system, which in turn regulate metabolic homeostasis, immune-inflammatory responses and insulin sensitivity[136,138,139]. Because of these actions, together with adipokines' direct association with adiposity, as well as the sexual dimorphism that characterizes body composition in aging and obesity, several controversies have raised in the past years regarding their beneficial/detrimental roles. This controversy has gained special attention with regard to the

development of aging- and obesity-associated metabolic, cardiovascular and musculoskeletal diseases[140]. The following subsections will explain the most relevant roles of the main adipokines in aging, obesity, and in women, which are summarized in **Table 1**.

2.4.1. Adiponectin

Adiponectin is the most abundant adipokine circulating in human serum[71,138] and is mainly produced in the adipose tissue, in which it also exerts local actions[141]. Adiponectin is the main antiinflammatory adipokine, and it has the ability to inhibit the synthesis of proinflammatory cytokines like IL-6, IL-18, and TNF-α by blocking nuclear factor-kappa B (NF-κB) activation[142,143]. Adiponectin also reduces macrophage infiltration[144] and promotes their polarization towards an anti-inflammatory phenotype[145], together with an induction of apoptotic cells removal[146]. Adiponectin also promotes hyperplasia in WAT, and upregulates fatty acid accumulation as triglycerides[144]. At the systemic level, adiponectin ameliorates insulin resistance and dyslipidemia in obesity[148,149], as well as protects against cardiovascular disease[150,151]. These effects are achieved by directly targeting glucose uptake in the liver, muscle and adipose tissue[152], and by regulating the muscle tone of the vascular system[153], respectively.

According to its beneficial effects, the levels of adiponectin decline with increasing BMI, while they increase with insulin sensitivity and with scWAT/vWAT ratio[154–156]. High adiponectin concentrations are associated with the metabolically healthy obese phenotype[157] and with lower risk of type 2 diabetes[158]. In turn, serum adiponectin raises after pharmacological and surgical reduction of adiposity in obese subjects[159,160].

Altogether, these findings led to hypothesize that the higher circulating levels of adiponectin found with aging and in centenarians could be due to an association with prolonged longevity[161]. However, there is an adiponectin paradox in the elderly, as it has also been associated with reduced physical functioning[161] and with cardiovascular mortality[162] in this population. Apparently, the controversy appears in subjects diagnosed with cardiovascular disease, in which the beneficial effects of higher levels of circulating adiponectin depends on the duration of the cardiovascular disease[163].

On the other hand, serum adiponectin levels are higher in women than in men[154]. Importantly, the sex-differences in high circulating adiponectin are also apparent when it comes to the mentioned adiponectin paradox. Thus, the described negative association between high circulating adiponectin and cardiovascular disease was more sizable in men compared to women[163]. Importantly, this negative association also displayed a sexual dimorphism in type 2 diabetic subjects, being it significant for men but not for women[164]. In fact, obese postmenopausal women with high circulating adiponectin show lower risk for newly onset hypertension, metabolic syndrome, as well as higher serum markers of bone formation[165–167]. By contrast, being overweight after menopause worsens the drop in adiponectin, and this favors the appearance of insulin resistance[168]. Altogether, these data point to a beneficial role of adiponectin in obesity and in aging. However, the context of its increased circulating levels seems to mediate the beneficial actions exerted by this adipokine.

2.4.2. Leptin

Leptin was the first discovered adipokine[169], known for its essential roles in regulating the homeostasis of satiety, appetite, and energy expenditure at the hypothalamus. Indeed, congenital leptin deficiency leads to hyperphagia and severe obesity both in rodents and humans[170]. Leptin is produced mainly by scWAT[171] and

circulates in blood in proportion to total fat mass[170]. In fact, leptin action directly depends on adipose tissue mass. Thus, parallel declines in adiposity and circulating leptin results in an increased appetite, which in turn increases feeding to restore adipose tissue lipid levels and circulating leptin, that finally acts to diminish appetite[170].

However, obesity and aging are states in which higher circulating leptin levels fail to reduce appetite and increase energy expenditure, in a so-called leptin resistant state that has been observed in obese mice[170,172] and humans[173,174] , as well as in aged rodents[175,176]. Interestingly, it has been described that the effects of leptin on the sympathetic nervous system also involve the regulation of blood flow to several metabolic organs, including BAT. Hence, leptin resistance has been suggested to play a role also in the loss of BAT activity during aging in mice, due to a decreased irrigation to this adipose tissue[177].

In general, leptin acts in several organs (liver, vascular and immune system) to increase inflammatory factors, activate immune cells, and polarize them towards a proinflammatory phenotype. Therefore, leptin also correlates positively with insulin resistance[178,179]. However, aging subjects categorized in the quartile showing the lowest circulating leptin show an increased risk for all-cause mortality. Importantly, this association was observed in lean and in obese subjects. Thus, this result highlights the role of this adipokine in the maintenance of physiological functions in aging[177].

With regard to women, the circulating levels of leptin are higher than those observed in men, due to higher fat mass percentages but also to higher synthesis rates, since androgens exert suppressive effects on leptin synthesis[180,181]. Interestingly, age directly influences leptin levels in women, in which it induces an increment in circulating leptin that is independent from adiposity[182]. However, whether this increment is due to a compensatory mechanism to stop leptin resistance, or if it acts because of an augmented adiposity to increase the proinflammatory state, is yet to be unraveled.

2.4.3. Chemerin

Chemerin is a hormone secreted by adipose tissue that activates the chemokine-like receptor-1, also known as chemerin receptor 23 (ChemR23), to initiate innate and adaptive immune responses, and acts as a chemoattractant for the immune cells that express its receptor[183]. Chemerin circulating levels correlate with BMI and age, and may be an important link between excess adiposity and type 2 diabetes, since it promotes the secretion of adipokines that induce insulin resistance[184,185].

In fact, obese adipocytes secrete more chemerin than lean adipocytes[186], and chemerin secretion is induced by the proinflammatory cytokine TNF-α[187]. In older obese adults, weight loss induced by exercise lowers chemerin together with biomarkers of cardiometabolic risk, while improves insulin secretion[188]. Moreover, the inflammatory effects of chemerin exert direct detrimental actions on the advance of disc degeneration disease in older obese adults, in which chemerin is also correlated to BMI and to adverse lipid profiles[189].

Importantly, chemerin also regulates the maintenance of adipogenesis and a healthy adipocyte metabolism[190]. Indeed, it has been described that the knockout (KO) mice for the receptor ChemR23 develop spontaneous obesity. Interestingly, obesity was not exacerbated by a high-fat diet (HFD) in this mice[191], demonstrating its role in adipose tissue homeostasis. Conversely, a protective effect against adiposity was reported by another study in ChemR23 KO mice. However, HFD-fed ChemR23 KO mice showed a better glucose tolerance compared to their wild type counterparts when submitted to a glucose tolerance test[192]. These findings indicate that chemerin is involved in a complex regulation of adipogenesis and systemic

glucose homeostasis, exerting beneficial actions even under obesogenic conditions. This complex regulation may explain some unexpected associations described in the literature, such as the drop in circulating chemerin levels observed in type 2 diabetic men[193].

In the context of sex differences, it is worth mentioning that the explained inverse association between chemerin and type 2 diabetes was not observed in women[193]. Moreover, *CHEMERIN* gene expression has been described to be higher in scWAT than vWAT of obese women but not men. Interestingly, while its mRNA levels appeared increased in scWAT, they negatively correlated to serum levels[194]. Altogether, these results shed light on the fact that the controversy of chemerin actions is further aggravated by sex differences, since none of these observations were observed in men. Thus, a plausible sexual dimorphism in the controversial effects of chemerin in obesity and/or aging, cannot be discarded.

2.4.4. Interleukin-6 (IL-6)

IL-6 is a proinflammatory cytokine produced by several organs. In obese states, the adipose tissues largely contribute to its circulating form, producing up to 35% of its circulating levels[195,196]. In fact, IL-6 synthesis and circulating levels augment in parallel with increases in adiposity, glucose intolerance, and insulin resistance[188]. However, the effects of IL-6 on insulin signaling apparently depend on its differential actions in the main metabolic organs (liver *vs*. muscle), and also rely upon the different source of this interleukin (muscle *vs.* fat)[71].

Nevertheless, the adipose-derived IL-6 contributes to insulin resistance by means of impairing insulin sensitivity in liver[198]. Moreover, IL-6 signaling contributes to the accumulation of macrophages and glucose intolerance in scWAT from diet-induced obese (DIO) mice[199]. Importantly, adipose tissue has been established as the major site of IL-6 production in aging[200]. As a matter of fact, IL-6 secretion is one hallmark of the secretory pattern that characterizes adipose tissue senescent cells[63]. Accordingly, in older overweight and obese adults, decreases in IL-6 are observed with dietary and physical activity interventions, occurring together with decreased leptin secretion[201]. This is of special relevance since the increase circulating IL-6 levels is also related to a decline in skeletal muscle strength, quality, function, and training-induced beneficial adaptations in the aging population[202].

However, it seems that whether IL-6 is elevated chronically or acutely, and how high the experimental IL-6 circulating concentrations are, make a crucial difference (reviewed by Cron, Allen and Febbraio[203]). Indeed, treatment with recombinant human IL-6 has been shown, in different studies, to improves insulin sensitivity, lipolysis, and fat oxidation in humans, while augmenting insulin-stimulated glucose uptake.[203] By contrast. IL-6 antibody results in an increase in blood glucose and lipid levels in rheumatoid arthritis patients[203]. These results are supported by the fact that several animal studies in IL-6 KO mice develop mature-onset obesity and, when fed a HFD, reveal worse glucose tolerance, insulin resistance and pancreatic insulin secretion than their wild type counterparts (reviewed by Pal, Febbraio and Whitham[204]). Importantly, IL-6 actions on glucose and insulin metabolism were found to occur due to IL-6 production and secretion from muscle after exercise[203]. Moreover, a recent clinical trial in obese adults has demonstrated that muscle-derived IL-6 induced by exercise is able to reduce visceral fat mass, since this effect was abolished in the presence of IL-6 receptor blockade[205]. Thus, IL-6 is also an attractive myokine to stimulate via exercise training to improve obesity and aging associated insulin resistance.

Table 1. Summary of the explained associations observed for adiponectin, leptin, chemerin and IL-6 in women, in aging and in obesity.

Adipokine	Study population	Results	Ref.
Adiponectin	Women	• Increased levels in women compared to men	154
		• Not related to cardiovascular disease, in lean or T2DM women	163,164
		• Associated to a decreased risk of hypertension, Metabolic Syndrome and Osteoporosis in postmenopausal obese women	165-167
		• Its drop is more likely to occur in overweight/obese postmenopausal women, associating to higher IR risk	168
	Aging	• High levels negatively relate to physical function and cardiovascular mortality	161,162
		• High levels are proposed to be associated with longevity	161
		• High levels associated to cardiovascular mortality in men with cardiovascular disease, in association to the duration of the disease	163
	Obesity	• Negatively associated to BMI, associated to a metabolically healthy obese phenotype, insulin sensitivity and scWAT/vWAT ratio	154-157
		• Associated to lower risk for T2DM	158
		• Increased levels after surgical/pharmacological treatment of obesity	159,160
Leptin	Women	• Increased levels in women compared to men	179
		• Higher synthesis rates/fat mass, since androgens inhibit its synthesis	180
		• Higher in aging women in an independent manner from adiposity	181
	Aging	• Leptin resistance state, described only in mice	175,176
		• Lowest levels associate to mortality, independently from obesity	178
		• Associated to decreased BAT activity due to the regulation of its irrigation	182
	Obesity	• Leptin resistance state	173,174
		• Correlates with IR	177,178
		• Leptin deficiency leads to the development of obesity	170
Chemerin	Women	• Higher chemerin levels in scWAT than vWAT, that appear negatively correlated to serum levels	194
		• Do not show the drop in chemerin levels in T2DM observed in men	193
	Aging	• In older obese adults, exercise-induced weight loss lowers chemerin and cardiometabolic risk biomarkers, improving insulin secretion	188
		• In older obese adults, chemerin promotes the advance of disc degeneration disease, and is correlated to BMI and proatherogenic lipid profiles	189
	Obesity	• Increased secretion in obese adipocytes, promoted by TNF-α, induces IR	184-187
		• KO mice for its receptor (ChemR23) show lower adiposity levels than WT under HFD, but show worse glucose tolerance	192
		• KO mice for its receptor (ChemR23) develop spontaneous obesity	191
IL-6	Women	• Lower levels of IL-6 than men, also in aging	206,207
		• Higher IL-6 levels are more associated to T2DM in women than in men	208
	Aging	• The major site of IL-6 production in aging is adipose tissue	200
		• Hallmark of senescent cells secretory pattern in adipose tissue	63
		• Related to lower muscle strength, quality, function, and training effects	202
		• Decreases after dietary/exercise-induced weight loss in overweight and obesity	201
	Obesity	• Adipose-derived IL-6 contributes to IR	198
		• Contributes to accumulation of macrophages in scWAT in obese mice	199
		• IL-6 KO mice develop obesity, and when fed a HFD show worse glucose tolerance, IR and insulin secretion than WT	204
		• Muscle-derived IL-6 reduces visceral fat	205

Note. T2DM: Type 2 Diabetes Mellitus; IR: Insulin Resistance; scWAT/vWAT: Subcutaneous/Visceral White Adipose Tissue; TNF-α: Tumor Necrosis Factor alpha; KO: Knock-out; IL-6: Interleukin-6; ChemR23: Chemerin receptor; WT: Wild Type; HFD: High-Fat Diet.

IL-6 serum concentrations are higher in males than in females in all BMI groups[206], and higher IL-6 levels appear as age advances only in men[207]. However, IL-6 levels are more strongly associated with type 2 diabetes in women, suggesting that IL-6 mediated inflammatory processes might be of particular significance in the pathogenesis of type 2 diabetes in women[208]. Therefore, more increasing muscle-derived IL-6 and decreasing the production in adipose tissue may be an interesting strategy to ameliorate insulin resistance and systemic inflammation in middle-aged and aging overweight/obese women.

3. *n*-3 PUFA supplementation: from adipose tissue biology to systemic effects

In the background of the proinflammatory micro and macroenvironment that occurs in obesity and in aging, lifestyle interventions aiming to reduce such persisting inflammation have become of public interest in the last years. Among them, dietary *omega*-3 polyunsaturated fatty acids (*n*-3 PUFA)[209] have been broadly studied.

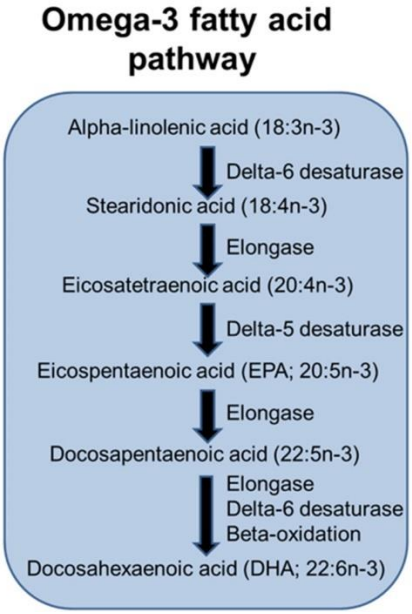

Figure 6. Pathway of *omega*-3 long-chain polyunsaturated fatty acid (*n*-3 PUFA) biosynthesis from essential fatty acid precursors. Figure from Miles *et al.*[210].

Amongst *n*-3 PUFA, docosahexaenoic acid (DHA, 22:6 *n*-3) and eicosapentaenoic acid (EPA, 20:5 *n*-3) are PUFA with potent antiinflammatory properties, that can be synthesized endogenously from its precursor α-linolenic acid (**Figure 6**). However, α-linolenic acid is an essential fatty acid that cannot be synthesized endogenously, due to the lack of the rate-limiting enzyme for the synthesis of its precursor, in humans and animals. Therefore, circulating levels of α-linolenic acid can only be achieved by dietary intake of its sources, namely green-leaf vegetables and nuts. However, the conversion rates of α-linolenic acid to EPA and DHA are extremely low in adult humans (reviewed by Calder *et al.*[211]). Hence, since DHA and EPA are from marine origin, and thus are naturally present in oily fishes, the consumption of fatty fishes is widely recommended in association to its beneficial antiinflammatory effects by several European and US health organizations[212–216].

Importantly, *n*-3 PUFA supplementation has been demonstrated to exert beneficial actions on weight excess and aging-associated comorbidities. In this sense, the *n*-3 status, which is the index of erythrocyte *n*-3 fatty acid content[217], is inversely associated with obesity and waist circumference in overweight and obese subjects[132]. Amongst the two marine origin *n*-3 PUFA, the erythrocyte content in DHA, but not in EPA, has been described to be inversely associated with the development of obesity-related metabolic comorbidities in the general population, including subjects diagnosed with metabolic syndrome[218].

Regarding the female population, it is worth mentioning that the inverse association between the *n*-3 status and obesity/waist circumference in older subjects is present only in women, but not in men[219]. Importantly, estrogens-dependent mechanisms for the synthesis of DHA have been suggested in women, thus mediating the higher levels of plasma DHA observed in women compared to men[220]. However, these mechanisms are active in pre-menopausal women, and accordingly, plasma DHA levels are lower in post than pre-menopausal women[221]. Most importantly, plasma DHA is also lower among postmenopausal women with cardiovascular disease and diabetes mellitus than in the healthy ones[221]. Moreover, plasma DHA levels have been inversely associated with all-cause mortality in older women[222].

In this context, it has been suggested that the current *n*-3 PUFA intake observed in young women may not be effective for achieving the needed *n*-3 status among those with obesity[223]. Considering the described observations, this ineffective *n*-3 PUFA intake might be more pronounced in middle-aged women, a population that would, in turn, highly benefit from an adequate *n*-3 PUFA intake.

3.1. *n-3* PUFA effects on adipose tissue

The beneficial effects of *n-3* PUFA on the prevention of obesity-related metabolic disorders occur through their actions on several metabolic organs, including the adipose tissue[224]. However, these actions depend on the adipose cell type and therefore on the fat depot[132], and will be explained in the following subsections.

3.1.1. n-3 PUFA effects on white and beige adipose cells

Growing evidence shows that the beneficial effects of *n-3* PUFA on obesity and associated comorbidities are mediated by inducing a recovery of adipose tissue functionality and antiinflammatory status (summarized in **Figure 7**)[70]. Since adipose tissue plasticity is impaired in obesity, several studies in rodent models of obesity supplemented with *n-3* PUFA have been conducted, revealing that EPA and DHA are able to modulate both lipogenic and lipolytic genes in WAT. Indeed, fatty acid synthase (*Fasn*) gene expression was reduced in WAT when *n-3* PUFA were administered, decreasing lipogenesis and fat accumulation[225]. Furthermore, downregulation of main lipolytic genes *Hsl* and *Atgl* in obese WAT was reversed[226]. Regarding glucose metabolism, several investigations have revealed that *n-3* PUFA also have insulin-sensitizing properties on adipose tissue in animal models of obesity-induced insulin resistance. Although the underlying mechanisms are not completely understood, an increase in *Glut4* mRNA, together with an improve in the insulin response and signal transduction mediated by an increase in insulin receptors have been reported[227,228].

Furthermore, current evidence in rodents shows that reduction of obesity and visceral fat deposition after *n-3* PUFA administration is not mediated by a reduction in food intake, but probably by stimulating adaptive thermogenesis in BAT (see the following subsection *3.1.2*) and oxidative metabolism in WAT[229]. Such evidence implies that *n-3* PUFA are able to increase mitochondrial biogenesis in WAT[230–232]. Therefore, *in*

vivo supplementation with *n-3* PUFA upregulated transcription factors for mitochondrial biogenesis *Pgc1a* and *Nrf1* in white fat depots from obese rodents, as well as genes encoding mitochondrial proteins involved in oxidative phosphorylation. Moreover, *n*-3 PUFA have been shown to upregulate *Cpt1a,* and therefore free fatty acids oxidation, in epididymal WAT[230,232]. Nevertheless, the induction of a more oxidative phenotype in WAT is not limited to the mitochondria, since also *Acox1* (peroxisomal fatty acid oxidation) has been found to be upregulated after n.3 PUFA supplementation in obese mice[232]. These effects on glucose and fatty acid metabolism have been proposed to be mediated by the AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptors (PPARs) (reviewed by Martínez-Fernández et al.[224])

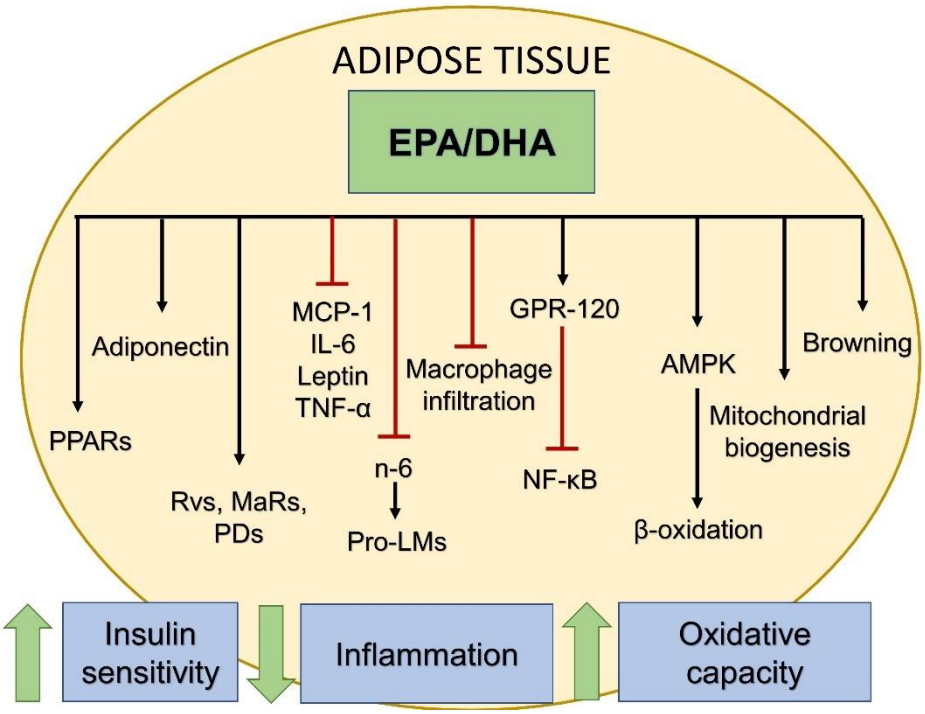

Figure 7. Summary of the beneficial effects of omega-3 polyunsaturated fatty acids EPA and DHA on adipose tissue in obesity, adapted from Martínez-Fernández *et al.*[224]. PPARs: Peroxisome Proliferator-Activated Receptors; Rvs: Resolvins; MaRs: Maresins; PDs: Protectins; MCP-1: Monocyte Chemoattractant Protein 1; IL-6: Interleukin-6; Pro-LMs: Proinflammatory Lipid Mediators; GPR120: G-coupled Protein Receptor 120; AMPK: AMP-Activated Protein Kinase; NF-κB: Nuclear Factor kappa B.

The ability of *n-3* PUFA to induce UCP1 in WAT, however, remains controversial[233]. It has been suggested that *n-3* PUFA could stimulate mitochondrial oxidative capacity in white fat from obese mice independently of UCP1[230,231]. However, a study revealed that *in vivo* EPA administration induced genes for mitochondrial biogenesis, and increased *Ucp1*, *Ucp2*, *Ucp3* and *Cidea* (a brown-specific protein) during the differentiation of adipocytes. Since these effects were not observed when EPA was given to mature adipocytes, this finding suggests that EPA could induce inguinal fat browning during differentiation[234]. However, a study of our group in subcutaneous mature adipocytes from overweight/obese subjects, revealed that EPA downregulated lipogenic genes and upregulated those involved in free fatty acids oxidation and mitochondrial biogenesis[235]. Moreover, EPA also upregulated the mRNA levels of *PRDM16*, a main factor required for the browning of scWAT, together with the beige adipocytes specific genes *TBX1* and *CD137*[235], suggesting that the appearance of beige adipocytes can also occur *via* transdifferentiation of mature fat cells.

In addition to the actions previously described, the beneficial metabolic effects of *n-3* PUFA on obesity could rely on their ability to reduce adipose tissue inflammation, both in humans and rodents, by modulating the secretion of adipokines and the infiltration of immune cells[236,237]. Indeed, the decrease in adiponectin that occurs in obesity has been shown to be reversed in rodents[227,238,239] and humans[240,241] after *n-3* PUFA supplementation. In terms of *n-3* PUFA effects on the secretion of proinflammatory adipokines, the vast majority of studies have shown the ability of *n*-3 PUFA to downregulate proinflammatory cytokines (*Lep* -leptin-, *Ccl2* -MCP-1*, Resistin*, *Il6* and *Tnf* mRNA levels), in mice with dietary and genetic obesity[239,242,243]. Moreover, *n*-3 PUFA supplementation has shown to promote a reduction in macrophage infiltration and/or to a polarization towards an M2 antiinflammatory phenotype in WAT of obese mice[244–246]. Furthermore, the antiinflammatory effects of *n-3* PUFA on adipose tissue have been shown to be mediated by the G-coupled protein receptor 120 (GPR120), which exerts potent antiinflamatory and insulin sensitizing actions in the obese WAT[247].On the other hand, other mechanism by which *n*-3 PUFA exert these antiinflammatory actions include an increased production of specialized proresolving lipid mediators (SPMs, further explained in section 3.1.3) while inhibiting the production of *omega*-6 (*n*-6) derived proinflammatory lipid mediators[248], even in obese mice[227].

In general, current evidence indicates that *n*-3 PUFA are able to induce a more insulin sensitive, oxidative, thermogenic and antiinflammatory profile in WAT even in the context of obesity. However, few studies have investigated these effects in aging and in obesity. Amongst them, Luo *et al*. revealed that DHA is able to reduce inflammation and angiogenesis in WAT of 14 months old DIO mice in a SIRT-1 dependent mechanism[249]. However, further studies are needed to confirm if the explained actions on WAT fatty acid and glucose metabolism, as well as on its ability to undergo browning, are also evident in aging.

3.1.2. n-3 PUFA effects on brown adipose tissue

Compared to WAT, there is less evidence regarding the effects of *n-3* PUFA supplementation on BAT[133]*.* However, studies in rodents have shown that the anti-obesity properties of *n-3* PUFA could be also mediated by stimulating adaptive thermogenesis in BAT[233,250], which has been proposed to occur both with and without induction of UCP1[250,251].

Hence, Takahashi and Ide[250] described that dietary *n-3* PUFA supplementation did not increase BAT mass, but upregulated *Ucp1* mRNA levels. More recently, an increase in UCP1 protein content was found by Crescenzo *et al*.[252] in rats refed with a HFD + *n-3* PUFA rich oil after 2 weeks of semi-starvation. Moreover, several studies have described that, after dietary supplementation with *n-3* PUFA, mice showed an increase of energy expenditure, oxygen consumption, and rectal temperature accompanied by mitochondrial increase of UCP1 and/or citrate synthase mRNA and protein content[253]. Furthermore, several markers associated with thermogenic function were upregulated by *n-3* PUFA supplementation in these studies, including mRNA and/or protein levels of *Ucp1*, *Pgc1a*, *Cpt1b*, *Cidea*, *Prdm16*, *Fgf21*, *β3AR*, *Gpr120*, *Ppara*, and *Pparg* both in lean and DIO animals with different genetic backgrounds[254–257]*.*

Therefore*,* several mechanisms have been proposed to explain the signaling pathways that led to these thermogenic effects of *n-3* PUFA. Thus, an *n-3* PUFA-enriched HFD promotes an increase in BAT mass and activity possibly caused by increased FGF21 levels, which increases the p-AMPK/AMPK ratio[253]. The binding of *n-3* PUFA to its receptor GPR120 has been proposed to mediate *n-3* PUFA effects on the adipocytes' thermogenic program[131,258]. However, Bjursell *et al*.[254], reported that *n-3* PUFA exhibited similar

effects on BAT mass, energy expenditure, respiratory exchange ratio, and core body temperature in WT and *Gpr120* KO mice fed a supplemented HFD. These findings suggest that other receptors/mechanisms could contribute to the beneficial effects of *n-3* PUFA apart from GPR120. In this context, it has been proposed that sympathetic activation mediates the effects on *n-3* PUFA on BAT and iWAT activation[257]. Interestingly, this study also proposed that these *n-3* PUFA effects occurred *via* TRPV1 (Transient Receptor Potential Vanilloid 1), as TRPV1 KO mice did not show the enhanced oxygen consumption, *Ucp1* or *β3AR* upregulation observed in BAT and iWAT of their wild type littermates[257]. The need for β3 adrenergic stimuli to observe beneficial effects of n3-PUFA was also supported by the recent investigations of Ghandour *et al*.[259].

Apart from few exceptions, most studies agree that *n-3* PUFA increase UCP1 content in BAT of lean and of DIO animals[133]. However, studies carried out in *Ucp1* KO mice showed that the beneficial effects of *n-3* PUFA supplementation can be partly achieved independently of UCP1 activity. In this sense, Pahlavani *et al*.[260] showed that, in the absence of UCP1, EPA-enriched fish oil mediates an increase of *Pgc1a*, oxygen consumption, and mitochondrial DNA content. Similarly, Oliveira *et al*.[256] found that fish oil supplementation protects from obesity and glucose intolerance both in wild type and *Ucp1* KO mice. These effects were attained by increasing energy expenditure, although the mechanisms involved remains unknown.

Regarding *n-3* PUFA effects on BAT antiinflammation, the results are scarce. However, a recent study found that *n-3* PUFA supplementation upregulated UCP1 and genes involved in mitochondrial biogenesis in BAT. These actions were exerted in parallel to an increase in the number of M2 polarized macrophages, as well as AMPK activation and incremented FGF21 concentrations in BAT[253].

Finally, it is important to note that the association of BAT with *n-3* PUFA also goes the other way round. Regardless of the dietary approach used to induce obesity, the supplementation of *n-3* PUFA leads to higher incorporation in BAT of DHA than EPA[261,262]. This drives the establishment of a fatty acid profile that differentiates BAT from WAT[263]. In fact, brown adipocytes can synthesize *de novo* DHA in vitro[264]. These findings, taken together with the ability of BAT to secrete fatty acids, hormones and growth factors[265], are especially interesting given that plasma DHA and EPA are increased after BAT activation by cold stimuli in humans[266]. On the other hand, basal plasma concentrations of DHA and EPA (prior cold-stimulation of BAT) are also associated to BAT activity[266], reinforcing the fact that BAT activity and marine *n*-3 PUFA concentrations influence each other.

In summary, the mentioned investigations reveal that *n-3* PUFA are key regulators of beige and brown adipogenesis and thermogenic function that could mediate the beneficial actions of *n-3* PUFA supplementation. Further studies are needed to better characterize the link between these thermogenic properties with its anti-inflammatory/immunomodulatory effects in BAT, and to characterize the potential role of *n-3* PUFA derived lipid mediators.

3.1.3. A role for n-3 PUFA derived specialized proresolving lipid mediators in adipose tissue function

Specialized proresolving lipid mediators (SPMs) are PUFA-derived bioactive lipid mediators that can resolve the inflammation at concentrations much lower (range of μg/μL) than those needed for *n-3* PUFA to exert such antiinflammatory actions (range of mg/μL). Hence, it was recently discovered that EPA and DHA can be converted into these SPMs, named resolvins (E-series and D-series Rvs), maresins (MaRs) and

protectins (PDs), by cyclooxygenase and lipoxygenase enzymes (COX and LOX), present in several tissues including mouse and human WAT (reviewed in Spite *et al*.[267]). The main SPMs present in adipose tissue, as well as their precursors and pathways, are summarized in the **Figure 8**.

With the identification of these proresolving mediators, the evidence that resolution of inflammation is an active programmed response that is "turned on", instead of being a passive process of diluting chemoattractant gradients, was provided by the investigations conducted by Prof. Serhan's research group[268]. This discovery opened the investigation field to elucidate SPMs actions to resolve inflammation in several chronic inflammatory diseases including obesity, since they are able to reduce the inflammatory milieu in several WAT depots with concomitant reductions in systemic inflammation and the consequent improvement in insulin sensitivity[269].

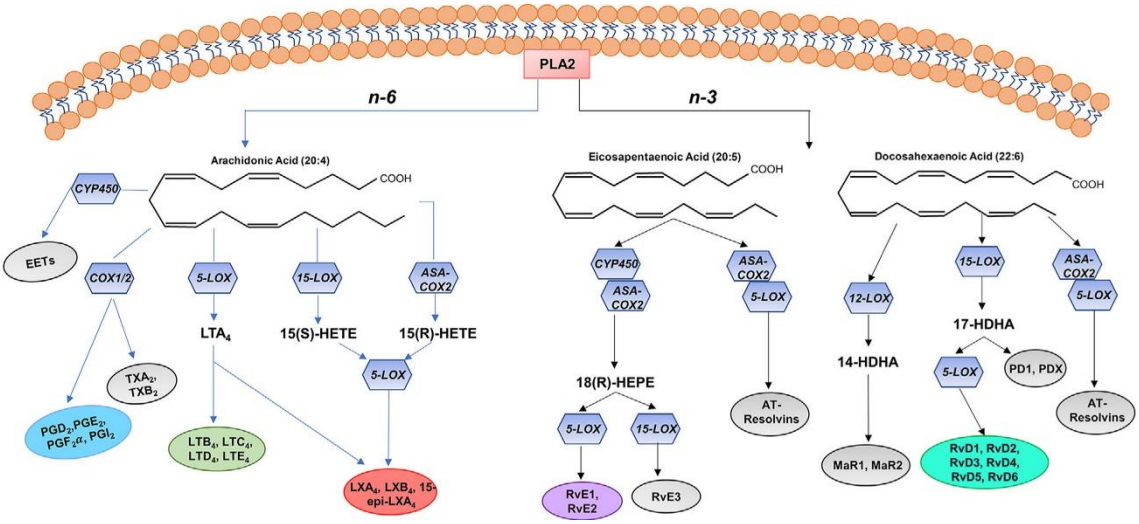

Figure 8. Principal specialized proresolving lipid mediators (SPMs) are derived from PUFA, both *n*-3 EPA and DHA as well as *n*-6 arachidonic acid (AA), and are produced mainly by oxidative reactions by several enzymes of the cyclooxygenase (COX) and lipoxygenase (LOX) families. Figure from Crouch, Al-Shaer and Shaikh[270]. EETs: Epoxyeicosatrienoic acids; PGs: Prostaglandins; TXs: Thromboxanes; LTs: Leukotrienes; HETEs: Hydroxyeicosatetraenoic acids; LXs: Lipoxins; HEPEs: Hydroxyeicosapentaenoic acids; Rvs: Resolvins; HDHAs: Hydroxy-docosahexaenoic acids; MaRs: Maresins; PDs: Protectins.

However, both aging and obesity lead to a reduction in SPMs production in WAT. It has been reported that mice consuming a HFD show a decrease in several SPMs (Protectin D1, PD1) and precursors (17-HDHA) levels in the gonadal WAT after 4 and 14 days of high fat feeding[271]. In addition, also the *n*-3 derived SPM Resolvin D1 (RvD1) and SPMs precursors (18-HEPE, 14-HDHA) were decreased when the HFD was prolonged for 18 weeks[271]. These results highlight that the reduced production of *n*-3 PUFA derived SPMs is worsened when high-fat feeding is prolonged in the long term. Moreover, aging *per se* increases the time needed for the process of resolution of inflammation to be effectively completed[272,273]. Importantly, SPMs were found in several human adipose depots, including the subcutaneous and the perivascular fat depots[269]. In fact, there is also evidence that SPMs are lower in obese subjects, including primary adipocytes from human obese scWAT, and leukocytes derived from obese subjects[274,275].

Furthermore, the obese WAT could be facilitating the conversion of SPMs to inactive oxidized metabolites (oxo-resolvins) or PUFA into inactive diols, which do not allow for SPMs conversion[276]. Thus, it is likely that

the decrease in SPMs concentrations contributes to the inflammation that takes place in obesity and in aging. Importantly, there is already evidence suggesting sex-differences in the loss of SPMs and their precursors in obesity, as obese male but not female mice display lower levels of splenic DHA-derived PD1 and SPMs precursors 17-HDHA and 14-HDHA[277].

Conversely, *n*-3 PUFA derived SPMs can counteract the subacute chronic inflammation that occurs in obesity[227,245,246,278]. Not only on inflammation, but as a consequence, *n*-3 PUFA derived lipid mediators have demonstrated their actions on improving insulin sensitivity and glucose homeostasis in obese mice[227,271,279]. There is evidence of Resolvin D1 (RvD1) promoting the switch in macrophage polarization towards an M2-like phenotype and nonphlogistic phagocytosis in WAT of obese mice[245]. Similarly, Maresin 1 (MaR1) promotes a reduction in the infiltration of macrophages to WAT of obese mice, together with lower markers of M1 proinflammatory macrophages and proinflammatory genes (*Cd11c, Tnf, Il1b)* and increased antiinflammatory *Il10*[246]. In fact, MaR1 has also been shown its effectiveness in ameliorating liver steatosis in obese mice[280–282]. Likewise, Resolvin E1 (RvE1) induces the expression of glucose transporters and insulin signaling genes in adipose tissue of *ob*/*ob* mice, in parallel with an increase in *Adipoq* and a decrease in liver F4/80 staining[227]. Moreover, SPMs beneficial actions on insulin sensitivity also involve positive regulations of the myokine-liver IL-6 glucoregulatory axis by Protectin DX (PDX)[283].

Animal studies have revealed that dietary marine origin *n*-3 PUFA supplementation promote an increment of the synthesis of *n*-3 PUFA–derived SPMs and their precursors in adipose tissue of obese mice[246,271,284]. Also *n*-3 PUFA supplementation is able to raise SPMs levels in humans in plasma and in adipose tissue among others[285]. Interestingly, *n*-3 PUFA supplementation significantly increased the production of some *n*-3 PUFA-derived SPMs, including RvE1, 17-HDHA, PD1, and RvD1 in vWAT, in parallel with the reduction of local and systemic inflammation, in severely obese-nondiabetic patients [286]. Increments of 600% have been reported in the DHA, EPA and arachidonic acid (AA)-derived lipid mediators concentrations, including 17-HDHA and 18-HEPE (RvE1 precursor), in plasma of subjects supplemented with DHA[287]. Moreover, this increment occurred in a time-dependent manner over the course of 12 weeks[287]. Importantly, these results can be achieved also in a dose-dependent manner when EPA and DHA supplementation are given in doses similar to 1, 2 or 4 portions of oily fish per week, underscoring the potential of this therapeutic approach to be translated to the clinical practice[288].

No study has investigated the profile of BAT SPMs, under lean or obesogenic conditions, during aging. However, recent studies have revealed the role of BAT as a secretory organ of bioactive *n*-3 PUFA-derived metabolites including several SPMs precursors, both in rodents and in humans[289]. Hence, an increase in BAT secretion of 12-LOX enzyme-derived 12-HEPE, 14-HDHA, 12-HETE was observed after cold stimuli, occurring together with an increased glucose uptake and metabolism in BAT and skeletal muscle[84]. Similarly, humans with active BAT revealed an increase in 12-HEPE and other *n-3* PUFA derived lipid mediators in blood after cold stimuli, in parallel to an increase in energy expenditure[289]. Moreover, BAT profiling of oxylipins under conditions of thermoneutrality revealed that some of the SPMs precursors are amongst the most abundant lipid mediators contributing to differentiate BAT lipid profile compared to that of WAT[290]. Altogether, these findings point to the fact that SPMs may also play a relevant role in BAT function and metabolism that is yet to be elucidated.

Finally, the *n*-6 PUFA AA is also a substrate to produce SPMs. Hence, despite this classically proinflammatory PUFA was believed to be converted only to proinflammatory lipid mediators, it also produced SPMs termed Lipoxins (LXs). In obesity, vWAT from obese patients shows an upregulation of both

AA-derived proinflammatory lipid mediators and LXs. However, the ratio of LXs to proinflammatory lipid mediators is significantly reduced[291], thus revealing an increased production of proinflammatory than antiinflammatory AA-derived lipid mediators in obesity. Moreover, obese mice show an increase in Leukotriene B_4 (LTB_4) levels in several tissues including the vWAT, in which LTB_4 acts to promote inflammation. Conversely, mice lacking the LTB_4 receptor show a reduced inflammation in adipose tissue and a protection against insulin resistance[292,293]. On the other hand, *n*-3 PUFA supplementation leads to a reduction in AA-derived proinflammatory eicosanoids in BAT when it is stimulated with a β3 agonist[259], revealing a role for these lipid mediators in BAT inflammatory status. Unexpectedly, some AA-derived proinflammatory prostaglandins have demonstrated to induce browning in WAT[294,295], together with a reduction in its inflammatory status[296]. Interestingly, similar browning and antiinflammatory properties have been described for AA-derived LXs in mice[297]. Hence, further studies aiming to identify the whole lipid mediator signature and function, including the AA-derived ones, in the different adipose depots are needed to fully understand the role of SPMs in obesity- and aging-associated inflammation and metabolic disorders.

3.2. Systemic effects in obesity and metabolic syndrome

Despite effects on adipocyte and adipose tissue function have been described, the effects for *n-3* PUFA on regulating body weight and body composition are not clear. Some studies have described associations between plasma DHA and lower risk for central obesity[298] and metabolic syndrome[218] in older subjects. It is likely that these associations are mediated by the effects of *n*-3 PUFA supplementation on reducing abdominal obesity measured by waist circumference, as revealed by previous meta-analyses on lean, overweight and obese subjects[299,300]. Nevertheless, these studies have also revealed no significant effects on fat mass or lean mass for *n*-3 PUFA supplemented subjects compared to the placebo supplemented ones[299,300]. Moreover, there are no studies showing body weight lowering effects for *n-3* PUFA administration in obese middle-aged or aged populations.

Similarly*, n*-3 PUFA effects on glucose uptake and insulin resistance are still under review, and it seems that they effects are dependent on the experimental approach and the targeted population. Indeed, n-3 PUFA supplementation have been described to induce improvements in glucose metabolism when the supplementation was accompanied by weight loss programs[301], without effects for *n*-3 PUFA alone. Moreover, a meta-analysis has found that *n*-3 PUFA exert lowering effects on the levels of fasting insulin and on insulin resistance in type 2 diabetic patients when the treatment is prolonged for at least 8 weeks[302]. However, when mixing diabetic and non-diabetic subjects, these effects are limited to a reduction in the HOMA-IR index, without improvements in other indexes/parameters of insulin sensitivity/resistance or an increase in glucose tolerance, not even when population subgroups (diabetic *vs*. non-diabetic) are compared[303].

Regarding their lipid lowering effects, *n*-3 PUFA have well-defined effects on lowering blood triglycerides in a dose-dependent manner on hypertrigliceridemic and healthy subjects[301]. Indeed, some studies have stated that the increased consumption of fish oil is the one related to lower total-cholesterol and LDL-cholesterol, with larger LDL-cholesterol size[304]. Nevertheless, the dose-dependent effect of *n*-3 PUFA supplementation are not limited to triglycerides, but also to blood pressure[305]. In fact, most of the studies investigating the effects of *n*-3 PUFA supplementation have demonstrated a myriad of actions on improving the lipid profile and on regulating blood pressure, that has led to their recommendation for concomitant treatment of cardiovascular disease, hyperlipidaemia and diabetes by the main health organizations for these conditions,

the American Diabetes Association and the American Heart Association[212,306]. However, the controversy of *n*-3 PUFA supplementation in cardiovascular disease emerged in the 2000s[213] and continues nowadays, since the US Food and Drug Administration (FDA) agency recently re-estated that there is not sufficient evidence yet to establish that *n*-3 PUFA supplementation prevents coronary heart disease[214,215].

Importantly, both EPA and DHA have also revealed an ability to reduce the C-reactive protein (CRP), IL-6 and TNF-α in lean and obese, normoglycemic and type 2 diabetic subjects[307,308]. In this sense, the effects on lowering leptin and increasing adiponectin have been observed in non-obese subjects and type 2 diabetic subjects, indicating a plausible interaction between metabolic organs that affects the concentration of these two adipokines[309,310].

Interestingly, recent studies in humans have also looked at the possibility of differential effects of DHA and EPA supplementation. In this regard, the studies from Allaire *et al*. are of special interest since they point out higher effects for DHA on improving the cardiometabolic risk than EPA. In this way, a recent study highlighted that DHA, more than EPA, increases the proportion of beneficial large LDL-cholesterol particles[311–313]. Moreover, these studies revealed that DHA had greater effects than EPA on increasing the *n*-3 status, as well as circulating adiponectin and HDL-cholesterol, while lowering IL-18, triglycerides and the atherogenic total cholesterol/HDL-cholesterol ratio, in middle-aged adults with abdominal obesity[311,313]. Regarding their effects on blood pressure, the lowering actions of *n*-3 PUFA are also differential for EPA (systolic blood pressure) and DHA (diastolic blood pressure), but similar lowering effects were described on circulating CRP, IL-6 and TNF-α[311,314]. Nevertheless, the lack of quality evidence regarding *n*-3 PUFA effects on lowering markers of inflammation and cardiovascular health has been highlighted[315], and more studies are needed to establish if these effects can be warranted for the general population.

It is important to note that sex-differences also emerge in this area. For example, a study has described that *n*-3 PUFA supplementation induces an amelioration in insulin resistance in women, but not in men[316]. Moreover, the detrimental effects observed in some studies for increases in LDL-cholesterol after DHA supplementation have been observed in men, but not in women[311]. Moreover, it has also been shown that DHA and AA contribute to plasma PUFA in a higher proportion in women than in men[317].

Moreover, few studies have analyzed the effects of *n*-3 PUFA supplementation specifically in older subjects. A recent randomized trial conducted by Bischoff-Ferrari *et al.*[318] revealed no effects for 3 years of *n*-3 PUFA supplementation in aged subjects (above 70 years old) on triglycerides, blood pressure, cognitive/functional tests, or fracture incidence. Indeed, most of the studies conducted in this population are related to the prevention of frailty and cognitive decline and, in contrast to the mentioned study[318], have described positive effects for *n*-3 PUFA supplementation on limiting cognitive decline[319,320]. Nevertheless, anti-inflammatory effects have been described also in this population, with reduced IL-6, IL-1β, TNF-α and CRP in blood[321,322].

Overall, current evidence suggests that the actions of n-3 PUFA supplementation on the recovery of the function of adipose function, with concomitant improvements in the systemic lipid profile and inflammatory status, that appear to be more pronounced for DHA than EPA supplementation. However, further studies are needed to confirm these actions, and to unravel what are the effects on systemic insulin sensitivity

4. Exercise training to treat obesity and aging-associated inflammation

Exercise training is now widely recognized as a non-pharmacological strategy to prevent and treat metabolic diseases, including type 2 diabetes mellitus[323], rheumatoid diseases[324] and cancer cachexia[325]. Thus, health organizations have made statements for the use of physical exercise in the maintenance of mobility in aging adults[326], as well as for the prevention and treatment of several pathologies including overweight and obesity[327], and their associated metabolic complications such as cardiovascular disease[328] and diabetes mellitus[329,330].

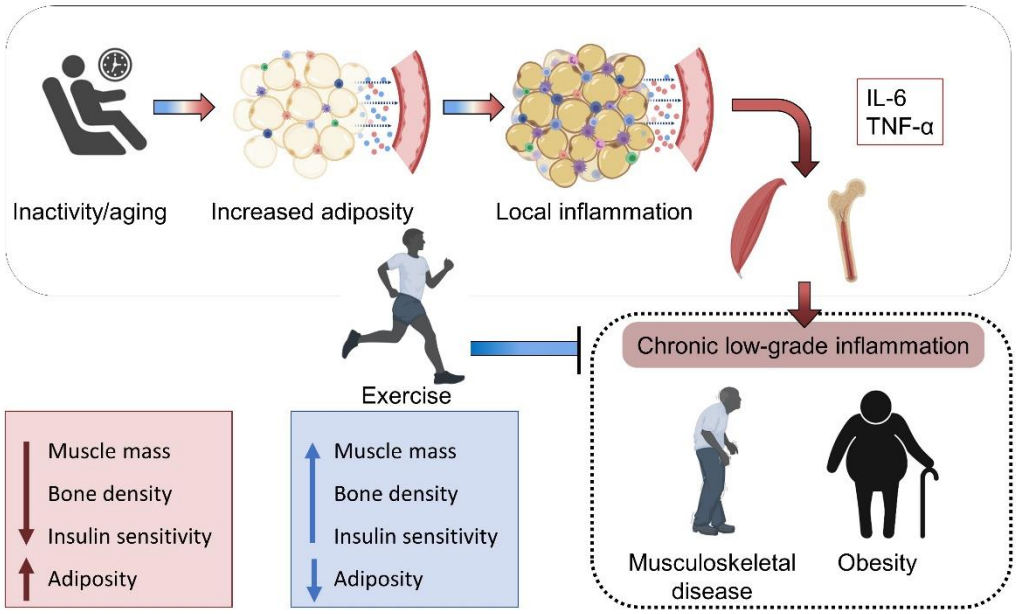

Figure 9. Summary of the effects of inactivity and aging on adipose tissue dysfunction and the derived systemic detrimental effects, as well as the potential actions of exercise to counteract them. Adapted from Kirk *et al.*[339]. IL-6: Interleukin-6; TNF-α: Tumor Necrosis Factor-alpha.

The direct measure of exercise training in relation to an individual's health status is physical fitness. Physical fitness is not a calculable score, but an abstract concept instead, that includes at least one cardiorespiratory, muscular, motor, morphological, and metabolic component[331]. In this regard, several studies have highlighted that aging women with higher levels of cardiorespiratory fitness and muscular strength show a lower incidence of insulin resistance, metabolic syndrome and cardiovascular disease[332–334]. On the other hand, other investigations have established that excessive fat is inversely related to a lower functional capacity in older subjects, mediating its association with frailty in older women, but not in men[335,336]. Moreover, lower self-reported physical functioning is inversely related to the appearance of advanced glycation end products, which are markers of chronic diseases in aging, only in women[337]. Indeed, a consistent "female disadvantage" in physical performance has been described among older adults, suggesting that preventive strategies aimed to enhance a healthy, active aging are specially necessary in women[338].

It is well-established that exercise-induced physical fitness is achieved thanks to specific adaptations to several organs (summarized in **Figure 9**) that result in whole-body metabolic improvements[340]. While exercise principal actions are exerted on the main metabolic tissues, the skeletal muscle[341] and the cardiovascular system[342], several studies have recently determined that exercise training also results in adaptations to adipose tissue that improve whole-body metabolism, also in overweight and obese subjects[343,344]. Importantly, these effects involve an increase in insulin sensitivity, in cardiorespiratory fitness, and a decrease in intra-abdominal adipose tissue[344]. Exercise beneficial actions include the downregulation of inflammatory pathways involving macrophage and adipokine transcripts (leptin and adiponectin) in WAT, that significantly correlate with their plasma concentrations[343]. Evidence of exercise-induced adaptations in aging subjects, as expected, is more associated to the prevention of frailty. However, recent studies have highlighted the role of exercise in improving the inflammatory pathways and macrophage infiltration in WAT of middle-aged and older aged individuals[345].

4.1. Effects of exercise on adipose tissue in obesity and aging

The effects of exercise in adipose tissue have been widely studied, also in animal models of obesity and aging during the recent years. Lately, research has become more specific, and focused also on which training type and what training periods are best to achieve beneficial metabolic outcomes in obesity and in aging[346–348]. In this sense, it is worth mentioning that recent studies have revealed that the actions of exercise training on adipose tissue biology are also depot specific[349], outlining the relevance of characterizing the distinct contributions of the adipose depots to the exercise-induced metabolic adaptations.

4.1.1. Effects on white adipocytes and beige induction

The first role of WAT under exercise stimuli was established to be fuel supply, reliant on WAT free fatty acids release to the blood stream from the stored triglyceride in white fat cells' lipid droplets[350]. However, nowadays it is known that exercise induces several metabolic adaptations to WAT in both rodents and humans, including the lipid and glucose metabolism, regulation of inflammatory pathways, mitochondrial biogenesis and thermogenesis, as well as endocrine adaptations[128,129]. These adaptations will be explained throughout this section and are summarized in **Figure 10**.

Regarding lipid metabolism, exercise has been shown to induce an increase in whole body lipolysis and fatty acid oxidation during exercise in DIO animals[351,352]. However, few studies have analyzed the effects of exercise on aged obese animals, and those who have mainly focused on vWAT due to its relation to worse metabolic phenotypes in aging[352–354]. Thus, studies investigating the effects of exercise in epidydimal WAT of aged obese animals, reported a partial recovery in fatty acid oxidation mediated by ACOX1 upregulation, and a marginal increase in phosphorylated monoglyceride lipase (MGL) but not in hormone-sensitive lipase (HSL)[353]. By contrast, scWAT and epidydimal WAT of exercised adult DIO mice displayed smaller adipocytes with increased HSL-mediated lipolysis[346]. However, depot-specific adaptations to exercise have been described in scWAT but not in vWAT depending on the duration of the training, namely when it was prolonged to the long-term[346]. Conversely, other studies have observed this depot-specificity only in vWAT, and not in scWAT, also in exercised adult DIO anials[355]. Furthermore, the increase in lipolysis has been reported to be dependent also on the type of exercise in adult DIO animals[346]. Moreover, studies in humans have suggested that these lipolytic actions occur only when long-term training periods are used (6

months)[356], while 3-12 weeks were not enough to induce significant changes in the lipid transcriptome of scWAT[357,358]. In summary, exercise-induced adaptions to lipid metabolism in scWAT of DIO animals require further investigations, both in the aging and in the obesity research fields.

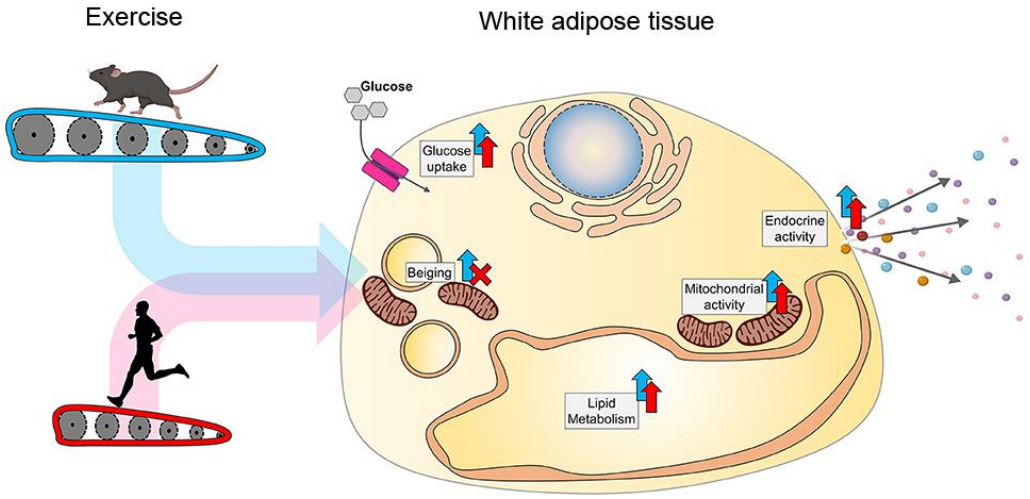

Figure 10. Exercise-induced adaptations to white adipose tissue in rodents and humans. Adapted from Vidal and Stanford[128].

With regard to the effects of exercise on glucose metabolism, it is known that exercise improves whole body glucose homeostasis in rodents and humans, partially by increasing the insulin sensitivity of scWAT[130], and by inducing genes and proteins related to glucose metabolism, both in scWAT and vWAT[349]. In humans, 6 months of exercise upregulates the expression of genes involved in glucose metabolism in lower-body scWAT[356]. The depot specificity also seems to affect these adaptations, since two weeks of exercise increased insulin-stimulated glucose uptake in lower-body scWAT, but not in upper-body scWAT or vWAT[359]. These data indicate that scWAT and vWAT also have distinct adaptations in glucose metabolism to exercise that affect scWAT modulation even depending on its location. Interestingly, humans with a higher proportion of upper-body WAT are more likely to show an impaired glucose tolerance, while humans with a higher proportion of lower-body WAT are associated with healthier glucose levels[360]. Therefore, this suggests that the plasticity of glucose metabolism adaptations to exercise in scWAT could be a target for exercise interventions in overweight and obesity. In fact, transplantation of scWAT from exercised mice to sedentary mice on a HFD improved glucose tolerance and insulin resistance on sedentary DIO mice[130]. This is also of particular relevance to aging, since the first alterations that appear in the aged scWAT are related to an impairment in the phosphorylation of insulin receptors and their downstream signaling cascade[361]. However, no study has looked at the effects of exercise on glucose metabolism in aged obese mice.

The adaptations to exercise in scWAT also involve a favorable modulation of the pro/antiinflammatory cytokine and adipokine signaling in animal models of obesity. However, these effects have been studied mainly in vWAT due to the harmful metabolic effects of this fat depot. Hereby, there are some inconsistencies across studies investigating the effects of exercise in scWAT from adult or aged DIO animals. Thus, exercise has been shown to induce a decrease in mRNA levels of *Lep* and *Adipoq* in scWAT from DIO rodents, together with concomitant changes in their circulatory forms[362,363]. However, in adult DIO animals, exercise training also reduces total macrophages markers (F4/80), while decreasing *Mcp1* and increasing *Adipoq* in

scWAT, in parallel to inducing a decline in circulating MCP-1, TNF-α, and IL-1β[348,364]. By contrast, other studies in adult DIO animals reported that exercise induced an upregulation of some inflammatory genes (*Ccl2*), while age-matched lean animals revealed an increase in M2 macrophages and in M2/M1 ratio, together with unaltered mRNA levels of several cytokines and adipokines (*Il10*, *Tnf*, *Il6*, *Adipoq, Icam*)[347,365]. Importantly, the ability of exercise to reduce WAT depots inflammation has been related to a decrease in senescence markers (*p53*, *p21*) in scWAT of 1 year old DIO mice[366]. These data demonstrate the close link between exercise-induced improvements of the inflammatory status in aging and obesity.

On the other hand, data on browning in aged DIO mice after exercise is scarce. Thus, exercise training has been shown to increase the browning of scWAT in adult DIO mice, with an increased number of lipid droplets, higher multilocularity, and the induction of a thermogenic and beige-specific program, in scWAT[367–369]. However, only two studies in aged animals can be found in the literature, which showed contrasting results to those observed in young mice. Thus, increased *Ucp1*, *Prdm16*, *Tfam* and *Pgc1a* were found in scWAT of 30 weeks old DIO mice, without changes in *Nrf1*. However, the effects were not similar for the two types of exercise conducted[348]. Indeed, endurance training was more effective for a healthier beige phenotype than high intensity interval training (HIIT), as it also induced higher increments in *Adipoq* than HIIT[348]. Conversely, *Ucp1* was upregulated in mice submitted to HIIT, but not in those undergoing endurance training[348]. Interestingly, increments in the thermogenic *Pgc1a* have been observed also in vWAT of 10 and 23 months old lean mice, without upregulation of *Ucp1*[347]. These data indicate a complex regulation of thermogenesis and mitochondrial biogenesis by exercise, by which they may not necessarily occur in parallel.

Importantly, the observed effects of exercise training in rodents' WAT have been also observed in scWAT from middle-aged, aged, and obese adult humans. However, these actions remain controversial. Indeed, middle-aged obese subjects submitted to combined training (aerobic + resistance) did not show changes in scWAT genetic profile, as revealed by a panel of inflammatory, immune cell, antiinflammatory and thermogenic genes, studied in abdominal scWAT biopsies[357]. By contrast, a similar combined training approach downregulated macrophage markers transcripts in young obese subjects, that were associated with inflammatory and immune-related pathways[343]. Interestingly, these transcripts were upregulated in dysglycaemic *vs.* normoglycemic subjects, while their levels were similar in both groups after the exercise intervention. Moreover, inverse associations were found between these transcripts and insulin sensitivity at baseline, after the intervention, and for the change between baseline and after the intervention, thus revealing an ability to predict exercise-induced improvements on insulin sensitivity[343]. Furthermore, the levels of adipokines (leptin and adiponectin) in scWAT, but not in skeletal muscle, were significantly correlated with corresponding plasma adipokine concentrations[343]. On the other hand, exercised older women revealed lower mRNA levels of inflammation and oxidative stress markers, relative content of $CD36^+$ macrophages, and higher number of T-cells subtypes in WAT when compared with untrained women[345]. Again, the content of $CD36^+$ cells, T-cells, and mRNA expression of several inflammatory and oxidative stress markers correlated to insulin sensitivity and cardiorespiratory fitness[345]. Altogether, these data reveal the crosstalk between the immune adipose organ, its inflammatory status and insulin sensitivity, as well as their modulation by exercise training also in humans.

Few studies have studied the potential effects of exercise training in human WAT, and those who have mainly addressed the potential browning of human scWAT in response to exercise[128]. Nevertheless, the exercise-induced browning of WAT has been suggested to be influenced by temperature, as thermoneutrality impedes the exercise-induced browning of scWAT in lean[67] and in DIO animals[370], adding

more difficulty to the study of browning of scWAT in humans. Nevertheless, a recent study revealed adaptations in mitochondria after 12 weeks of combined training in young obese women, that occurred together with an improved insulin sensitivity[371]. Importantly, the adaptations were depot-specific to an increase in mitochondrial respiration in scWAT and not gonadal WAT that correlated with the decrease in body fat and the increase in soleus and hepatic fat content[371]. These results underscore the role of exercise on inducing whole body metabolic adaptations, mediated by scWAT increased mitochondrial activity in obese older individuals.

In summary, it appears that all the mentioned processes are regulated by exercise in adult obese mice, acting to improve adipose tissue biology and with potential systemic beneficial effects. However, aging apparently exerts some type of interaction with obesity that leads to lower or even inverse exercise adaptations in WAT. Hence, more studies are warranted to better elucidate the effects of exercise on WAT adaptations during aging and obesity.

4.1.2. Effects on brown adipocytes recruitment and activation

The effects of exercise on BAT are, as one would expect, not so studied. Therefore, the exercise training-induced adaptions to BAT have not been well established. For example, the thermogenic adaptations induced in BAT after exercise training in rodents seem to rely on the housing temperature[67,372]. Thus, both *in vivo* thermogenesis and glucose uptake have been discarded in BAT after exercise training in conditions of thermoneutrality, which are the temperatures (28-32 ºC) at which BAT thermogenesis is inactivated[372]. This finding contrasts to the number of studies showing that exercise induces thermogenesis and glucose uptake in BAT by gene studies[128].

Despite BAT UCP1 is not increased when exercise is performed at thermoneutrality[370], *Pgc1a* does increase. This indicates the appearance of exercise-induced effects on mitochondrial thermogenesis independent of UCP1, similar to those observed in WAT. Nevertheless, the lack of changes after exercise in BAT thermogenesis or brown fat gene markers, including *Pgc1a* or *Prdm16,* has also been revealed to occur at the standard rooms' temperatures[373]. In fact, the actions of exercise on BAT mitochondria have been proposed to be, so far, affected by the exercise modality and the animal model, *i.e*. a swimming protocol that would induce exercise adaptations to BAT, but again likely mediated by cold exposure[128].

Nevertheless, exercise can induce an increase in the number of brown adipocyte progenitors in BAT of young (3 months old) DIO mice exercised for 8 weeks[373]. In DIO rodents, exercise is also able to induce the appearance of smaller adipocytes that also display a reduced lipid droplet size[374,375]. Regarding lipogenesis and lipolysis, a study in adult DIO animals has observed an increase in cAMP (cyclic Adenosine Monophosphate) in BAT, that could involve an increase in lipolysis[376]. However, a decrease in palmitate oxidation has also been described *ex vivo* in BAT from DIO exercised animals[377]. On the other hand, studies in lean animals have revealed an increment in fatty acid oxidation, with downregulation of lipolysis in BAT[349]. These data reveal a complex response of BAT lipolysis to exercise, and highlight the need for more studies on BAT fatty acid oxidation/lipolysis pathways after exercise in obesity and in aging.

The effects of exercise on glucose uptake in rodents' BAT are also conflicting. On one hand, some studies have shown that 2-8 weeks of exercise upregulates the expression of genes involved in insulin signaling, glucose and fatty acid oxidation in BAT[349,374]. However, another study indicated that 6 weeks of exercise did not affect *in vivo* glucose uptake in BAT at room temperature or at thermoneutrality, as measured by insulin-

stimulated 3H-2DG ([3H]-2-Deoxyglucose) uptake[372]. These data seem to indicate that, like those results observed in BAT thermogenesis, exercise results in an upregulation of genes involved in glucose metabolism that is not translated to *in vivo* increments in glucose uptake in BAT.

In humans, the evidence is also conflicting. Higher exercise intensities have been associated with BAT density[378,379], while conversely, low BAT activity has been described in trained subjects compared to the sedentary ones, as objectively measured by PET/CT (positron emission tomography/computed tomography) scans[380]. Regarding studies on lipid and glucose metabolism, apparently glucose uptake is unaltered in BAT after different training programs approaches and durations, both in lean and overweight adult and middle-aged men[359,381]. However, there are no studies in the current literature investigating the potential differential effects of exercise on BAT in women, nor in the concomitant effects in glucose or in lipid metabolism.

Despite collectively, these data suggest that there might be no adaptations in BAT thermogenesis and metabolism after exercise training, recent studies have revealed the ability of exercise to induce the secretion of lipid-derived *batokines* by BAT, namely the diol 12,13-diHOME that acts directly on fatty acid uptake by the skeletal muscle, without acting on glucose homeostasis[78]. These data suggest that the effects of exercise in BAT could involve a modulation of its role as a secretory organ of batokines that act systemically to improve whole-body metabolism[83,84,265], more than modulating BAT thermogenic activity and metabolism.

4.2. Systemic effects of aerobic *vs.* resistance exercise

As explained above, exercise training is of special relevance in the aging population due to its beneficial actions at whole-body composition and physiology. Amongst this population, postmenopausal women might specially benefit from the mentioned effects. In fact, postmenopausal women show a decay in physical activity that begins from midlife, contributing to the lower energy expenditure and thus to weight gain after menopause[19]. Interestingly, some studies have pointed that this reduced physical activity is not a contributor, but the main cause for weight gain during menopause[382]. Therefore, physical activity is related to lower risk for cardiovascular disease, metabolic syndrome and sarcopenia in this population[273,382,383].

In general, exercise can be broadly divided into endurance (aerobic) and resistance (strength) training (RT). Some studies have investigated the different adaptations to both types of training in skeletal muscle[341,384], but this is not the case for adipose tissue. Recently, a meta-analysis concluded that RT is the one needed to achieve improvements in body composition and physical performance in individuals with sarcopenic obesity, a condition which is likely to be present in obese older subjects[385]. In this sense, recent data has revealed that RT increases the resting metabolic rate compared to aerobic and combined exercise training, highlighting its potential beneficial effects in whole-body metabolism[386]. Importantly, it has been established that weight loss strategies have significant effects on body composition and inflammatory markers that are related to improvements in insulin resistance/sensitivity and other cardiometabolic risk factors in obese older individuals. However, sustained improvements in insulin sensitivity are only achieved when exercise training is used[387].

On the other hand, while combining aerobic and RT seems to be the most effective to reduce fat mass[388], RT has a constellation of more relevant beneficial effects to aging, mainly with regard to the prevention of muscle mass loss[389,390]. These effects are likely to be the cause for the improvements in frailty, which is at the core of fracture risk in aging individuals[391], and are of special interest in aging obese adults, since intentional weight loss in this population leads to a loss of bone mineral density (BMD) that persists even

after later weight regain[392]. Even when weight loss is limited to reductions in adiposity, careful approaches must be used, as fat mass losses are also directly related to BMD losses in this population[393].

Interestingly, the increase in muscle mass has been associated to the ability of RT to reduce CRP in aging subjects[394]. However, while both resistance and aerobic exercise are able to reduce also IL-2, TNF-α, the HOMA-IR index and HbA1c (glycated hemoglobin), apparently aerobic exercise has more beneficial impacts than RT on reducing inflammatory markers, both in type 2 diabetic individuals and in obese subjects[395,396]. For example, the effects on reducing leptin and increasing adiponectin are more pronounced for aerobic than RT in overweight and obese individuals[396]. Nevertheless, the lowering effects of exercise training on leptin has been described also in middle-aged and old obese patients in which, by contrast, RT seems to have a higher potential to reduce its circulating levels[397]. Regarding chemerin, older obese subjects show a reduction after aerobic exercise training that is associated with increments in insulin sensitivity[188]. However, no study has investigated the role of chemerin in this population after RT.

There is a great bias in sex-specific trainings and thus, in the study of sex differences regarding exercise-induced adaptations in men and women. Indeed, the vast majority of the studies investigating effects of exercise in women have targeted bone metabolism[398–400] and therefore, RT and combined aerobic + RT have been extensively studied on peri and postmenopausal women, since they are established to induce the greatest improvements on bone mineral density[399,400]. However, in this population group RT has also demonstrated to decrease inflammatory markers like circulating CRP, IL-6 and TNF- α[401,402], as well as adipose tissue derived adipokines including visfatin, vaspin, and leptin levels, together with an increase in adiponectin[189]. Noteworthy, RT reduces body fat percentage and fat mass in overweight and obesity[405] also in healthy obese postmenopausal women[402,406] and in those with sarcopenic obesity[407]. These results occur together with lower circulating levels of fasting glucose, and an improve in blood lipids by increasing HDL-cholesterol[402,408].

In summary, aerobic exercise and combined aerobic + resistance exercise have been suggested to be the best training approaches for the general population, regarding the achieved fat mass losses and the associated improvements in the inflammatory milieu. However, in middle-aged and aged subjects, RT appears to be the best approach, as it accounts for more systemic and whole-body composition improvements. Despite this, there is a lack of exhaustive studies in aging obese subjects aiming to elucidate the effects of RT on body composition, glucose metabolism, markers of cardiovascular and inflammatory status, as well as adipose tissue contributions to such adaptations[409]. Because the heterogeneity of studies involving RT is likely to be higher due to the number of factors influencing the experimental approach, including frequency, volume, intensity, and exercises that determine the training program, more studies are needed in this population.

5. Combining *n*-3 PUFA and exercise for an enhanced healthy effect

The most popular effects regarding *n*-3 PUFA in the context of exercise training are their role as ergogenic aids, which are broadly defined as techniques or substances used for the purpose of enhancing performance[410]. In fact, *n*-3 PUFA have the ability to promote muscle adaptation to exercise, regulate its energy metabolism, as well as facilitate muscle recovery and prevent injury by reducing muscle soreness[411]. Very recently, a study described their potential anabolic activity in older (60-85 years) subjects[412].

Importantly, these effects were found to be potentiated after only one session of RT[413]. Thus, these findings led to specific population-based interventions to combine RT and *n*-3 PUFA supplementation.

Therefore, several studies combining both interventions have been conducted also in postmenopausal women, revealing some beneficial effects in muscle metabolism. For example, in lean women aged 65-70 years, increasing dietary *n*-3 PUFA intake by following a specific isocaloric, healthy diet (36% of energy from fat, mainly monounsaturated fatty acids (MUFA) and PUFA, with a ratio *n*-3/*n*-6 < 2) in combination with a RT program of 24 weeks, led to greater increments in muscle strength and leg lean mass compared to subjects trained without the dietary intervention[414]. Later on, this same study revealed that this experimental approach also increased muscle cross sectional area, by inducing an increment in Type IIA muscle fibers[415]. Increments in several parameters of muscle strength and responsiveness tests were found to be higher after 90 days (almost 13 weeks) of RT and fish oil supplementation (2 g/d providing ~0.4 g EPA and 0.3 g DHA/d) in overweight women aged 64 ±1 years[416]. On the other hand, overweight women between aged 65-75 years revealed increments in muscle strength and quality, but not in its cross-sectional area, after 18 weeks of RT and *n*-3 PUFA supplementation (2.1 g/d EPA + 0.6 g/d DHA)[417]. Since the latter trial was performed in overweight women, it suggests that excessive fat mass might halt the anabolic effects of exercise in older women. Moreover, this study also found that greater increments in strength and muscle quality with *n*-3 PUFA supplementation occurred in women, but not in men, suggesting, like previous reports, that this result might be sex dependent[418]. Indeed, Cornish *et al.*[419] found no synergistic effects in any of these parameters after 3 g/d *n*-3 PUFA and RT for 12 weeks in overweight middle-aged men. Importantly, all these results were accompanied by a reported significant increase in plasma DHA and/or EPA[413,414,416,417,419].

It is important to note that few of these studies have investigated the effects of the combination of both treatments in circulating metabolic or inflammatory biomarkers, with no additive nor synergistic effects observed in the groups receiving both treatments. Strandberg *et al.*[414] described no synergistic effects on CRP nor in IL-6. Similarly, Da Boit *et al*.[417] found no synergistic effects on IL-6, TNF-α, nor in blood glucose, insulin, triglycerides, or blood pressure. Studies in older men have neither observed synergistic effects for RT and *n*-3 PUFA in circulating IL-6 and TNF-α[419]. In fact, one study even revealed a significant increase in the CRP in the fish oil group compared to the placebo-supplemented group[420]. From the inflammatory point of view, the reported decrease in muscle soreness that occurs after *n*-3 PUFA supplementation in exercise must entail a decrease in local inflammation. However, it has been pointed by several studies that this research field is lacking of more studies combining exercise and *n*-3 PUFA, and that this lack may confound the current available results[421,422].

Indeed, other studies combining *n*-3 PUFA with different approaches of exercise and supplementation have proven beneficial effects in the aging population. In overweight women aged 60-76 years consuming 5 g/d of fish oil (2 g EPA and 1 g/d DHA), increments in the resting metabolic rate and energy expenditure during exercise (cycling), as well as increased fat oxidation in both conditions, were found in fish oil compared to placebo supplemented women[420]. Moreover, the trained + fish oil group revealed lower triglycerides and increased functional capacity compared to baseline values. Similarly, a recent trial has reported improvements in functional tests, in older women supplemented with canola oil and submitted to a combined exercise program (aerobic + RT) for 16 weeks, compared to the placebo trained group[423].

Nevertheless, no study has thoroughly investigated the metabolic changes after RT and *n*-3 PUFA supplementation on metabolic parameters such as glucose tolerance or insulin resistance, nor revealed effects on blood lipids, adipokines, or adipose tissue biology in postmenopausal women. Importantly, very

recently Bischoff-Ferrari *et al.*[318] showed that *n*-3 PUFA supplementation and RT started in the old age have no effects on triglycerides, blood pressure, highlighting that interventions at younger aging stages might be more effective to achieve clinical outcomes for a healthy aging.

In this background, a recent study of Brezinova *et al.*[424] revealed an increment in insulin sensitizing PAHSAS (palmitic acid esters of hydroxy stearic acids), bioactive lipids with antiinflammatory and antidiabetic effects, in serum and adipose tissue of overweight aged women supplemented with canola oil (230 mg/d EPA and DHA) and following a combined exercise training program for 4 months. Among the increased oxylipins, the DHA-derived 17-HDHA, precursor of DHA-derived SPMs, could be found. Regarding the possible mechanisms underlying these beneficial effects of the combination of both treatments in older ages, recent studies have shown a preventive effect in the loss of muscle myogenic capacity, together with lower fat mass gains in aged obese rats after chronically combining alpha-linolenic acid and RT[425]. Moreover, in young obese rats, the combination of alpha-linolenic acid and RT led to improved cardiovascular markers including diastolic blood pressure[426].

Therefore, the potential combination of both treatments seems to have effects mainly at the adipose tissue level in aging women with overweight/obesity, and has been demonstrated to induce some beneficial actions on muscle anabolic response yet to be confirmed by an increase in muscle mass. Therefore, more studies are needed to elucidate if the combination of both *n*-3 PUFA and RT could have beneficial actions on adipose tissue inflammatory status, together with concomitant reductions on systemic inflammation, as well as with improvements in the gluco-lipid metabolism, and body composition.

Hypothesis and Objectives

1. Hypothesis

Obesity and aging are characterized by an chronic low-grade inflammation[427]. Current evidence suggests that adipose tissue dysfunction is at the core of the development and instauration of such inflammatory state in both processes, favoring the development of their associated comorbidities[30,99]. Such dysfunction affects both white and brown adipose tissues, and therefore their respective roles in metabolic homeostasis and thermogenesis. Furthermore, the ability of white adipose tissue to acquire a thermogenic phenotype (beige) is impaired in aging and in obesity[31,50,99,136]. Since adipose tissue is an endocrine organ, local inflammation eventually becomes systemic, affecting the function of other metabolic organs[30,99].

In this regard, several studies have suggested beneficial effects for marine origin *n*-3 PUFA, both EPA and DHA, on obesity-related metabolic disorders in rodents and humans, in part by acting on adipose tissue biology[133,224]. Importantly, both EPA and DHA also serve as substrates for the production of lipid mediators that act directly to resolve inflammation, also in adipose tissue[267,271]. On the other hand, physical exercise has been demonstrated to be an effective lifestyle intervention to prevent obesity and promote a healthy aging[428,429]. Similar to *n*-3 PUFA supplementation, exercise-based interventions have demonstrated to improve systemic and adipose tissue metabolic status in rodents and humans[128,129]. However, there is a lack of studies characterizing if long-term exercise or *n*-3 PUFA supplementation can ameliorate adipose tissue dysfunction and inflammation in aging, specifically in an obesogenic context.

In this sense, postmenopausal women constitute a population at higher risk of developing or worsening the obesity and aging associated comorbidities, since adipose tissue undergoes unfavorable changes in its mass, distribution, and biology during the menopausal transition[16,430]. Therefore, they would specially benefit from *n*-3 PUFA and exercise training-based interventions, both locally and systemically,

Based on these premises, our **hypothesis** is that antiinflammatory nutritional and lifestyle interventions, including DHA supplementation and physical exercise, would help to prevent/restore white and brown adipose tissues dysfunction, as well as the inflammatory status that accompanies aging and obesity. These actions would improve systemic inflammation and metabolic health in aged obese female mice, and in postmenopausal women with overweight/obesity.

2. Objectives

The **general objective** of this study was to determine the ability of DHA dietary supplementation and exercise programs, alone or in combination, to modulate the adipose tissue dysfunction and its implications in systemic metabolic health in obese aged female mice and in overweight/obese postmenopausal women.

For this purpose, the specific objectives were:

1. To study the effects of chronic DHA supplementation to restore subcutaneous white adipose tissue plasticity, through the characterization of morphological and functional changes on genes/proteins controlling lipid metabolism, inflammatory status, and browning process in 18 months old obese female mice (*Chapter 1*).
2. To characterize the changes in specialized proresolving lipid mediators' signature in brown adipose tissue of young (2 months old) and aged (18 months old) lean and obese female mice and to investigate the effects of long-term dietary supplementation with a DHA-rich fish oil concentrate, as well as their potential associations with brown adipose tissue function markers (*Chapter 2*).
3. To perform a comparative study in subcutaneous white *vs.* brown adipose tissues, on the effects of a long-term treadmill running program initiated in adulthood on genes/proteins controlling fat accumulation, inflammatory status, and thermogenic function in 18 months-old obese female mice (*Chapter 3*).
4. To explore the effects of a DHA-rich fish oil-derived supplement and a resistance training program, alone or in combination, for 16 weeks on fat mass, body weight, serum biomarkers of glucose and lipid metabolism and on muscle strength and quality markers in overweight and obese postmenopausal women (*Chapter 4*).
5. To examine the effects of DHA supplementation (DHA-rich fish oil concentrate) and a resistance training program, alone or in combination, for 16 weeks on serum biomarkers of systemic inflammation and on the expression of subcutaneous white adipose tissue genes related to inflammatory status and browning markers in overweight and obese postmenopausal women (*Chapter 5*).

3. Hipótesis

La obesidad y el envejecimiento se caracterizan por una inflamación crónica subyacente de bajo grado[427]. La evidencia actual sugiere que la disfunción del tejido adiposo está en el centro del desarrollo e instauración de dicha inflamación en ambos procesos, favoreciendo el desarrollo de sus comorbilidades asociadas[30,99]. Esta disfunción afecta tanto al tejido adiposo blanco como al pardo y, por tanto, a sus respectivas funciones en la regulación de la homeostasis metabólica y la termogénesis. Además, la capacidad del tejido adiposo blanco de adquirir un fenotipo termogénico (beige) esta alterada en la obesidad y en el envejecimiento[31,50,99,136]. Dado que el tejido adiposo es un órgano endocrino, la inflamación local eventualmente se vuelve sistémica y afecta a la función de otros órganos metabólicos[30,99].

En este sentido, varios estudios han sugerido efectos beneficiosos para los ácidos grasos polinsaturados omega-3 (AGPI *n*-3) de origen marino, tanto el EPA como el DHA, sobre los trastornos metabólicos relacionados con la obesidad en roedores y humanos, en parte al actuar sobre la biología del tejido adiposo[133,224]. Es importante destacar que el EPA y el DHA también sirven como sustratos para la producción de mediadores lipídicos que actúan directamente para resolver la inflamación, también en el tejido adiposo[267,271]. Por otro lado, el ejercicio físico ha demostrado ser una intervención eficaz en el estilo de vida para prevenir la obesidad y promover un envejecimiento saludable[428,429]. De manera similar a la suplementación con AGPI *n*-3, las intervenciones basadas en el ejercicio han demostrado mejorar el estado metabólico sistémico y del tejido adiposo en roedores y humanos[128,129]. Sin embargo, hay una falta de estudios que caractericen si el ejercicio o la suplementación con AGPI *n*-3 a largo plazo pueden mejorar la disfunción del tejido adiposo y la inflamación en el envejecimiento, específicamente en un contexto obesogénico.

En este sentido, las mujeres posmenopáusicas constituyen una población con mayor riesgo de desarrollar o empeorar las comorbilidades asociadas al envejecimiento y a la obesidad, ya que el tejido adiposo sufre cambios desfavorables en su masa, distribución y biología durante la transición menopáusica[16,430]. Por lo tanto, las mujeres de mediana edad se beneficiarían especialmente de las intervenciones basadas en el entrenamiento físico y los AGPI *n*-3, tanto a nivel local como sistémico.

Partiendo de estas premisas, nuestra **hipótesis** es que las intervenciones antiinflamatorias nutricionales y de estilo de vida, incluida la suplementación con DHA y el ejercicio físico, ayudarían a prevenir/restaurar la disfunción de los tejidos adiposos blanco y pardo, así como el estado inflamatorio que acompaña al envejecimiento y la obesidad. Estos efectos mejorarían la inflamación sistémica y salud metabólica en ratones hembra obesos envejecidos, y en mujeres postmenopáusicas con sobrepeso/obesidad.

4. Objetivos

El **objetivo general** de este estudio fue determinar la capacidad de la suplementación dietética con DHA y programas de ejercicio físico, solos o en combinación, para modular la disfunción del tejido adiposo y sus implicaciones en la salud metabólica a nivel sistémico en ratones hembra obesos y en mujeres posmenopáusicas con sobrepeso/obesidad.

Para ello, los objetivos específicos fueron:

1. Estudiar los efectos de la suplementación crónica con DHA para restaurar la plasticidad del tejido adiposo blanco subcutáneo, mediante la caracterización de cambios morfológicos y funcionales en genes/proteínas que controlan el metabolismo de lípidos, el estado inflamatorio y el proceso de pardeamiento en ratones hembra obesos de 18 meses (*Capítulo 1*).
2. Caracterizar los cambios en el perfil de mediadores lipídicos proresolutivos de la inflamación en el tejido adiposo pardo inducidos por la edad y/o la obesidad en ratones hembra jóvenes (2 meses) y envejecidos (18 meses) e investigar los efectos de la suplementación de la dieta con DHA, así como sus posibles asociaciones con marcadores de función del tejido adiposo pardo (*Capítulo 2*).
3. Realizar un estudio comparativo en el tejido adiposo subcutáneo blanco vs. el tejido adiposo pardo de los efectos de un programa de ejercicio en cinta rodante iniciado en la edad adulta sobre genes/proteínas que controlan la acumulación de grasa, el estado inflamatorio y la función termogénica en ratones hembra obesos de 18 meses de edad (*Capítulo 3*).
4. Explorar los efectos de un suplemento derivado de aceite de pescado rico en DHA y de un programa de entrenamiento de resistencia, solos o en combinación, durante 16 semanas sobre la grasa y el peso corporal, biomarcadores séricos del metabolismo de la glucosa y los lípidos, así como sobre la fuerza y marcadores de calidad muscular en mujeres posmenopáusicas con sobrepeso/obesidad (*Capítulo 4*).
5. Examinar los efectos de la suplementación dietética con DHA (concentrado de aceite de pescado rico en DHA) y de un programa de entrenamiento de resistencia, solos o en combinación, durante 16 semanas sobre biomarcadores séricos de inflamación sistémica y sobre la expresión de genes del tejido adiposo blanco subcutáneo relacionados con el estado inflamatorio, la acumulación de grasa, y marcadores de pardeamiento en mujeres posmenopáusicas con sobrepeso/obesidad (*Capítulo 5*).

Materials and Methods

The experimental procedures of this thesis belong to the *OBELEX* research project: *Dysfunction of adipose tissue in obesity, inflammation and aging: mechanisms and effects of physical exercise and omega-3 fatty acids.* The OBELEX project carried out a translational research consisting of two different DHA supplementation and/or exercise interventions, conducted in DIO female mice aged up to 18 months old, or in overweight and obese postmenopausal women.

1. Studies in Animals

1.1. Animal experimental design and diets description for *Chapters 1* and *2* (DHA intervention)

The animal studies were conducted in 7 weeks-old female C57BL/6J mice purchased from Harlan Laboratories (Barcelona, Spain) and housed at the animal facilities of the University of Navarra under controlled conditions of light, temperature, and humidity (12-h light/12-h dark cycle, 22 ± 2 ºC, relative humidity 55% ± 10%).

After a period of 10-days acclimation, 10 animals were sacrificed to serve as a young control group (young CT). Then, two months-old mice were assigned to a standard chow diet (20% proteins, 67% carbohydrates, and 13% lipids as energy, Harlan Teklad Global Diets, Harlan Laboratories, Indianapolis, IN, USA) up to 18 months of age (*aged CT*), or a HFD (20% proteins, 35% carbohydrates, and 45% lipids as energy, Research Diets Inc., New Brunswick, N.J., USA) in order to induce obesity for 4 months. After this period, 6 months-old DIO animals were assigned to the following intervention subgroups:

- DIO group: fed with the standard HFD up to 18 months of age (*aged DIO*).
- DIOMEG group: DIO mice fed with a HFD + *n*-3 PUFA rich oil, replacing 15% of dietary fat (wt/wt) up to 18 months of age (*aged DIOMEG*).

The *n*-3 PUFA rich oil was a DHA-rich fish oil concentrate (SOLUTEX0063TG) containing 683.4 mg DHA/g and 46.7 mg EPA/g, with a total content of 838.9 mg of *n*-3 PUFA/g as triglycerides, provided by Solutex, Spain. Since the DHA-rich fish oil concentrate contained 2 mg/g of mixed tocopherols (Covi-ox® T-70EU) to preserve *n*-3 PUFA oxidation, the same amount was added to the HFD of the DIO mice that continued with the standard high-fat feeding during this experimental period.

The different HFDs were prepared by Research Diets Inc., vacuum sealed in 2.5 kg plastic bags and kept frozen (-20 °C) until used to avoid rancidity. Specific dietary compositions can be found in the Supporting Information at the end of this section.

1.1.1. Experimental design and assays for Chapter 1

In order to study the effects of diet-induced obesity and long-term feeding with the DHA-enriched diet on scWAT of aged obese female mice, the following experimental groups were included in *Chapter 1*: aged CT, aged DIO and aged DIOMEG mice (summarized in **Figure 11**).

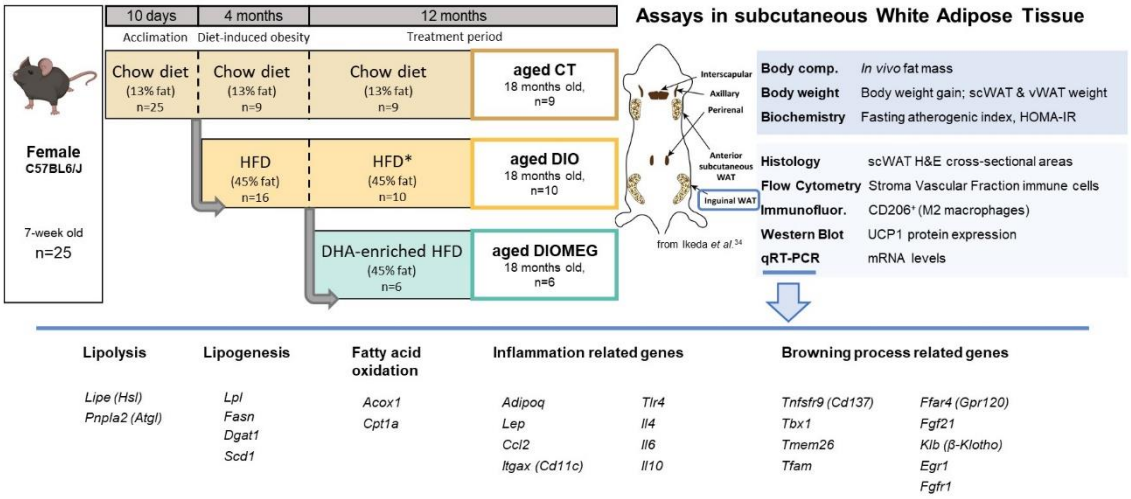

Figure 11. Animals, experimental design, and assays conducted in subcutaneous White Adipose Tissue (scWAT) of aged (18 months) lean mice (CT), diet-induced obese (DIO) mice fed a high-fat diet (HFD), and DIO mice fed HFD with 15% fat replaced by a DHA-rich *n*-3 PUFA oil. CT: Control; DIO: Diet-induced obese; DIOMEG: Diet-induced obese + omega-3; Body comp.: Body composition; vWAT: Visceral Adipose tissue; HOMA-IR: Homeostatic Model assessment for insulin resistance; Inmunofluor.: Immunofluorescence; qRT-PCR: quantitative Real Time Polymerase Chain Reaction.

In vivo body composition measurements were performed by nuclear magnetic resonance (EchoMRI-100-700; Echo Medical Systems). Fasting serum biochemical determinations (total cholesterol, HDL-cholesterol, TG, LDL-cholesterol, glucose and insulin) were used for calculations of the atherogenic ratio total cholesterol/LDL-cholesterol, and the HOMA-IR index as surrogates of animals' metabolic status.

Ex vivo experiments for scWAT inflammatory phenotyping included flow cytometry of immune cells in the stromal vascular fraction (SVF), and immunofluorescence of antiinflammatory macrophages ($CD206^+$) in scWAT. Histomorphometric analysis of scWAT was performed to determine adipocyte size in Hematoxylin & Eosin-stained samples and quantified with ImageJ 2.0 imaging suite (U.S. National Institutes of Health, Bethesda, MD, USA). Protein expression of UCP1 in scWAT was determined by Western blot, and mRNA levels of markers of scWAT metabolic pathways (lipolysis, lipogenesis, and fatty acid oxidation) and genes related to browning process and inflammatory status were evaluated by RT-PCR.

Detailed procedures can be found in the Materials and Methods subsection of *Chapter 1* of the Results.

1.1.2. Experimental design and assays for Chapter 2

In order to study the effects of aging, diet-induced obesity and long-term feeding with the DHA-enriched diet on iBAT function of female mice, the following experimental groups were included in *Chapter 2*: young CT, aged CT, aged DIO and aged DIOMEG mice (summarized in **Figure 12**).

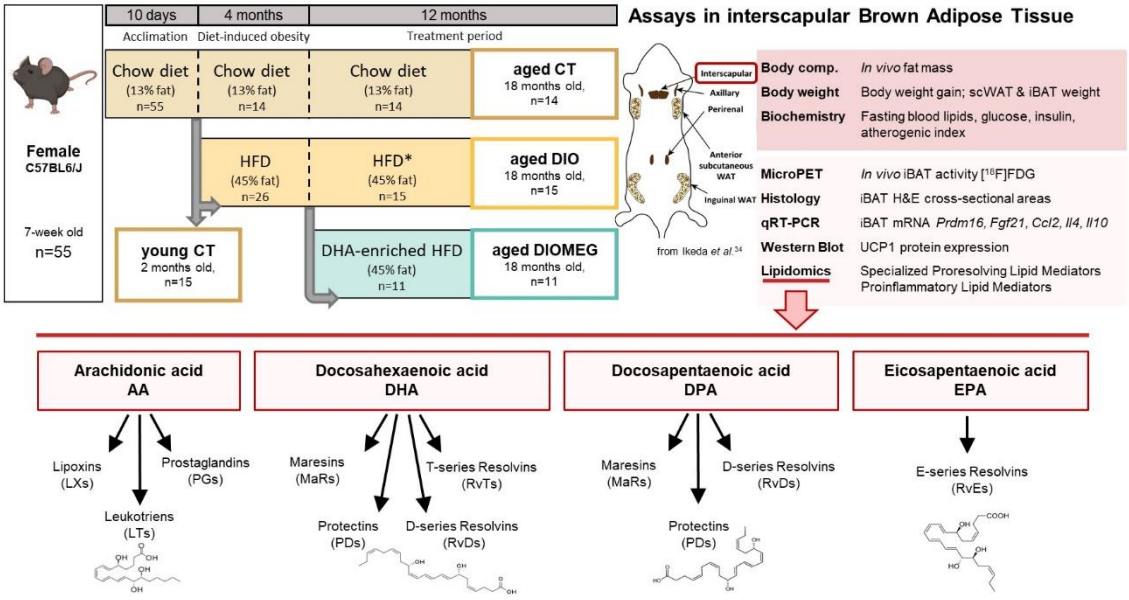

Figure 12. Animals, experimental design, and studies conducted in interscapular Brown Adipose Tissue (iBAT) of young CT (2 months) and aged CT (18 months) mice, as well as in aged DIO mice fed a standard HFD, and aged DIOMEG mice fed DHA-enriched HFD. CT: Control; DIO: Diet-induced obese; DIOMEG: Diet-induced obese + omega-3; Body comp.: Body composition; scWAT: Subcutaneous White Adipose Tissue; MicroPET: Micro positron emission tomography; [^{18}F]-FDG: ^{18}F-Fluoro-2-deoxy-2-D-glucose; SVF: stroma vascular fraction; qRT-PCR: quantitative Real Time Polymerase Chain Reaction.

In vivo experiments included iBAT activity assayed by [^{18}F]Fluoro-2-deoxy-2-D-glucose ([^{18}F]FDG) uptake measured by positron emission tomography in a small-animal dedicated imaging tomograph (Mosaic, Philips Electronics, Cleveland, OH, USA). Body composition was also determined *in vivo* in a small animal dedicated nuclear magnetic resonance system (EchoMRI-100-700; Echo Medical Systems, Houston, TX, USA).

Ex vivo experiments included serum biochemical determinations (fasting total cholesterol, LDL- cholesterol, HDL- cholesterol, ratio LDL- cholesterol /HDL- cholesterol, β-hydroxybutyrate, glucose and insulin), iBAT and WAT depots weight (studied as the sum of the subcutaneous, mesenteric, gonadal, and retroperitoneal WAT depots). Histological analysis of iBAT was performed in Hematoxylin & Eosin-stained preparations.

iBAT tissue samples were analyzed for proresolving and proinflammatory lipid mediator signatures, as well as iBAT morphology, protein expression (UCP1) and mRNA levels of specific markers of iBAT development and function (*Prdm16, Fgf21*) and inflammatory status (*Ccl2, Il4, Il10*).

Detailed procedures can be found in the Materials and Methods subsection of *Chapter 2* of the Results.

1.2. Experimental design and assays for the exercise intervention (*Chapter 3*)

The effects of a long-term exercise protocol in obese animals were compared on the scWAT and iBAT depots of 18 months old DIO mice. To do so, two months-old female mice were fed a HFD (45% energy) to induce obesity for 4 months. After this period, 6 months-old obese (DIO) mice were divided into two experimental groups: the DIO sedentary group, fed a HFD up to 18 months of age (*aged DIO*); and the DIO exercised (DIOEX) group, fed a HFD and assigned to chronic exercise running on a treadmill up to 18 months of age (*aged DIOEX*).

Hence, the treadmill (LE8710M; Panlab, Barcelona, Spain) training program was conducted from 6 months up to 18 months old. Prior to the beginning of the training program, mice adapted to treadmill by running for 10 minutes on 2 consecutive days (first day at 3 m min^{-1}, second day at 4.8 m min^{-1}). At 6 months of age, the DIOEX mice started the treadmill running protocol (3 m min^{-1} for 5 min, increased to 4.8 m min^{-1} for 5 min, reaching a maximum of 7.2 m min^{-1} for 20 min; 0% slope) conducted 3 days/week. At 10 months of age, the number of running sessions was increased to 5 days/week for 5 weeks, and the protocol was intensified (5 m min^{-1} for 5 min, followed by 8 m min^{-1} for 5 min, and 12 m min^{-1} for 20 min; 0% slope). During the next 7 months, sessions were decreased to 3 days/week and the program was maintained. During the exercise experimental period, the sedentary DIO mice were left on the treadmill for the same time (30 min) without running.

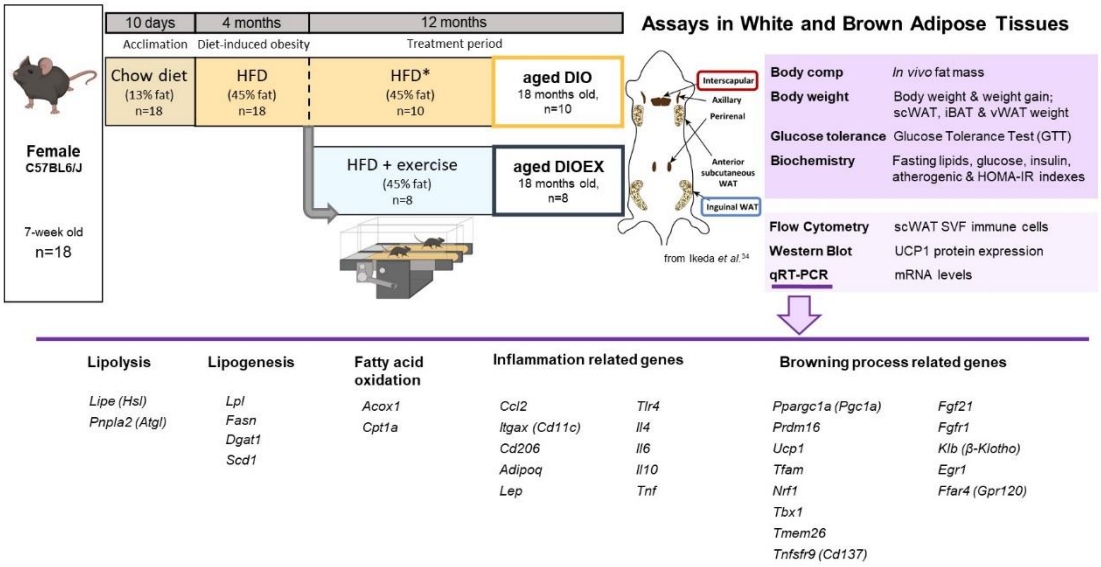

Figure 13. Animals, experimental design, and studies conducted in subcutaneous White Adipose Tissue (scWAT) and interscapular Brown Adipose Tissue (iBAT) of aged (18 months) sedentary diet-induced obese mice (DIO) mice or DIOEX mice: DIO mice under a treadmill exercise protocol from 6 to 18 months. Body comp.: Body composition; vWAT: Visceral Adipose tissue; GTT: Glucose tolerance test; HOMA-IR: Homeostatic model assessment for insulin resistance; SVF: Stromal vascular fraction; qRT-PCR: quantitative Real Time Polymerase Chain Reaction; UCP1: Uncoupling protein 1; vWAT: visceral White Adipose Tissue.

The experimental design and assays for *Chapter 3* are summarized in **Figure 13**. Animals underwent *in vivo* body composition measurement with a nuclear magnetic resonance system (EchoMRI-100-700; Echo Medical Systems) and an intraperitoneal glucose tolerance test (GTT) was performed previous sacrifice.

After sacrifice, blood was collected and fasting serum biochemical determinations were performed, including total cholesterol, LDL-cholesterol, HDL-cholesterol, β-hydroxybutyrate, glucose, and insulin. The HOMA-IR index was also calculated.

On the other hand, infiltration of immune cells was studied in the SVF of scWAT by flow cytometry. Both scWAT and iBAT were analyzed for UCP1 protein content and mRNA levels of lipogenic, lipolytic, fatty acid oxidation, pro and antiinflammatory cytokines, adipokines, and macrophages markers, as well as genes of brown adipocytes development and function. The process of browning of scWAT was also analyzed by studying the gene expression of specific markers of beige adipocytes and of *Fgf21* signaling.

Detailed procedures can be found in the Materials and Methods subsection of *Chapter 3* of the Results.

2. Human studies: the OBELEX trial

2.1. Participants and study design

The OBELEX trial was a randomized, placebo-controlled trial to study the effects of a DHA-rich *n*-3 PUFA supplementation, a resistance training (RT) program, or the combination of both in healthy overweight and obese postmenopausal women.

Four parallel intervention groups were designed: 1. Placebo group (P), supplemented with olive oil (3 g/day), 2. Omega-3 group (*n*-3), supplemented with a DHA-rich fish oil concentrate (3 g/day providing 1650 mg/day DHA and 150 mg/day EPA); 3. Placebo + Resistance training (P+RT), supplemented with olive oil (3 g/day) and attending RT sessions twice a week; and 4. Omega-3 + Resistance training (*n*-3 + RT) supplemented with a DHA-rich fish oil derived supplement (3 g/day providing 1650 mg/day DHA and 150 mg/day EPA) and attending RT sessions twice a week.

Taking fat mass percentage losses as the primary outcome, based on the results reported by previous studies on the placebo untrained group and the *n*-3 trained group[431,432], the calculated effect size was 1.185. With a bilateral alpha of 95%, a power calculation of 90% and an estimated dropout rate of 25%, the sample size calculation estimated 20 subjects per group for a total of 80 subjects.

According to the design of the study, participants of the OBELEX trial were postmenopausal women (at least 1 year of amenorrhea), aged 55-70 years old, with a BMI of 27.5-35 kg/m^2 and a stable weight (varying less than 3 kg) 3 months prior the start of the trial. Exclusion criteria were the use of regular prescription, including beta blockers, hypolipidemic drugs, antidiabetic drugs and hormone replacement therapy; recently changed/prescribed medication including hypotensive, thyroid, anxiolytic or antidepressant drugs; to suffer from any severe metabolic, hepatic, renal, cardiovascular, neuromuscular, arthritic, pulmonary or other debilitating diseases; a history of eating disorders, obesity-bariatric surgery or drug/alcohol abuse; or to follow any special diets in the 3 months prior to the start of the trial.

Volunteers were recruited from June 2017 to December 2018. After giving their signed consent, volunteers attended a screening visit (**Figure 14**) at the Metabolic Unit (MU) of the University of Navarra. The included

volunteers were randomly allocated to one of the four parallel experimental groups based on their age (middle aged, 55-64 years; older adult, 65-70 years) and BMI (overweight 27.5-29.99 kg/m^2, obese I 30-35 kg/m^2) according to WHO criteria[1,433] using MATLAB® software (The MathWorks™, Natick, MA, USA).

At baseline and at the end of the trial, subjects attended the MU of the University of Navarra in fasting conditions and fasting blood samples were obtained before undergoing a 2-hour oral glucose tolerance test (OGTT). Body composition was measured by Dual X-Ray Absorptiometry (Lunar iDXA, encore 14.5, Madison, WI, USA) and bioimpedance; anthropometric measurements were obtained including 7 body circumferences (neck, arm, waist, umbilical, hip, thigh, calf) and 3 fat skinfolds (arm, thigh, calf) following the International Society for the Advancement of Kinanthropometry (ISAK) guidelines at baseline and end of the trial[434]. Blood pressure was also registered. On a separate day, volunteers attended the Department of Plastic and Reconstructive Surgery at the Clínica Universidad de Navarra in fasting conditions, and a subcutaneous adipose tissue (SAT) biopsy (1-2 g) was obtained by liposuction of the periumbilical area under local anesthesia.

Once the baseline visit was completed, participants were given the corresponding supplements and dietary recommendations based on the guidelines from the Spanish Society for Communitarian Nutrition (SENC, 2016)[435]. Follow-up visits were scheduled every 2 weeks, and training sessions were organized twice a week in groups of 5-7 subjects. In every follow-up visit, untrained participants checked in at the MU of the University of Navarra and returned blisters, whether they were empty or not, to control for supplementation compliance by leftover pill count. The same procedure was followed for resistance trained participants at the training facilities of the Studies, Research and Sports Medicine Centre (*Centro de Estudios, Investigación y Medicina del Deporte*, CEIMD). A timeline of the intervention can be found in **Figure 14**.

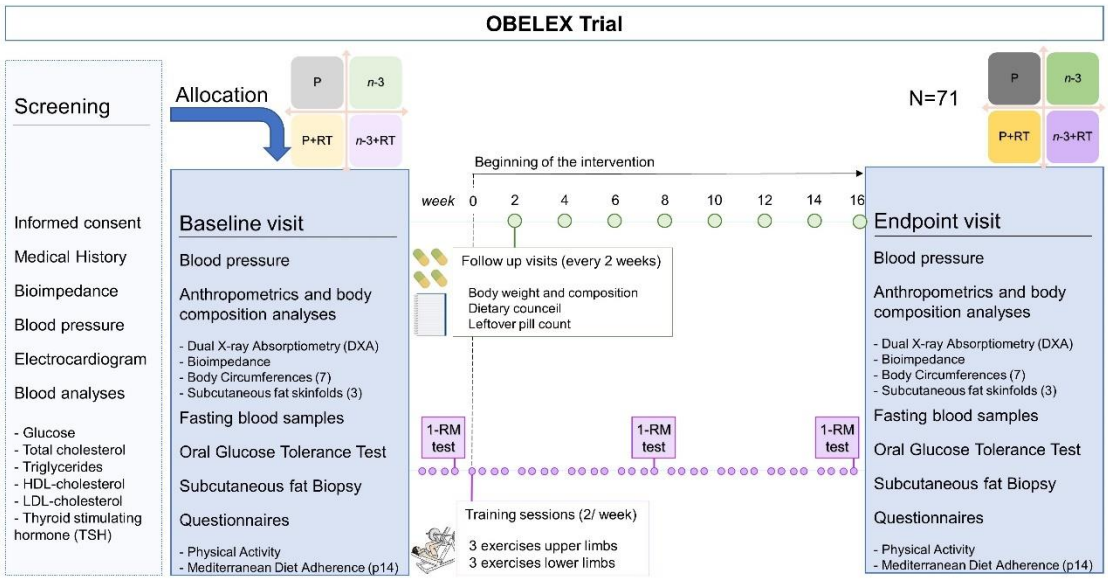

Figure 14. Experimental design of the OBELEX trial. P: Placebo group, *n*-3: DHA-rich *n*-3 PUFA supplemented group; P+RT: Placebo + resistance training group; *n*-3+RT: DHA-rich *n*-3 PUFA supplemented + resistance training group.

At baseline and at the end of the trial, participants also filled two questionnaires that served as control for the dietary and physical activity intervention: the p14 score for Adherence to Mediterranean Diet[436] and the

Seguimiento Universidad de Navarra (SUN) questionnaire of Physical Activity (PA)[437]. To better control for physical activity differences between groups, participants wore an accelerometer (ActiGraph GT3X, Actigraph Corporation, Pensacola, FL, US) during a random week of the study.

The intervention was approved by the Research Ethics Committee of the University of Navarra (140/2015mod2) and was performed in compliance with the Helsinki Declaration guidelines[438]. The study was registered at clinicaltrials.gov as NCT03300388.

2.2. Supplementation and Training Program interventions

The supplements given to the participants of the study were either olive oil placebo capsules (6 capsules of 0.5 g), or DHA-rich *n*-3 PUFA concentrate capsules (6 capsules of 0.5 g). Participants consumed two capsules with each meal (breakfast, lunch, and dinner), and were asked to report any symptom. The *n*-3 PUFA capsules contained a DHA-fish oil (DHA 55%) concentrate that provided 1650 mg/day of DHA and 150 mg/day of EPA. Both the placebo and the DHA-rich fish oil concentrate supplements were provided by Solutex (Madrid, Spain) in hard gelatin transparent liquid fill capsules similar in shape and size, whereas small differences could be appreciated in the viscosity/thickness and color of the oils.

The daily dose of DHA-rich *n*-3 PUFA supplement was selected based on previous studies[439–441], and in accordance to the of the U.S. FDA recommendations (3 g/day EPA and DHA maximum, with up to 2 g/day from dietary supplements)[213]. To fulfill these criteria, the consumption of fish was controlled depending on their *n*-3 PUFA's composition, according to the EFSA recommendations for a normal cardiac function maintenance (250 mg/day)[442], and based on Mataix-Verdú food composition tables[443] and online food composition databases (Easydiet® and Odimet® softwares). Consumption of *n*-3-PUFA enriched food and dietary supplements was not allowed during the study.

Familiarization (F) and test RM				Routine 1				Routine 2			
Week 0		Week 1		Week 2		Week 3		Week 4		Week 5	
F1-Routine 1	**F2-Routine 2**	**F3-Routine 1**	**TEST**	**15-12-15**	**15-12-15**	**15-12-15**	**15-12-15**	**15-12-12-15**	**15-12-12-15**	**15-12-12-15**	**15-12-12-15**
40%	40%	40%	RM	50-60-50	50-60-50	50-60-50	50-60-50	50-60-60-50	50-60-60-50	50-60-60-50	50-60-60-50

Routine 1				Routine 1				Routine 2			
Week 6		Week 7		Week 8		Week 9		Week 10		Week 11	
12-10-12	**12-10-12**	**12-10-12**	**12-10-12**	**TEST**	**12-10-10-12**	**12-10-10-12**	**12-10-10-12**	**10-8-10**	**10-8-10**	**10-8-10**	**10-8-10**
60-70-60	60-70-60	60-70-60	60-70-60	RM	60-70-70-60	60-70-70-60	60-70-70-60	70-80-70	70-80-70	70-80-70	70-80-70

Routine 1				Routine 2				Routine 2		
Week 12		Week 13		Week 14		Week 15		Week 16		
10-8-8-10	**10-8-8-10**	**10-8-8-10**	**10-8-8-10**	**12-10-10-12**	**12-10-10-12**	**12-10-10-12**	**12-10-10-12**	**12-10-10-12**	**TEST**	# Repetitions
70-80-80-70	70-80-80-70	70-80-80-70	70-80-80-70	60-70-70-60	60-70-70-60	60-70-70-60	60-70-70-60	60-70-70-60	RM	% of the RM

Figure 15. Progression of the resistance training program along the 16-week protocol.

On the other hand, the training program was developed at the CEIMD training facilities, where volunteers attended training sessions twice a week for 16 weeks of intervention. Eight exercises for upper and lower limbs were performed in machines (Technogym). Two routines were designed with six exercises each: leg press, chest press, knee extension and lat pulldown were maintained across the RT program, while shoulder press and hip extension (routine 1) and chest fly and leg curl (routine 2) were selected to complete each routine, changing every two weeks. Before testing and training, subjects attended three sessions for familiarization with the procedure of voluntary force production. The progression of the RT program is summarized in **Figure 15**.

Strength tests were performed using the 1-repetition maximum (1-RM) approach[444] at the beginning, midst, and at the end of the trial to obtain strength data and to individually adjust training loads. The training progression was established using the pyramidal training approach, so as 50% of intensity was selected to start the training program, and a maximum intensity of 80% was reached at week 10[445], with three to four series performed in each exercise and 8-15 repetitions adapting to training loads (see **Figure 15**). In each session, one of the researchers was present to direct and assist each subject towards ensuring adequate performance in each exercise (work rates, loads and ranges of motion) following the American College of Sports Medicine (ACSM) guidelines for older adults[445]. To control for strength gains/losses also in untrained groups, first and last follow-up visits were scheduled at the training facilities for subjects allocated to these groups to perform 1-RM tests with its corresponding familiarization session.

2.3. Studies on cardiovascular biomarkers, glucose metabolism, and body composition changes (*Chapter 4*)

Fasting serum samples were used for basic biochemical determinations (glucose, triglycerides, total-cholesterol, HDL-cholesterol) in an automated autoanalyzer (Pentra C200, HORIBA ABX, Madrid, Spain). Fasting insulin was measured using ELISA kits according to the manufacturer´s instructions in a Triturus ELISA Instrument (Grifols Diagnostic Solutions, Barcelona, Spain). LDL-cholesterol was calculated using the Friedewald equation[446], HOMA-IR and the Triglycerides to Glucose (TyG) indexes for insulin resistance were calculated as described previously[447,448]. The area under the curve (AUC) of the OGTT excursion curves was calculated. These results, alongside the changes in body composition, strength and muscle quality, and blood pressure, were considered the main results of the clinical trial in overweight and obese postmenopausal women after DHA supplementation and the RT program, and are further explained in *Chapter 4* of the results section.

2.4. Studies on systemic and subcutaneous adipose tissue inflammatory markers and adipose tissue browning (*Chapter 5*)

Markers of adipose, immune, and systemic inflammation were determined in blood samples and adipose tissue biopsies before and after the intervention. Fasting hematologic determinations (white blood cell and platelets counts) were measured in an automated hematology analyzer (Pentra 60, HORIBA ABX, Madrid, Spain). Fasting CRP, as well as key adipokines adiponectin, leptin and chemerin were measured using ELISA kits according to the manufacturer´s instructions in a Triturus ELISA Instrument (Grifols Diagnostic Solutions, Barcelona, Spain). The adiponectin/leptin ratio was estimated as a marker of adipose-systemic inflammation[449], and platelet-to-lymphocyte (PLR), neutrophil-to-lymphocyte ratios (NLR), and systemic immune-inflammation (PxN/L, SII) index were calculated as markers of immune inflammation[450]. SAT biopsies were used for gene expression analyses by qRT-PCR of adipokines, chemokines and macrophage markers (*ADIPONECTIN, CHEMERIN, LEPTIN*, *CCL2, CD11C, IL6*) and *browning* of WAT genes (*TBX1, TMEM26* and *UCP1*). These studies are detailed in the *Chapter 5* of the results.

Supporting Information

Supplementary Table 1. High fat diets used in the animal studies and their composition.

Diet	**Standard HFD**		**HFD Tocopherols***		**HFD + DHA-rich *n*-3 PUFA Marine Oil****	
Product #	**D12451**		**D16112302**		**D16112301**	
Macronutrient %	**g**	**kcal**	**g**	**kcal**	**g**	**kcal**
Protein	23.7	20	23.7	20	23.7	20
Fat	23.6	45	23.6	45	23.6	45
Carbohydrate	41.4	35	41.4	35	41.4	35
Total		100		100		100
kcal/g	4.73		4.73		4.73	
Ingredient %						
Casein	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12
Soybean Oil	25	225	25	225	25	225
Lard	177.5	1598	177.5	1598	147.1	1324
High DHA Marine Oil (Solutex)*	0	0	0	0	30.4	274
Corn Starch	72.8	291	72.8	291	72.8	291
Maltodextrin 10	100	400	100	400	100	400
Sucrose	172.8	691	172.8	691	172.8	691
Cellulose	50	0	50	0	50	0
Vitamin Mix V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
Mixed tocopherols	0	0	0.061	0	0	0

*A standard high-fat diet (HFD) including the same quantity of tocopherols contained in the HFD formulated with the DHA-rich *n*-3 PUFA concentrate (SOLUTEX). Diets were formulated and prepared by Research Diets Inc. **The DHA-rich *n*-3 PUFA concentrate contains 2 mg/g of mixed tocopherols to prevent oxidation.

Results

CHAPTER 1

Chronic docosahexaenoic acid supplementation improves metabolic plasticity in subcutaneous adipose tissue of aged obese female mice

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Abstract

This study aimed to characterize the potential beneficial effects of chronic docosahexaenoic acid (DHA) supplementation on restoring subcutaneous white adipose tissue (scWAT) plasticity in obese aged female mice. Two-month-old female C57BL/6J mice received a control (CT) or a high fat diet (HFD) for 4 months. Then, 6-month-old diet-induced obese (DIO) mice were distributed into the DIO and the DIOMEG group (fed with a DHA-enriched HFD) up to 18 months. In scWAT, the DHA-enriched diet reduced the mean adipocyte size and reversed the upregulation of lipogenic genes induced by the HFD, reaching values even lower than those observed in CT animals. DIO mice exhibited an up-regulation of lipolytic and fatty oxidation gene expressions that was reversed in DHA-supplemented mice except for *Cpt1a* mRNA levels, which were higher in DIOMEG as compared to CT mice. DHA restored the increase of proinflammatory genes observed in scWAT of DIO mice. While no changes were observed in total macrophage $F4/80^+/CD11b^+$ content, the DHA treatment switched scWAT macrophages profile by reducing the M1 marker *Cd11c* and increasing the M2 marker CD206. These events occurred alongside with a stimulation of beige adipocyte specific genes, and a recovery of the HFD-induced *Fgf21* upregulation. In summary, DHA supplementation induced a metabolic remodeling of scWAT to a healthier phenotype in aged obese mice by modulating genes controlling lipid accumulation in adipocytes, reducing the inflammatory status, and inducing beige adipocyte markers in obese aged mice.

Keywords: aging, obesity, inflammation, DHA, white adipose tissue, inflammation, beiging

1. Introduction

The prevalence of obesity is increasing worldwide, with one out of every two adults expected to suffer from obesity in developed countries by 2030[1], and being the prevalence now rising in low- and middle-income countries[2]. Aging occurs together with an increase in abdominal obesity and, in turn, obesity may accelerate the aging process[3]. Being the aged population predicted to rise over 1.5 billion by 2050[4], it is likely that a significant percentage of older adults present obesity and their associated comorbidities, including cardiovascular disease, diabetes mellitus, musculoskeletal disorders and certain types of cancer[2].

White adipose tissue (WAT) functions as an energy storage organ which is indispensable for maintaining metabolic homeostasis. WAT was classically categorized into subcutaneous WAT (scWAT), which constitutes the largest site of fat storage (nearly 80% of whole body fat); and visceral WAT (vWAT), which accounts for a small fraction of body fatness (approximately 20% of total body fat in men, and 5-8% in women)[5]. Fat depots differences are clinically relevant, as vWAT is highly related to insulin resistance and increased cardiometabolic risk, while scWAT seems to have a protective effect against metabolic syndrome[6]. However, scWAT undergoes significant changes in quantity and distribution during aging. Indeed, fat tissue mass increases through midlife and declines during the old age. Moreover, aging is associated with a redistribution of body WAT, characterized by an increased fat accumulation in vWAT, as well as ectopic fat deposition, with a decline in scWAT mass[7,8].

Certainly, WAT is the most plastic organ among the metabolically relevant tissues[9]. In WAT, the balance between lipogenesis, lipolysis, and fatty acid oxidation occurs depending on whole-body energetic status, in which WAT plasticity takes place by adapting to fasting and feeding conditions[10]. However, under obesogenic conditions and in aging, WAT metabolic flexibility is gradually lost. This is of special relevance in the scWAT depot, which loses its ability to buffer the excess of fat, leading to its ectopic accumulation in other organs and to the development of unhealthy phenotypes[11,12]. In advanced ages, vWAT has been reported as the most affected tissue and the responsible for the metabolic derangements. However, scWAT seems to be affected in the early aging stages, showing a defect in hypertrophy and/or hyperplasia, fibrosis, and inflammatory processes[13].

In addition to the classic thermogenic brown adipocytes, inducible brown-like adipocytes exist and reside mainly in scWAT. Termed as beige adipocytes, this type of fat cell has unique markers that differentiate them from white and brown adipocytes[14]. Growing evidence supports the induction of WAT browning (beige adipocytes formation) as a strategy to ameliorate the detrimental effects of excessive WAT accumulation and improve whole-body metabolic health[15]. Importantly, both obesity and aging have been related with a reduction in beige adipocyte formation[16–19].

Underlying both obesity and aging there is a chronic, low-grade systemic inflammation that has been related to the development of metabolic disorders such as insulin resistance and cardiovascular diseases, among others[20]. While this inflammatory state is primarily initiated in the dysfunctional WAT, it progressively becomes systemic. Under chronic exposure to excessive energy fuel or during aging, adipocytes become hypertrophic and exhibit an altered secretion of adipokines, chemokines and cytokines, characterized by reduced production of the antiinflammatory molecules (adiponectin, interleukin-10, IL-10) and increased release of proinflammatory signals (leptin, macrophage chemoattractant protein 1, MCP-1, IL-6 and IL-1β)[21–24]. On the other hand, growing evidence supports that an aberrant immune cell infiltration (macrophages, T

and B lymphocytes) within WAT stroma-vascular fraction also occurs in obesity and in aging, favoring the accumulation of a proinflammatory immune cell pool which also plays a relevant role in the development of the chronic subacute inflammatory status[25,26]. This inflammatory state, in turn, could also limit adipocytes function, leading to an impaired fat-handling capacity[24].

Dietary fat is an important nutritional component constituted by different types of fatty acids. In addition to fatty acids role in energy supply, they also constitute potent signaling molecules, even influencing transcriptional activity and triggering extensive physiological responses[27]. Thus, dietary fatty acids can specially modulate WAT function, as it is their main storage site[28]. Via WAT modulation, dietary fatty acids regulate metabolic health and play a key role in the promotion or prevention of obesity and inflammation during aging[29]. Indeed, saturated fatty acids stimulate inflammatory pathways through Toll like receptor 4 (TLR4) dependent and independent mechanisms, which interfere directly with insulin signaling and thus can lead to insulin resistance and metabolic disturbances in WAT[29]. By contrast, n-3 polyunsaturated fatty acids (n-3 PUFA) and especially those of marine origin, namely eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), have shown great potential to reduce inflammation and promote its resolution in WAT[30–36]. Therefore, n-3 PUFA have been proposed for the treatment of inflammatory processes, including obesity[37] and inflammaging-related disorders[38–40]. Besides their antiinflammatory effects, the ability of EPA and DHA to modulate white adipocytes and WAT function has been widely characterized, by our group and others, in rodents and humans[41]. In addition to the reduction in inflammation, EPA and DHA regulate adipogenesis[42], adipokine secretion[43–45], lipolysis, fatty acid oxidation[46–48], mitochondrial biogenesis, and adipose tissue browning[49–51]. However, these studies have been conducted in cultured systems and in young/middle-age mice. To our knowledge, there is no available data about the ability of n-3 PUFA to prevent WAT inflammation and metabolic inflexibility in aging, and especially under obesogenic conditions.

Importantly, several studies have characterized the differential effects between DHA and EPA in adipocytes, rodents and humans[41,42,52–54]. Interestingly, DHA and not EPA is inversely associated with fat mass independently from age, sex, ethnicity or cardiovascular disease history in large population studies like the National Health and Nutrition Examination Survey (NHANES)[42]. Moreover, the improvements in obesity-associated metainflammation have been suggested to occur mainly due to DHA supplementation[42,54].

Therefore, the aim of the current study was to characterize the potential effect of chronic DHA supplementation to restore scWAT plasticity in aged-obese female mice, through the study of changes in scWAT morphology and genes/proteins controlling scWAT function, inflammatory status, and beiging process.

2. Material and Methods

2.1. Animal experimental design

7 weeks old female C57BL/6J mice were obtained from Harlan Laboratories (Barcelona, Spain) and housed at the animal facilities of the University of Navarra under specific conditions (22 ± 2 °C, 12-h light/12-h dark cycle; relative humidity 55% ± 10%). After 10 days acclimation, animals were divided into the control group (CT group, n=9), fed a standard control diet up to 18 months of age (20% proteins, 67% carbohydrates, and 13% lipids; Harlan Teklad Global Diets, Harlan Laboratories, Indianapolis, IN, USA); and a diet-induced

obese (DIO, n=16) group, fed a high-fat diet (HFD, 20% proteins, 35% carbohydrates, and 45% lipids; Research Diets Inc., New Brunswick, NJ, USA) for 4 months. Thereafter, the 6-month-old DIO group was divided into 2 experimental groups: one continued with the HFD for 12 additional months (aged DIO group, 18 months old, n=10); and the other was fed with the HFD supplemented with a high DHA fish oil concentrate (aged DIOMEG group, 18 months old, n=6) replacing 15% w/w of dietary lipids for 12 additional months. The DHA-rich fish oil provided 683.4 mg DHA/g and 46.7 mg EPA/g, with a total of 838.9 mg n-3 PUFA/g as triglycerides (SOLUTEX0063TG, Solutex, Spain). Because it also contained 2 mg/g of mixed tocopherols (Covi-ox® T-70EU) to preserve n-3 PUFA from oxidation, the same amount was added to the HFD of the DIO mice that continued with the standard HFD during this experimental period. The different HFDs (prepared by Research Diets Inc., New Brunswick, N.J., USA) were vacuum sealed in 2.5 kg plastic bags and kept frozen (-20 °C) until used to avoid rancidity. Diets' compositions are specified in a previous study of our group[55].

All experimental groups were fed *ad libitum* and controlled for body weight changes 3 days/week during the whole experiment. Before sacrifice, whole-animal body composition was measured in live conscious animals with magnetic resonance technology (EchoMRI-100-700; Echo Medical Systems, Houston, TX, USA). At the end of the experiment, animals were fasted overnight, and fat depots (subcutaneous inguinal, mesenteric, gonadal, and retroperitoneal) were collected, weighted and frozen at -80 ºC. The visceral fat weight was calculated as the sum of mesenteric, gonadal, and retroperitoneal adipose depots. Prior to freezing, scWAT tissue samples were selected for histological analysis and isolation of stroma vascular fraction (SVF). Blood samples were collected, and serum was obtained and frozen at -80 ºC for biochemical determinations. All experiments were performed according to national animal care guidelines, and with the approval of the Ethics Committee for Animal Experimentation of the University of Navarra (protocol no. 113-15) in accordance with the EU Directive 2010/63/EU.

2.2. Biochemical analyses

Serum biochemical analyses were performed after a 12 h fasting period. Glucose, total cholesterol, and HDL-cholesterol serum levels were determined using a Pentra C200 autoanalyzer (HORIBA ABX, Madrid, Spain), following manufacturer's instructions. Insulin levels were determined with a commercially available ELISA kit (Mercodia, Uppsala, Sweden), according to manufacturer's guidelines. The HOMA-IR index was calculated as previously described[56].

2.3. Histological analyses

ScWAT tissue samples were extracted and fixed in formaldehyde 3.7 - 4.0% buffered to pH = 7 and stabilized with methanol for histology (Panreac Química S.L.U., Barcelona, Spain). Then, fat pads were embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Images were taken under a GFP-B filter (Ex 480/40; DM 505; BA 535/50) on a Nikon SMZ18 stereomicroscope equipped with a fluorescence system and a Nikon DS-Fi2 high-definition color camera (Nikon Instruments Inc., Tokyo, Japan). At least four different cross-sectional areas per sample were assessed for adipocyte area determination using ImageJ software (National Center for Biotechnology Information, Bethesda NCBI, MD, USA).

2.4. Stromal Vascular Fraction flow cytometry

scWAT SVF cells were isolated to analyze cell surface markers by flow cytometry. scWAT samples were cut into small pieces and digested with collagenase buffer (Sigma-Aldrich; St. Louis, MO, USA) during 45 min. Then, blood cells were lysed by the addition of ACK buffer (Gibco, Invitrogen Corporation; Carlsbad, CA, USA). For SVF isolation, cells were centrifuged at 300 *g* for 5 min and washed 3 times with DMEM-F12 medium (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Invitrogen).

The obtained SVF was disaggregated mechanically and filtered in 70 µm cell strainers (Falcon™, ref #352350, Corning, Arizona, USA). Then, 100 µl of the obtained cell fractions were incubated with the respective antibodies for 15 min at 4 °C. FcBlock was used to prevent non-specific binding of Fc receptor. The following antibodies were used to quantify immune cells by flow cytometry analyses: CD19 (B lymphocytes), CD3 (T lymphocytes), Ly6G/Ly6C (granulocytes) and F4/80 and CD11b (macrophages). To wash cells, PBS was added to each sample and centrifuged for 5 min at 1500 rpm. Then supernatant was decanted, and cells were stained with 7-AAD to assess cell viability (1/100 dilution in PBS) for 5 min at room temperature. Flow cytometry was performed in a FACSCantoII device (Becton Dickinson) and analyzed using the FlowJo software (TreeStar). A list of the antibodies used is shown in **Supplementary Table 1**.

2.5. CD206 Immunofluorescence

Paraffin-embedded fat tissue slides were deparaffinized and permeabilized by incubating for 10 min with PBS containing 0.25% Triton X-100 (PBST). Then, unspecific binding was blocked by incubating with 1% BSA in PBST for 30 min at room temperature. After that, samples were incubated with anti-CD206 (Proteintech, Rosemont, IL, USA) in 1% BSA in PBST for 1 h at room temperature and detected with the fluorescent secondary goat anti-rabbit Alexa Fluor™ 488 (Molecular Probes, Inc., Eugene, OR, USA). Finally, tissue samples were incubated with DAPI (Sigma-Aldrich, Darmstadt, Germany) during 1 min for nuclei staining. After a quick wash, the coverslip was mounted with mounting medium (Citifluor LTD, London, UK) and sealed to the slide. Images were acquired on a ZOE Fluorescent Cell Imager (Bio-Rad Laboratories, Hercules, CA, USA). Quantification of CD206 fluorescence was obtained by subtracting DAPI fluorescence, with at least three different cross-sectional areas per tissue sample using ImageJ software (NCBI).

2.6. Western Blot Analyses

ScWAT samples were homogenized with lysis buffer (Pierce® RIPA Buffer, Thermo Fisher Scientific) with 10 mM ethylenediaminetetraacetic acid and 100X protease and phosphatase inhibitor cocktail (Halt™, Thermo Fisher Scientific), and centrifuged at 20000 *g* for 15 min (4º C) to obtain the supernatant fraction containing the proteins. The protein extract concentrations were quantified with the BCA protein assay kit (Thermo Fisher Scientific).

Proteins extracts (40-60 µg) were resolved by electrophoresis on 12% SDS-polyacrylamide gel electrophoresis gels and electroblotted onto a polyvinylidene difluoride membrane (Amersham™ Hybond™, GE Healthcare Life Science, Freiburg, Germany), which was blocked with 1% BSA in TBS (Sigma-Aldrich) for 1 h at room temperature. Then, membranes were incubated with primary antibodies for UCP1 (Abcam, Cambridge, UK) and β-Actin (Sigma-Aldrich) overnight at 4 °C. After incubation, secondary goat anti-rabbit

IgG HRP (Bio-Rad) was used for 1 h at room temperature. The immunoreactive proteins were detected with enhanced chemiluminescence (Thermo Fisher Scientific) and quantified by densitometry analysis (Imagen Studio Lite; LI-COR Biosciences, Lincoln, Ne., USA). The results are expressed in relation to the CT group mean value, which was set to 1. **Supplementary Table 1** also lists the references to antibodies used for western blot analyses.

2.7. Gene expression analyses

For gene expression assays, total RNA was extracted with QIAzol lysis reagent® protocol (Qiagen; Venlo, Limburg, The Netherlands) and eluted in Rnase-free DEPC-treated water (Thermo Fisher Scientific) using the Rneasy MinElute Cleanup Kit (Qiagen; Venlo, Limburg, The Netherlands). RNA quality and quantity were measured (Nanodrop Spectrophotometer ND1000; Thermo Fisher Scientific) and then incubated (2 µg) for 30 min at 37 °C with Dnase I (Thermo Fisher Scientific) and reverse transcribed to cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturers' instructions.

Real-time PCR was performed using the Touch Real-Time PCR System (C1000 + CFX384, BIO-RAD, Hercules, CA, USA). Gene expression was analyzed using Taqman Universal Master Mix (Applied Biosystems) methodology with predesigned Taqman Assays-on-Demand; or by using Power SYBR® Green PCR (Bio-Rad). Primer-Blast software (NCBI) was used to design the Sybr Green primers.

Supplementary Tables 2-3 show oligonucleotides sequences : *Fatty acid synthase* (*Fasn), Diacylglycerol O-acyltransferase 1* (*Dgat1), Stearoyl-Coenzyme A desaturase 1 (Scd1), Lipoprotein lipase (Lpl*), *Patatin-like phospholipase domain containing 2* (*Pnpla2*, symbol *Atgl), Lipase, hormone sensitive (Lipe*, symbol *Hsl*), *Carnitine palmitoyltransferase 1a, liver (Cpt1a), Acyl-Coenzyme A oxidase 1, palmitoyl (Acox1), Toll-like receptor 4 (Tlr4), Interleukin 4 (Il4), Interleukin 6 (Il6), Interleukin 10 (Il10), Chemokine (C-C motif) ligand 2 (Ccl2), Integrin alpha X (Itgax*, symbol *Cd11c,), Adiponectin, C1Q and collagen domain containing (Adipoq), Leptin (Lep), Tumor necrosis factor receptor superfamily, member 9 (Tnfrsf9*, symbol *Cd137), T-box 1 (Tbx1), Transmembrane protein 26 (Tmem26), Transcription factor A, mitochondrial (Tfam), Free fatty acid receptor 4 (Ffar4,* symbol *Gpr120), Fibroblast growth factor 21 (Fgf21), klotho beta (Klb,* symbol *β-Klotho), Fibroblast growth factor receptor 1 (Fgfr1) and Early growth response 1 (Egr1).* Relative expression was determined by the $2^{-\Delta\Delta Ct}$ method[57] after normalization to *36b4* gene expression.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 (GraphPad software, La Jolla, CA, USA). Comparisons between groups were analyzed using 1-way ANOVA or Kruskal–Wallis test followed by post-hoc test for multiple groups-comparisons after testing the normality with Shapiro-Wilk tests. Differences were considered significant at a two-sided *P* value <0.05.

3. Results

3.1. Metabolic phenotype of aged, obese mice chronically supplemented with DHA

Body weight gain and body composition are shown in **Table 1**, along with fat depots weights in control (CT), DIO and DIOMEG mice at 18 months of age. As expected, DIO mice presented significantly higher body weight gain and whole-body fat mass. The increase in WAT weight was observed in both scWAT and vWAT depots compared to the aged lean mice. The DHA-enriched HFD tended to reduce vWAT (P=0.087) and scWAT (P=0.079), but no significant differences were observed as compared to the DIO group (**Table 1**).

Table 1. Body weight gain and body fat mass, subcutaneous and visceral fat depots weights, as well as atherogenic and insulin resistance indexes in the three experimental groups.

	CT	DIO	DIOMEG
Body weight gain (g)	6.61 ± 0.92	25.48 ± 3.15***	20.48 ± 1.66***
Fat mass (g)	8.47 ± 0.84	27.69 ± 2.16***	22.53 ± 1.31***
scWAT (g)	0.50 ± 0.07	2.41 ± 0.24***	1.76 ± 0.18***,†
vWAT (g)	1.30 ± 0.12	5.75 ± 0.59***	4.25 ± 0.22*,†
Total cholesterol/HDL-cholesterol	2.04 ± 0.03	2.51 ± 0.07***	1.78 ± 0.03$^{***,\#\#\#}$
HOMA-IR index	0.75 ± 0.07	1.53 ± 0.19**	1.24 ± 0.13**

CT: control; DIO: Diet-induced obese; DIOMEG: Diet-induced obese + DHA; scWAT: subcutaneous white adipose tissue; vWAT: visceral white adipose tissue. Data are expressed as mean ± SEM. (n=5-10).

$^{**}P$<0.01, $^{***}P$<0.001 *vs*. CT group, $^{\#\#\#}P$<0.001 *vs*. DIO group, $^{\dagger}P$=0.079-0.087 *vs*. DIO group.

DIO mice showed a significant increase in the atherogenic index (total cholesterol/HDL-cholesterol) and in HOMA-IR index as compared to age-matched CT mice (**Table 1**). The long-term feeding with the DHA-rich diet was able to decrease the atherogenic index even in the background of a HFD. However, the HOMA-IR was non-significantly reduced in the DHA-supplemented group, suggesting that DHA could be useful to prevent the lipid-related atherogenic risk, but less effective in controlling insulin resistance when associated to obesity and aging.

3.2. Effects of long-term DHA supplementation on subcutaneous WAT morphology and genes related to lipid accumulation/mobilization in aged obese mice

The histomorphological analysis of scWAT sections showed a predominance of enlarged hypertrophic adipocytes in the DIO group with almost absence of small size adipocytes as compared to the CT group. However, the DIOMEG group showed a mix of small, median, and big size adipocytes. Consequently, the analysis of the adipocytes size in the three experimental groups showed a dramatically higher average adipocyte area in the aged DIO mice in comparison with the aged CT mice, while the average adipocyte size was reduced in the DHA-supplemented mice even in the background of a HFD when compared to the DIO mice (**Figure 1A**). This indicates that the type and not only the amount of dietary fat play a key role in determining the accumulation of triglycerides in adipocyte fat droplets and, therefore, adipocyte size.

To gain a better insight of how dietary fat types can modulate the processes controlling adipocyte lipid turnover in scWAT, the expression of genes involved in lipogenesis, lipolysis and fatty acid oxidation was analyzed (**Figure 1B**). The long-term feeding with a HFD rich in saturated fat induced a marked increase in the mRNA levels of some of the main genes promoting fat accumulation in the adipocytes (*Fasn, Dgat1, Scd1 and Lpl*). However, the DHA-enriched HFD caused a dramatic downregulation of these lipogenic genes even to levels lower than those observed in the aged CT mice.

Curiously, the expression of lipolytic (*Hsl and Atgl*) and fatty acid oxidation genes (*Cpt1a and Acox1*) was also significantly increased in the DIO group as compared to the CT group (**Figure 1B**) and was reversed by the DHA-enriched HFD. Indeed, DIOMEG mice exhibited levels of *Hsl*, *Atgl* and *Acox1* similar or lower to those observed in the CT group, and only *Cpt1a* was significantly higher in the DIOMEG group as compared to CT mice (**Figure 1B**). Taken together, these observations suggests than DHA mainly prevent triglycerides accumulation in adipocytes of aged mice by downregulating the expression of lipogenic genes.

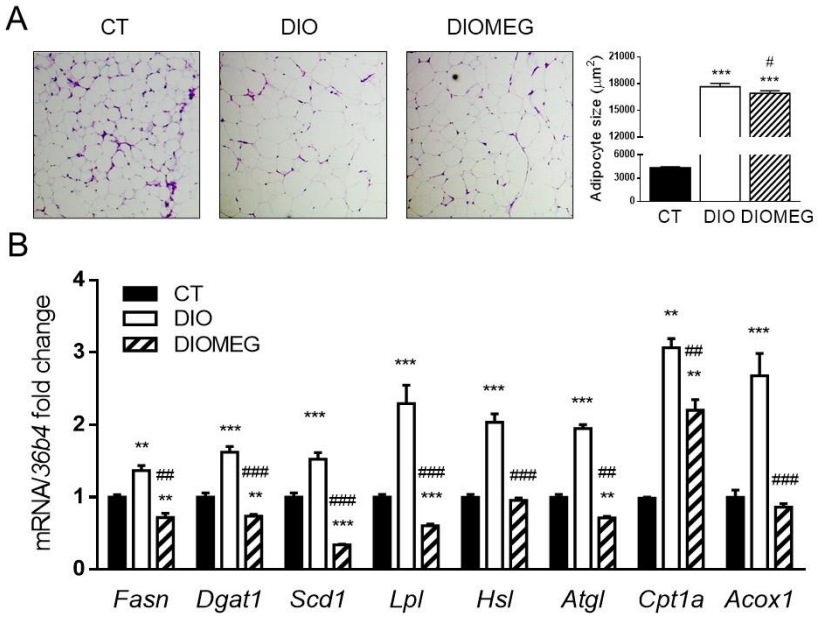

Figure 1. Effects of chronic DHA supplementation on subcutaneous white adipose tissue (scWAT) morphology and genes involved in adipocyte lipid turnover in 18 months old diet-induced obese (DIO) female mice. **A.** Representative H&E-stained paraffin-embedded sections of scWAT from aged CT, DIO and DIOMEG mice (left panel). Quantification of adipocytes area expressed as average (right panel). **B.** mRNA levels of lipogenic, lipolytic and fatty acid oxidation genes. Data are presented as mean ± SEM. (n=4-6). $^{**}P$<0.01, $^{***}P$<0.001 *vs.* CT group, $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 *vs.* DIO group. CT: Control; DIO: Diet-induced obese; DIOMEG: Diet-induced obese + DHA.

3.3 Subcutaneous WAT inflammatory status after chronic DHA supplementation in aged obese mice

Next, we also aimed to characterize if the chronic DHA supplementation could reduce the inflammatory status that characterizes scWAT in aging, and especially under obesogenic conditions. **Figure 2A** shows that the mRNA levels of mediators of the inflammatory signaling, such as *Tlr4*, the macrophage chemoattractant protein gene *Ccl2*, and the proinflammatory adipokine leptin (*Lep*) were all significantly upregulated in the aged obese mice compared to the aged CT group, while their levels were reverted to

those of the CT group in mice fed with the DHA-supplemented HFD. *Il6* mRNA expression did not change in the DIO nor in the DIOMEG group. Surprisingly, the expression levels of the anti-inflammatory interleukins *Il4* and *Il10*, as well as the antiinflammatory adipokine adiponectin (*Adipoq*), were also higher in DIO mice vs. CT mice. A similar stimulatory effect on *Il4* and *Il10* was observed in DHA-supplemented mice, while *Adipoq* mRNA returned to levels even lower than those found in CT mice (**Figure 2A**).

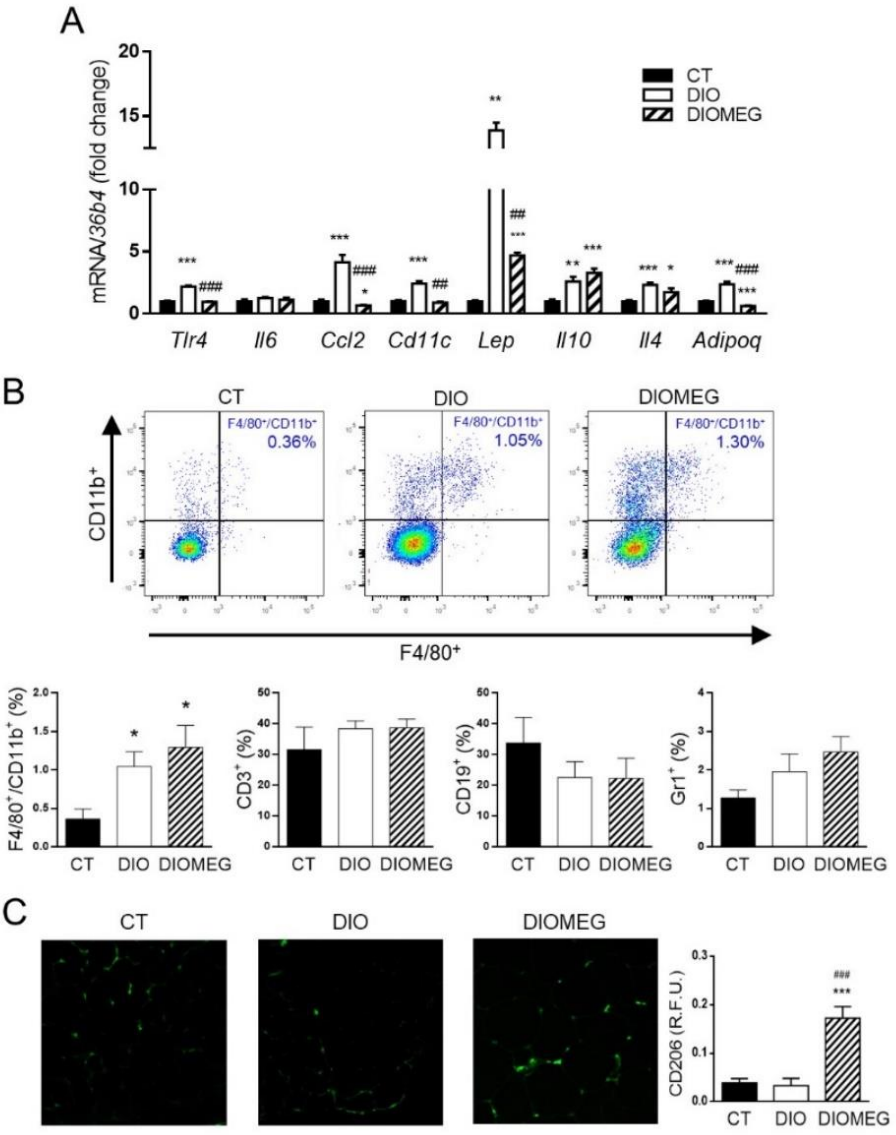

Figure 2. Effects of chronic DHA supplementation on the inflammatory profile of subcutaneous white adipose tissue (scWAT) in 18 months old diet-induced obese (DIO) female mice. **A.** mRNA levels of pro and anti-inflammatory makers (receptors, adipocytokines and chemokines) in scWAT. **B.** Characterization of immune cells in the stromal vascular fraction of scWAT by flow cytometry: macrophages ($F4/80^+/CD11b^+$), T lymphocytes ($CD3^+$), B lymphocytes ($CD19^+$), and granulocytes ($Gr1^+$). **C.** Representative images of immunofluorescence (IHF) labelling of CD206 in scWAT (left), and quantification of immunoreactive signals (right panel). Data are mean ± SEM. (n=4-8). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* CT group, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* DIO group. CT: Control; DIO: Diet-induced obese; DIOMEG: Diet-induced obese + DHA.

Because both diet-induced and age-related obesity can modify scWAT immune cell infiltration and thus contribute to scWAT inflammation[58,59], we also evaluated total macrophages (F4/80$^+$/CD11b$^+$ cells), T and B lymphocytes (CD3$^+$ and CD19$^+$, respectively), and granulocytes (Gr1+) populations by flow cytometry on WAT SVF. As shown in **Figure 2B**, the DIO group showed a significant increase in the total number of macrophages as compared to aged CT mice. A similar increment was observed in the DIOMEG group. No relevant changes were observed neither in T and B lymphocytes or in granulocytes subpopulations among the three experimental groups.

Regarding macrophages subpopulations, scWAT of DIO mice exhibited an increase in the mRNA levels of *Cd11c,* a marker of M1 macrophages (**Figure 2A**), while immunofluorescence-based analysis of scWAT confirmed no changes in the number of CD206$^+$ M2 anti-inflammatory macrophages (**Figure 2C**). These results indicated that long-term feeding with a HFD rich in saturated fat promotes the recruitment and infiltration of M1 proinflammatory macrophages in scWAT of aged DIO mice. In contrast, although the DHA-enriched HFD did not reduce the total macrophage content, it decreased the mRNA levels of *Cd11c* (**Figure 2A**) and highly increased the quantity of CD206$^+$ M2 anti-inflammatory macrophages (**Figure 2C**). These data suggest that 18 months old obese mice exhibited a decreased inflammatory status in scWAT that included the switch from M1 to M2 macrophages.

3.4. Beiging of subcutaneous WAT after chronic DHA supplementation in aged obese mice

Aging is also associated with a loss of beige adipocytes, which has been also related to the increased inflammatory state in WAT[60]. In order to address if chronic DHA supplementation could promote the beige phenotype in scWAT of aged mice, beige adipocytes-specific markers (*Cd137*, *Tbx1* and *Tmem26)*[14] were measured by qRT-PCR. Noteworthy, the expression of *Cd137* and *Tbx1* was downregulated by chronic high-fat feeding in DIO mice as compared to CT mice, and upregulated in DIOMEG mice fed with the DHA-enriched HFD. Moreover, the DHA-supplemented group also exhibited a marked upregulation of *Tmem26* and *Tfam*, a transcription factor involved in mitochondrial biogenesis. All these data point that long-term DHA supplementation prevents the loss of beige adipocytes induced by the obesogenic diet in aged mice. Moreover, the protein levels of the thermogenic UCP1 were downregulated in DIO mice, while they returned to values similar to those of CT mice, in DIOMEG animals (**Figure 3B**).

We also analyzed the expression levels of *Fgf21*, a relevant regulator of adipose tissue metabolism that has been shown to be involved in the browning of scWAT[61]. Our data show that *Fgf21* was significantly upregulated in DIO mice, while it was drastically downregulated in DHA-supplemented mice, even to lower levels than those of aged CT mice. The FGF21 signaling pathway was also evaluated by the assessment of gene expression levels of both components of its receptor (*Fgfr1* and *β-Klotho*) and a canonical target gene (*Egr1*)[62]. Hence, DIO mice exhibited a moderate upregulation of *β-Klotho* without changes in *Fgfr1* and *Egr1,* while both FGF21 receptor components and *Egr1* were significantly downregulated in DIOMEG mice fed with the DHA-enriched diet (**Figure 3C**). Finally, we also tested the expression levels of *Grp120*, a lipid sensor involved in the anti-inflammatory actions of n-3 PUFA[36] that also promotes scWAT browning and FGF21 release from adipocytes[50]. Our data show that *Grp120* mRNA levels followed a similar pattern that the observed for *Fgf21*, being upregulated in DIO animals, and downregulated in DIOMEG mice (**Figure 3C**). Hence, these data suggest that neither *Fgf21* nor *Gpr120* are involved in the stimulation of beige adipocyte markers induced by the DHA-enriched HFD in obese aged mice.

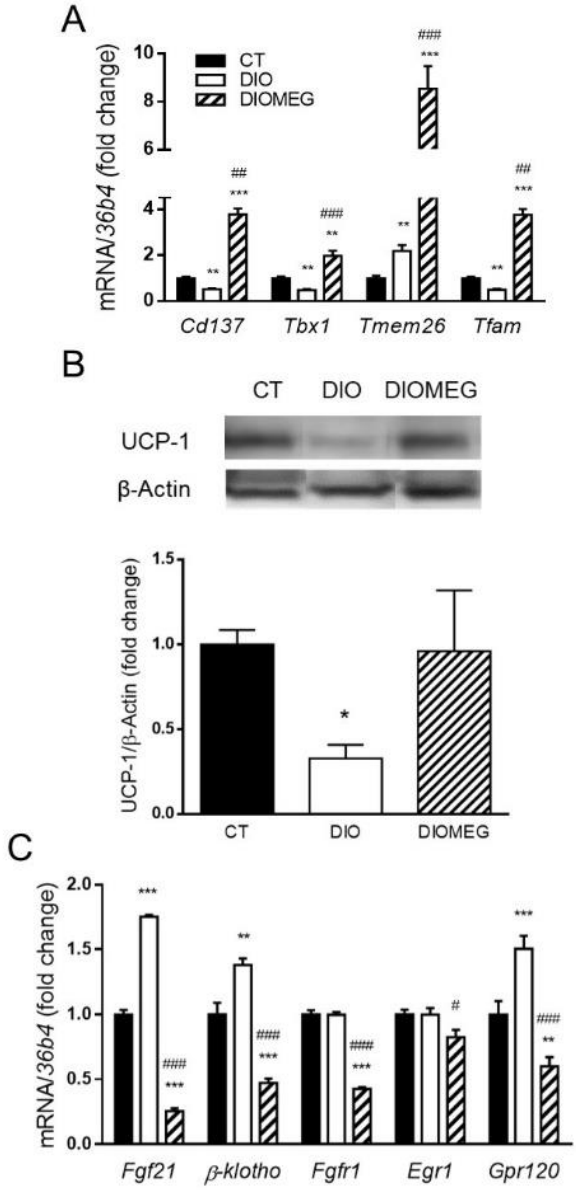

Figure 3. Effects of chronic DHA supplementation on gene characteristic of beige adipocytes and on the *Fgf21* signaling pathway in subcutaneous white adipose tissue (scWAT) in 18 months old diet-induced obese (DIO) mice. **A.** mRNA levels of gene characteristics of beige adipocytes and mitochondrial biogenesis. **B.** UCP1 protein content: representative western blot images and densitometric analysis. **C.** mRNA levels of *Gpr120*, *Fgf21* and of genes of the *Fgf21* signaling pathway. Data are mean ± SEM. (n=4-6). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* CT group, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* DIO group. CT: control; DIO: Diet-induced obese; DIOMEG: Diet-induced obese + DHA.

4. Discussion

Our data show that long-term dietary supplementation with DHA induces a metabolic remodeling in scWAT of aged, obese female mice by reducing adipocytes size and downregulating lipogenic genes, as well as through modulation of pro/antiinflammatory signals, and in parallel to an upregulation in beige-specific genes.

The accumulation of triglycerides in adipocytes depends on the balance between lipogenesis (triglycerides synthesis) and lipolysis/fatty acid oxidation (triglycerides breakdown). Triglycerides storage inside the lipid droplets starts with fatty acid uptake and subsequent reesterification into triglycerides or *de novo* fatty acid biosynthesis (*de novo* lipogenesis). In this context, LPL is the master regulator of fatty acids entrance into adipocytes, being its main function the lipolysis of chylomicron- and VLDL-derived triglycerides in adipose tissue capillaries. Our current data show that DHA supplementation reversed the upregulation of *Lpl* observed in DIO mice, which would reduce the access of fatty acids to adipocytes and their subsequential esterification into triglycerides. Indeed, adipose-specific *Lpl* knockout mice showed reduced adiposity when exposed to a HFD[63]. Moreover, the DHA-enriched diet also reduced *Dgat1,* one of the acyltransferases responsible for triglycerides esterification, according to previous studies showing reduced *Dgat1* expression in fat depots of rats fed with n-3 PUFA for 7 weeks[64]. Previous studies in different animal models have also suggested that n-3 PUFA supplementation could modulate adipocytes size through downregulation of *Fasn* and *Scd1*, key enzymes catalyzing the rate-limiting steps of *de novo* synthesis of fatty acid in WAT, but some contradictory data were found depending on the fat depot and the experimental conditions, including the animal model, the treatment duration and the n-3 PUFA formulation[64–66]. Our data show that chronic DHA feeding could inhibit both *Fasn* and *Scd1*, suggesting that inhibition of *de novo* lipogenesis may have accounted for a reduced fat accumulation in scWAT adipocytes of obese aged mice.

A striking finding was the fact that the aged DIO animals showed not only an increase in lipogenic genes[67], but also in lipolytic genes *Hsl* and *Atgl* in scWAT. In contrast, other studies have shown reduced HSL and ATGL in vWAT of mice chronically fed a HFD[68]. However, despair results can be found in the literature concerning the HFD-induced adaptations in the lipolytic pathway, with both upregulating[69] and downregulating effects[46] on *Hsl* and/or *Atgl* mRNA levels. The differential outcomes may be due to depot-specific metabolic responses to the HFD. In fact, large adipocytes were proved to present higher lipolytic rates than smaller adipocytes from the same subject[70]. Importantly, one of the mechanisms proposed to underlie the increased lipolysis that occurs in hypertrophic adipocytes is the proinflammatory microenvironment, with higher levels of proinflammatory cytokines that induce lipolysis[69]. The scWAT of our obese aged mice exhibited a significant increase in proinflammatory genes and increased infiltration of macrophages, which probably contributed to the upregulation observed in lipolytic genes[69]. By contrast, the scWAT of DHA-treated mice showed less hypertrophic adipocytes, an attenuated inflammatory status and, in turn, decreased levels of lipolytic genes. This hypothesis is in agreement with previous findings showing that n-3 PUFA reversed the lipolytic effect of proinflammatory cytokines in cultured adipocytes[46]. In parallel with the upregulated lipolytic genes, aged DIO mice also showed higher expression of genes involved in fatty acid oxidation (*Cpt1a* and *Acox1*), which could be an adaptive mechanism to reduce the fatty acid overload in scWAT. It is worth of mention that the DHA-enriched HFD also promoted an increase in *Cpt1a*, which facilitates fatty acids access to the mitochondria for oxidation, as compared to CT mice. This suggests a specific activation of mitochondrial fatty acid oxidation since *Acox1*, rate-limiting enzyme of peroxisomal fatty acid β-oxidation, was markedly lower in DIOMEG mice as compared to DIO mice and to CT mice. *Cpt1a* upregulation has been previously observed in epididymal WAT of younger C57BL/6J male mice fed with an n-3 PUFA-enriched diet, although accompanied with upregulated *Acox1*[71].

Our data support the efficiency of chronic DHA supplementation to ameliorate the scWAT inflammation associated to obesity and aging. This anti-inflammatory action occurred mainly *via* reduction of proinflammatory genes (*Tlr4, Ccl2 and Lep*), according to previous studies characterizing the anti-

inflammatory properties of n-3 PUFA in WAT of younger mice and rats[30,31,36]. An unexpected finding, however, was the upregulation of the well-known anti-inflammatory adipocytokines *Adipoq*, *Il4* and *Il10* mRNA levels in scWAT of DIO aged mice[72–74]. Nevertheless, other studies have also observed this upregulation in *Adipoq* and *Il10* in WAT in response to a HFD[34,73,75,76], suggesting that the increase in anti-inflammatory adipocytokines could represent a counteracting mechanism to balance the action of the proinflammatory ones[34,73,76]. These data could also explain the increased adiponectin levels observed in older people, which could entail a compensatory response to halt inflammaging and metabolic disorders[77,78]. In this way, the reduced *Adipoq* levels in animals fed with the DHA-enriched HFD could also be a consequence of the reduced inflammation observed in scWAT of these mice. These findings could also explain the paradox with studies reporting increased adiponectin levels after n-3 PUFA treatment in young mice[33].

On the other hand, mice fed with the DHA-enriched HFD revealed mRNA levels of *Il4* and *Il10* similar to those observed in DIO mice, but elevated when compared to CT mice, suggesting a direct stimulatory action of DHA on these cytokines. This occurred in parallel with the increase in the M2 macrophage marker CD206 and the reduction in the M1 marker *Cd11c*, which suggests that chronic DHA promoted the macrophages switch towards an M2 antiinflammatory phenotype, without significantly altering the content of total macrophages. These observations support the ability of DHA to reduce the ratio of M1-to-M2 macrophages which was described to be increased by aging or obesity[24,79]. Our finding in old obese mice also agree with those previously observed in younger mice, showing that n-3 PUFA favor a reduction in macrophage infiltration or promote the polarization towards an M2 antiinflammatory phenotype in vWAT[80]. We did not find changes in B and T lymphocytes nor in granulocytes populations in the scWAT SVF of DIO or DIOMEG mice, which contrast with other studies that have shown alterations in WAT B and T lymphocytes, as well as neutrophils, during obesity and/or aging[25,79,81]. This discrepancy could be due to the fact that these studies were carried out in vWAT and in male mice, which have described to be more prone to inflammation than female mice[82].

Studies analyzing the crosstalk between adipose tissue macrophages and adipocytes revealed that the recruitment of M2 macrophages plays a key role in the browning process of WAT[74,83]. In this way, the increase in M2 macrophages observed in scWAT of DHA-supplemented mice occurred in parallel with an increased gene expression of beige adipocytes markers (*Cd137, Tbx1, Tmem26*)[14] and with the upregulation of the main thermogenic marker UCP1. Noteworthy, this finding agrees with previous studies showing the browning properties of n-3 PUFA in WAT of younger mice[42,84,85]. A previous study has shown that G-Protein Coupled Receptor 120 (GPR120) is required for the thermogenic effects of EPA and for FGF21 induction and release on inguinal adipocytes[50]. However, our current data suggest that neither *Gpr120* nor *Fgf21* were mediating the stimulatory effects of DHA on genes and proteins involved in browning of scWAT. It is important to note that this study showed differential effects between EPA and DHA, which had barely significant effects[50]. Here, we show that the DHA-enriched diet clearly downregulated the upregulation of *Fgf21* observed in aged DIO mice. FGF21 has been demonstrated to be an important metabolic regulator with beneficial effects mediated by adiponectin on glucose and lipid metabolism[86]. However, other studies have suggested that FGF21 could promote the development of metabolically unhealthy adipocytes[87]. Obesity and aging have been associated to increased circulating levels of FGF21, which could entail a FGF21-resistant state[88,89]. This could explain the higher expression of *Fgf21* observed in scWAT of aged DIO mice, according to previous studies in obese mice[90]. However, we did not observe an impairment in the

expression of *Fgfr1* or *Egr1* in scWAT, suggesting that the signaling pathway of FGF21 was not altered at least in this fat depot. Nevertheless, we cannot rule out alterations at the protein level or defective signaling in other fat depots and/or tissues. FGF21 has been also characterized as a stress-induced hormone that plays a key role in the adaptive response to different stressors such as nutrient excess[91] and inflammation[92]. Therefore, the reduced *Fgf21* expression observed in scWAT of DHA-treated mice could be secondary to the reduction in the proinflammatory status and the decrease in lipid overload. Moreover, DHA also caused a significant reduction in the mRNA levels of both genes for FGF21 receptor components, *Fgfr1* and *β-klotho,* as well as in the downstream *Egr1*. The study by Villarroya *et al.*[93] showed that FGF21 target genes and receptors were not differentially affected by a n-3 PUFA-enriched HFD, suggesting that increased FGF21 levels were not mediating the beneficial effects of n-3 PUFA on high-fat feeding-associated metabolic disorders. Furthermore, it is important to mention that *Egr1* KO mice developed beige adipocytes in scWAT[94], and thus the reduced *Egr1* observed in DIOMEG mice could be a plausible mechanism contributing to the increase in beige adipocytes-markers in our aged, DHA-supplemented obese mice.

GPR120 has been demonstrated to exert beneficial antiinflammatory, antiobesity and insulin sensitizing effects in WAT[36,95]. Yet, controversial outcomes have been found concerning the role of GPR120 in mediating the beneficial effects of n-3 PUFA in WAT[96,97]. Our current data argue against a role of GPR120 in the beneficial actions of DHA in WAT, since a marked reduction in the expression of *Gpr120* was observed in our DHA-treated animals. Actually, GPRs are highly and rapidly regulated by many signals, including nutritional and inflammatory[97]. In this background, GPR120 has been recently shown to bind also to proinflammatory omega-6 (n-6) PUFA in culture[98], and to be correlated with inflammatory signals in inflammatory diseases like osteoarthritis[99].

Interestingly, the remodeling caused by the long-term DHA supplementation on scWAT could also contribute to the beneficial metabolic effects observed at systemic level such as the reduction observed on atherogenic index as estimated by total cholesterol/HDL-cholesterol ratio. Future studies should also focus in characterizing the transcriptomics and functional changes[100] induced by DHA on vWAT in obese aged mice, since vWAT accretion is a hallmark of aging[101]. Moreover, a sex-specific regulation of WAT function during aging and obesity[102,103] and a possible differential response to dietary supplementation cannot be discarded, and thus further studies that confirm the observed findings in aged male mice are needed to achieve a better understanding for the general population.

In summary, our study suggests that chronic feeding with a DHA-enriched HFD improves metabolic homeostasis in scWAT of aged obese female mice, by ameliorating inflammation, controlling metabolic remodeling of lipid pathways, and beiging of white adipocytes. These actions of DHA on scWAT metabolic plasticity could also contribute to the systemic beneficial effects observed in these aged mice, including the reduction of atherogenic index. Thus, we provide evidence that DHA could be a beneficial dietary fat to prevent/treat fat metabolic disturbances and inflammatory status associated to obesity and aging.

5. Conclusions

In summary, our study suggests that chronic feeding with a DHA-enriched HFD improves metabolic homeostasis in scWAT of aged obese female mice, by ameliorating inflammation, controlling metabolic remodeling of lipid pathways, and beiging of white adipocytes. These actions of DHA on scWAT metabolic plasticity could also contribute to the systemic beneficial effects observed in these aged mice, including the

reduction of atherogenic index. Thus, we provide evidence that DHA could be a beneficial dietary fat to prevent/treat fat metabolic disturbances and inflammatory status associated to obesity and aging.

References

1. Zachary J. Ward, Sara N. Bleich, Angie L. Cradock, et al. Projected U.S. state-level prevalence of adult obesity and severe obesity. *N Engl J Med*. 2019;381:2440-2450. doi:10.1056/NEJMsa1909301
2. World Health Organization. Obesity and overweight. 2020. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight
3. Salvestrini V, Sell C, Lorenzini A. Obesity may accelerate the aging process. *Front Endocrinol (Lausanne)*. 2019;10:266. doi:10.3389/fendo.2019.00266
4. United Nations Department of Economic and Social Affairs. World population prospects 2019: highlights.
5. Amaia Rodriguez, Victoria Catalan, Javier Gomez-Ambrosi, Gema Fruhbeck. Visceral and subcutaneous adiposity: are both potential therapeutic targets for tackling the metabolic syndrome? *Curr Pharm Des*. 2007;13:2169-2175. doi:10.2174/138161207781039599
6. Kwok KHM, Lam KSL, Xu A. Heterogeneity of white adipose tissue: molecular basis and clinical implications. *Exp Mol Med*. 2016;48:e215. doi:10.1038/emm.2016.5
7. Zamboni M, Nori N, Brunelli A, Zoico E. How does adipose tissue contribute to inflammageing? *Exp Gerontol*. 2021;143:111162. doi:10.1016/j.exger.2020.111162
8. Tchkonia T, Morbeck DE, Von Zglinicki T, et al. Fat tissue, aging, and cellular senescence. *Aging Cell*. 2010;9:667-684. doi:10.1111/j.1474-9726.2010.00608.x
9. Lee MJ, Wu Y, Fried SK. Adipose tissue heterogeneity: Implication of depot differences in adipose tissue for obesity complications. *Mol Aspects Med*. 2013;34:1-11. doi:10.1016/j.mam.2012.10.001
10. Zechner R, Zimmermann R, Eichmann TO, et al. Fat signals - lipases and lipolysis in lipid metabolism and signaling. *Cell Metab*. 2012;15:279-291. doi:10.1016/j.cmet.2011.12.018
11. Masoodi M, Kuda O, Rossmeisl M, Flachs P, Kopecky J. Lipid signaling in adipose tissue: Connecting inflammation & metabolism. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2015;1851:503-518. doi:10.1016/j.bbalip.2014.09.023
12. Sertié RAL, Caminhotto RDO, Andreotti S, et al. Metabolic adaptations in the adipose tissue that underlie the body fat mass gain in middle-aged rats. *Age*. 2015;37:87. doi:10.1007/s11357-015-9826-5
13. Corrales P, Vivas Y, Izquierdo-Lahuerta A, et al. Long-term caloric restriction ameliorates deleterious effects of aging on white and brown adipose tissue plasticity. *Aging Cell*. 2019;18:e12948. doi:10.1111/acel.12948
14. Wu J, Boström P, Sparks LM, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell*. 2012;150:366-376. doi:10.1016/j.cell.2012.05.016.Beige
15. Rabiee A. Beige fat maintenance; toward a sustained metabolic health. *Front Endocrinol (Lausanne)*. 2020;11:634. doi:10.3389/fendo.2020.00634
16. Berry DC, Jiang Y, Arpke RW, et al. Cellular aging contributes to failure of cold-induced beige adipocyte formation in old mice and humans differential anti-adipogenic effects of eicosapentaenoic and docosahexaenoic acids in obesity. *Cell Metab*. 2017;25:166-181. doi:10.1016/j.cmet.2016.10.023
17. Rogers NH, Landa A, Park S, Smith RG. Aging leads to a programmed loss of brown adipocytes in murine subcutaneous white adipose tissue. *Aging Cell*. 2012;11:1074-1083. doi:10.1111/acel.12010
18. Li H, Shen L, Zhang L, et al. Reduced beige adipogenic potential in subcutaneous adipocytes derived from obese Chinese individuals. *Diabetes, Metab Syndr Obes Targets Ther*. 2020;13:2551-2562. doi:10.2147/DMSO.S248112
19. Zoico E, Rubele S, De Caro A, et al. Brown and beige adipose tissue and aging. *Front Endocrinol (Lausanne)*. 2019;10:368. doi:10.3389/fendo.2019.00368

20. Livshits G, Kalinkovich A. Inflammaging as a common ground for the development and maintenance of sarcopenia, obesity, cardiomyopathy and dysbiosis. *Ageing Res Rev*. 2019;56:100980. doi:10.1016/j.arr.2019.100980

21. Rodríguez A, Ezquerro S, Méndez-Giménez L, Becerril S, Frühbeck G. Revisiting the adipocyte: a model for integration of cytokine signaling in the regulation of energy metabolism. *Am J Physiol - Endocrinol Metab*. 2015;309:E691-E714. doi:10.1152/ajpendo.00297.2015

22. Rojas JXS, Frutos MGS, Horrillo D, et al. Differential development of inflammation and insulin resistance in different adipose tissue depots along aging in wistar rats: effects of caloric restriction. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2016;71:310-322. doi:10.1093/gerona/glv117

23. Villarroya F, Cereijo R, Gavaldà-Navarro A, Villarroya J, Giralt M. Inflammation of brown/beige adipose tissues in obesity and metabolic disease. *J Intern Med*. 2018;284:492-504. doi:10.1111/joim.12803

24. Trim W, Turner JE, Thompson D. Parallels in immunometabolic adipose tissue dysfunction with ageing and obesity. *Front Immunol*. 2018;9:169. doi:10.3389/fimmu.2018.00169

25. Daniela Frasca, Diaz A, Romero M, Vazquez T, Blomberg BB. Obesity induces pro-inflammatory B cells and impairs B cell function in old mice. *Mech Ageing Dev*. 2017;162:91-99. doi:10.1016/j.mad.2017.01.004.

26. Shirakawa K, Yan X, Shinmura K, et al. Obesity accelerates T cell senescence in murine visceral adipose tissue. *J Clin Invest*. 2016;126:4626-4639. doi:10.1172/JCI88606

27. Ertunc ME, Hotamisligil GS. Lipid signaling and lipotoxicity in metaflammation: Indications for metabolic disease pathogenesis and treatment. *J Lipid Res*. 2016;57:2099-2114. doi:10.1194/jlr.R066514

28. Petrus P, Arner P. The impact of dietary fatty acids on human adipose tissue. *Proc Nutr Soc*. 2020;79:42-46. doi:10.1017/S0029665119000624

29. Glass CK, Olefsky JM. Inflammation and lipid signaling in the etiology of insulin resistance. *Cell Metab*. 2012;15:635-645. doi:10.1016/j.cmet.2012.04.001

30. Todoric J, Löffler M, Huber J, et al. Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia*. 2006;49:2109-2119. doi:10.1007/s00125-006-0300-x

31. Pérez-Matute P, Pérez-Echarri N, Martínez JA, Marti A, Moreno-Aliaga MJ. Eicosapentaenoic acid actions on adiposity and insulin resistance in control and high-fat-fed rats: role of apoptosis, adiponectinand tumour necrosis factor-α. *Br J Nutr*. 2007;97:389-398. doi:10.1017/S0007114507207627

32. Pérez-Echarri N, Pérez-Matute P, Marcos-Gómez B, et al. Differential inflammatory status in rats susceptible or resistant to diet-induced obesity: effects of EPA ethyl ester treatment. *Eur J Nutr*. 2008;47:380-386. doi:10.1007/s00394-008-0738-3

33. González-Périz A, Horrillo R, Ferré N, et al. Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB J*. 2009;23:1946-1957. doi:10.1096/fj.08-125674

34. Martínez-Fernández L, González-Muniesa P, Laiglesia LM, et al. Maresin 1 improves insulin sensitivity and attenuates adipose tissue inflammation in ob/ob and diet-induced obese mice. *FASEB J*. 2017;31:2135-2145. doi:10.1096/fj.201600859R

35. Huerta AE, Prieto-Hontoria PL, Sáinz N, Martínez JA, Moreno-Aliaga MJ. Supplementation with α-lipoic acid alone or in combination with eicosapentaenoic acid modulates the inflammatory status of healthy overweight or obese women consuming an energy-restricted diet. *J Nutr*. 2016;146:889S-896S. doi:10.3945/jn.115.224105

36. Oh DY, Talukdar S, Bae EJ, et al. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell*. 2010;142:687-698. doi:10.1016/j.cell.2010.07.041

37. Lorente-Cebrián S, Costa AG V, Navas-Carretero S, Zabala M, Alfredo Martínez J, Moreno-Aliaga MJ. Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: a review of the evidence. *J Physiol Biochem*. 2013;69:633-651. doi:10.1007/s13105-013-0265-4

38. Chen J, Wei Y, Chen X, Jiao J, Zhang Y. Polyunsaturated fatty acids ameliorate aging via redoxtelomere- antioncogene axis. *Oncotarget*. 2017;8:7301-7314. doi:10.18632/oncotarget.14236

39. Guo X fei, Li K lei, Li J mei, Li D. Effects of EPA and DHA on blood pressure and inflammatory factors: a meta-analysis of randomized controlled trials. *Crit Rev Food Sci Nutr*. 2019;59:3380-3393. doi:10.1080/10408398.2018.1492901

40. Troesch B, Eggersdorfer M, Laviano A, et al. Expert opinion on benefits of long-chain omega-3 fatty acids (DHA and EPA) in aging and clinical nutrition. *Nutrients*. 2020;12:2555. doi:10.3390/nu12092555

41. Saínz N, Fernández-Galilea M, Costa AGV, Prieto-Hontoria PL, Barraco GM, Moreno-Aliaga MJ. N-3 polyunsaturated fatty acids regulate chemerin in cultured adipocytes: role of GPR120 and derived lipid mediators. *Food Funct*. 2020;11:9057-9066. doi:10.1039/d0fo01445a

42. Zhuang P, Lu Y, Shou Q, et al. Differential anti-adipogenic effects of eicosapentaenoic and docosahexaenoic acids in obesity. *Mol Nutr Food Res*. 2019;63:e1801135. doi:10.1002/mnfr.201801135

43. Pérez-Matute P, Marti A, Martínez JA, et al. Eicosapentaenoic fatty acid increases leptin secretion from primary cultured rat adipocytes: role of glucose metabolism. *Am J Physiol - Regul Integr Comp Physiol*. 2005;288:R1682-8. doi:10.1152/ajpregu.00727.2004

44. Pérez-Echarri N, Pérez-Matute P, Marcos-Gómez B, Martínez JA, Moreno-Aliaga MJ. Effects of eicosapentaenoic acid ethyl ester on visfatin and apelin in lean and overweight (cafeteria diet-fed) rats. *Br J Nutr*. 2009;101:1059-1067. doi:10.1017/S0007114508048307

45. Lorente-Cebrián S, Bustos M, Marti A, Martinez JA, Moreno-Aliaga MJ. Eicosapentaenoic acid up-regulates apelin secretion and gene expression in 3T3-L1 adipocytes. *Mol Nutr Food Res*. 2010;54:104-111. doi:10.1002/mnfr.200900522

46. Lorente-Cebrián S, Bustos M, Marti A, Fernández-Galilea M, Martinez JA, Moreno-Aliaga MJ. Eicosapentaenoic acid inhibits tumour necrosis factor-α-induced lipolysis in murine cultured adipocytes. *J Nutr Biochem*. 2012;23:218-227. doi:10.1016/j.jnutbio.2010.11.018

47. Soni N, Ross AB, Scheers N, Nookaew I, Gabrielsson BG, Sandberg AS. The omega-3 fatty acids EPA and DHA, as a part of a murine high-fat diet, reduced lipid accumulation in brown and white adipose tissues. *Int J Mol Sci*. 2019;20:5895. doi:10.3390/ijms20235895

48. Flachs P, Rossmeisl M, Kuda O, Kopecky J. Stimulation of mitochondrial oxidative capacity in white fat independent of UCP1: A key to lean phenotype. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2013;1831:986-1003. doi:10.1016/j.bbalip.2013.02.003

49. Laiglesia LM, Lorente-Cebrián S, Prieto-Hontoria PL, et al. Eicosapentaenoic acid promotes mitochondrial biogenesis and beige-like features in subcutaneous adipocytes from overweight subjects. *J Nutr Biochem*. 2016;37:76-82. doi:10.1016/j.jnutbio.2016.07.019

50. Quesada-López T, Cereijo R, Turatsinze JV, et al. The lipid sensor GPR120 promotes brown fat activation and FGF21 release from adipocytes. *Nat Commun*. 2016;17:13479. doi:10.1038/ncomms13479

51. Bargut TCL, Martins FF, Santos LP, Aguila MB, Mandarim-de-Lacerda CA. Administration of eicosapentaenoic and docosahexaenoic acids may improve the remodeling and browning in subcutaneous white adipose tissue and thermogenic markers in brown adipose tissue in mice. *Mol Cell Endocrinol*. 2019;482:18-27. doi:10.1016/j.mce.2018.12.003

52. Tishinsky JM, Ma DWL, Robinson LE. Eicosapentaenoic acid and rosiglitazone increase adiponectin in an additive and PPARγ-dependent manner in human adipocytes. *Obesity*. 2011;19:262-268. doi:10.1038/oby.2010.186

53. Oster RT, Tishinsky JM, Yuan Z, Robinson LE. Docosahexaenoic acid increases cellular adiponectin mrna and secreted adiponectin protein, as well as pparg mrna, in 3T3-L1 adipocytes. *Appl Physiol Nutr Metab*. 2010;35:783-789. doi:10.1139/H10-076

54. Allaire J, Couture P, Leclerc M, et al. A randomized, crossover, head-to-head comparison of eicosapentaenoic acid and docosahexaenoic acid supplementation to reduce inflammation markers in men and women: the Comparing EPA to DHA (ComparED) Study. *Am J Clin Nutr*. 2016;104:280-287. doi:10.3945/ajcn.116.131896

55. Yang J, Sáinz N, Félix-soriano E, et al. Effects of long-term DHA supplementation and physical exercise on non-alcoholic fatty liver development in obese aged female mice. *Nutrients*. 2021;13:501. doi:10.3390/nu13020501

56. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28:412-419. doi:10.1007/BF00280883

57. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402-408. doi:10.1006/meth.2001.1262

58. Srikakulapu P, Mcnamara CA. B lymphocytes and adipose tissue inflammation. *Arterioscler Thromb Vasc Biol*. 2020;40:1110-1122. doi:10.1161/ATVBAHA.119.312467

59. Garg SK, Delaney C, Shi H, Yung R. Changes in adipose tissue macrophages and T cells during aging. *Crit Rev Immunol*. 2014;34:1-14. doi:10.1615/CritRevImmunol.2013006833

60. Villarroya F, Cereijo R, Villarroya J, Gavaldà-Navarro A, Giralt M. Toward an understanding of how immune cells control brown and beige adipobiology. *Cell Metab*. 2018;27:954-961. doi:10.1016/j.cmet.2018.04.006

61. Trellakis S, Rydleuskaya A, Fischer C, et al. Low adiponectin, high levels of apoptosis and increased peripheral blood neutrophil activity in healthy obese subjects. *Obes Facts*. 2012;5:305-318. doi:10.1159/000339452

62. Martínez-Fernández L, González-Muniesa P, Sáinz N, et al. Maresin 1 regulates hepatic FGF21 in diet-induced obese mice and in cultured hepatocytes. *Mol Nutr Food Res*. 2019;63:e1900358. doi:10.1002/mnfr.201900358

63. Bartelt A, Weigelt C, Cherradi ML, et al. Effects of adipocyte lipoprotein lipase on de novo lipogenesis and white adipose tissue browning. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2013;1831:934-942. doi:10.1016/j.bbalip.2012.11.011

64. Rokling-Andersen MH, Rustan AC, Wensaas AJ, et al. Marine n-3 fatty acids promote size reduction of visceral adipose depots, without altering body weight and composition, in male Wistar rats fed a high-fat diet. *Br J Nutr*. 2009;102:995-1006. doi:10.1017/S0007114509353210

65. Hensler M, Bardova K, Jilkova Z, et al. The inhibition of fat cell proliferation by n-3 fatty acids in dietary obese mice. *Lipids Health Dis*. 2011;10:128. doi:10.1186/1476-511X-10-128

66. Raclot T, Groscolas R, Langin D, Ferré P. Site-specific regulation of gene expression by n-3 polyunsaturated fatty acids in rat white adipose tissues. *J Lipid Res*. 1997;38:1963-1972. doi:10.1016/S0022-2275(20)37127-3

67. Bargut TCL, Souza-Mello V, Mandarim-De-Lacerda CA, Aguila MB. Fish oil diet modulates epididymal and inguinal adipocyte metabolism in mice. *Food Funct*. 2016;7:1468-1476. doi:10.1039/c5fo00909j

68. Jones JEC, Rabhi N, Orofino J, et al. The adipocyte acquires a fibroblast-like transcriptional signature in response to a high fat diet. *Sci Rep*. 2020;10:2380. doi:10.1038/s41598-020-59284-w.

69. Varghese M, Griffin C, Mckernan K, et al. Sex differences in inflammatory responses to adipose tissue lipolysis in diet-induced obesity. *Endocrinology*. 2019;160:293-312. doi:10.1210/en.2018-00797

70. Laurencikiene J, Skurk T, Kulyté A, et al. Regulation of lipolysis in small and large fat cells of the same subject. *J Clin Endocrinol Metab*. 2011;96:2045-2049. doi:10.1210/jc.2011-1702

71. Flachs P, Horakova O, Brauner P, et al. Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat. *Diabetologia*. 2005;48:2365-2375. doi:10.1007/s00125-005-1944-7

72. Singh P, Sharma P, Sahakyan KR, et al. Differential effects of leptin on adiponectin expression with weight gain versus obesity. *Int J Obes*. 2016;40:266-274. doi:10.1038/ijo.2015.181

73. Fujisaka S, Usui I, Bukhari A, et al. Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. *Diabetes*. 2009;58:2574-2582. doi:10.2337/db08-1475

74. Qiu Y, Nguyen KD, Odegaard JI, et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell*. 2014;157:1292-1308. doi:10.1016/j.cell.2014.03.066

75. da Cunha de Sá RDC, Cruz MM, de Farias TM, et al. Fish oil reverses metabolic syndrome, adipocyte dysfunction, and altered adipokines secretion triggered by high-fat diet-induced obesity. *Physiol Rep*. 2020;8:e14380. doi:10.14814/phy2.14380

76. Ludwig T, Worsch S, Heikenwalder M, Daniel H, Hauner H, Bader BL. Metabolic and immunomodulatory effects of n-3 fatty acids are different in mesenteric and epididymal adipose tissue of diet-induced obese mice. *Am J Physiol - Endocrinol Metab*. 2013;304:1140-1156. doi:10.1152/ajpendo.00171.2012

77. Kizer JR, Arnold AM, Strotmeyer ES, et al. Change in circulating adiponectin in advanced old age: determinants and impact on physical function and mortality. the cardiovascular health study all stars study. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2010;65:1208-1214. doi:10.1093/gerona/glq122

78. Schautz B, Later W, Heller M, Peters A, Müller MJ, Bosy-Westphal A. Impact of age on leptin and adiponectin independent of adiposity. *Br J Nutr*. 2012;108:363-370. doi:10.1017/S0007114511005605

79. Lumeng CN, Liu J, Geletka L, et al. Aging Is Associated with an Increase in T Cells and Inflammatory Macrophages in Visceral Adipose Tissue. *J Immunol*. 2011;187:6208-6216. doi:10.4049/jimmunol.1102188

80. Titos E, Rius B, González-Périz A, et al. Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. *J Immunol*. 2011;187:5408-5418. doi:10.4049/jimmunol.1100225

81. Watanabe Y, Nagai Y, Honda H, et al. Bidirectional crosstalk between neutrophils and adipocytes promotes adipose tissue inflammation. *FASEB J*. 2019;33:11821-11835. doi:10.1096/fj.201900477RR

82. Varghese M, Griffin C, McKernan K, Eter L, Abrishami S, Kanakadurga S. Female adipose tissue has improved adaptability and metabolic health compared to males in aged obesity. *Aging (Albany NY)*. 2020;12:1725-1746. doi:10.18632/aging.102709

83. Nguyen KD, Qiu Y, Cui X, et al. Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. *Nature*. 2011;480:104-108. doi:10.1038/nature10653

84. Kim M, Goto T, Yu R, et al. Fish oil intake induces UCP1 upregulation in brown and white adipose tissue via the sympathetic nervous system. *Sci Rep*. 2015;5:18013. doi:10.1038/srep18013

85. Bargut TCL, Silva-e-Silva ACAG, Souza-Mello V, Mandarim-de-Lacerda CA, Aguila MB. Mice fed fish oil diet and upregulation of brown adipose tissue thermogenic markers. *Eur J Nutr*. 2016;55:159-169. doi:10.1007/s00394-015-0834-0

86. Lin Z, Tian H, Lam KSL, et al. Adiponectin mediates the metabolic effects of FGF21 on glucose homeostasis and insulin sensitivity in mice. *Cell Metab*. 2013;17:779-789. doi:10.1016/j.cmet.2013.04.005

87. Berti L, Irmler M, Zdichavsky M, et al. Fibroblast growth factor 21 is elevated in metabolically unhealthy obesity and affects lipid deposition, adipogenesis, and adipokine secretion of human abdominal subcutaneous adipocytes. *Mol Metab*. 2015;4:519-527. doi:10.1016/j.molmet.2015.04.002

88. Fisher FM, Chui PC, Antonellis PJ, et al. Obesity is a fibroblast growth factor 21 (FGF21)-resistant state. *Diabetes*. 2010;59:2781-2789. doi:10.2337/db10-0193

89. Villarroya J, Gallego-Escuredo JM, Delgado-Anglés A, et al. Aging is associated with increased FGF21 levels but unaltered FGF21 responsiveness in adipose tissue. *Aging Cell*. 2018;17:e12822. doi:10.1111/acel.12822

90. Muise ES, Azzolina B, Kuo DW, et al. Adipose fibroblast growth factor 21 is up-regulated by peroxisome proliferator-activated receptor γ and altered metabolic states. *Mol Pharmacol*. 2008;74:403-412. doi:10.1124/mol.108.044826

91. Kim KH, Lee M-S. FGF21 as a stress hormone: the roles of FGF21 in stress adaptation and the treatment of metabolic diseases. *Diabetes Metab J*. 2014;38:245-251. doi:10.4093/dmj.2014.38.4.245

92. Feingold KR, Grunfeld C, Heuer JG, et al. FGF21 is increased by inflammatory stimuli and protects leptin-deficient ob/ob mice from the toxicity of sepsis. *Endocrinology*. 2012;153:2689-2700. doi:10.1210/en.2011-1496

93. Villarroya J, Flachs P, Redondo-Angulo I, et al. Fibroblast growth factor-21 and the beneficial effects of long-chain n-3 polyunsaturated fatty acids. *Lipids*. 2014;49:1081-1089. doi:10.1007/s11745-014-3948-x

94. Milet C, Bléher M, Allbright K, et al. Egr1 deficiency induces browning of inguinal subcutaneous white adipose tissue in mice. *Sci Rep*. 2017;7:16153. doi:10.1038/s41598-017-16543-7

95. Ichimura A, Hirasawa A, Poulain-Godefroy O, et al. Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. *Nature*. 2012;483:350-354. doi:10.1038/nature10798

96. Bjursell M, Xu X, Admyre T, et al. The beneficial effects of n-3 polyunsaturated fatty acids on diet induced obesity and impaired glucose control do not require Gpr120. *PLoS One*. 2014;9:e114942. doi:10.1371/journal.pone.0114942

97. Tan JK, Mckenzie C, Mari E, Macia L, Mackay CR. Metabolite-sensing G Protein-coupled receptors-facilitators of diet-related immune regulation. *Annu Rev Immunol*. 2017;35:371-402. doi:10.1146/annurev-immunol-051116-052235.

98. Mobraten K, Haug TM, Kleiveland CR, Lea T. Omega-3 and omega-6 PUFAs induce the same GPR120-mediated signalling events, but with different kinetics and intensity in Caco-2 cells. *Lipids Health Dis*. 2013;12:101. doi:10.1186/1476-511X-12-101

99. Chen Y, Zhang D, Ho KW, et al. GPR120 is an important inflammatory regulator in the development of osteoarthritis. *Arthritis Res Ther*. 2018;20:163. doi:10.1186/s13075-018-1660-6

100. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet*. 2012;13:227-232. doi:10.1038/nrg3185

101. Huffman DM, Barzilai N. Role of visceral adipose tissue in aging. *Biochim Biophys Acta - Gen Subj*. 2009;1790:1117-1123. doi:10.1016/j.bbagen.2009.01.008

102. Priego T, Sánchez J, Picó C, Palou A. Sex-differential expression of metabolism-related genes in response to a high-fat diet. *Obesity*. 2008;16:819-826. doi:10.1038/oby.2007.117

103. Van Den Beukel JC, Grefhorst A, Hoogduijn MJ, et al. Women have more potential to induce browning of perirenal adipose tissue than men. *Obesity*. 2015;23:1671-1679. doi:10.1002/oby.21166

Supporting information

Supplementary Table 1. Antibodies used for Flow Cytometry, Immunofluorescence, and Western-Blot analyses.

Antibody target	Experiment	Supplier	Catalog number
beta-Actin	Western Blot	Sigma-Aldrich	SAB5600204
CD11b FITC	Flow cytometry	Biolegend	Clone M1/70
CD19-APC-cy7	Flow cytometry	Biolegend	Clone 6D5
CD206	Immunofluorescence	Proteintech	18704 1-AP
CD3-PE-cy7	Flow cytometry	Biolegend	Clone 17A2
F4/80-PE	Flow cytometry	Biolegend	Clone BM8
IgG (HRP)	Western Blot	Bio-Rad	1705046
IgG (H+L)	Immunofluorescence	Alexa Fluor 488	A-11034
Ly6G/Ly6C-APC	Flow cytometry	Biolegend	Clone RB6-8C5
UCP1	Western Blot	Abcam	23841
7-AAD	Flow cytometry	Invitrogen	A1310

Supplementary Table 2. NCBI primer blast-designed oligonucleotides used for qRT-PCR analyses.

Gene symbol	Forward sequence	Reverse sequence
Acox1	5'-CTATGGGATCAGCCAGAAAG-3'	5'-AGTCAAAGGCATCCACCAA-3'
Adipoq	5'-AAGGGAGAGAAAGGAGATGC-3'	5'-TACACATAAGCGGCTTCTCC-3'
Ccl2	5'-AGCACCAGCCAACTCTCACT-3'	5'-TCATTGGGATCATCTTGCTG-3'
Cpt1a	5'-CACCAACGGGCTCATCTTCTA-3'	5'-CAAAATGACCTAGCCTTCTATCGAA
Dgat1	5'-GAGGCCTCTCTGCCCCTATG-3'	5'-GCCCCTGGACAACACAGACT-3'
Egr1	5'-GTCCTTTTCTGACATCGCTCTGA-3'	5'-CGAGTCGTTTGGCTGGGATA-3'
Fasn	5'-GCTGCGGAAACTTCAGGAAAT-3'	5'-AGAGACGTGTCACTCCTGGACTT
Ffar4 (Gpr120)	5'-GTGCCGGGACTGGTCATTGTG-3'	5'-TTGTTGGGACACTCGGATCTGG-3'
Fgf21	5'-CCTCTAGGTTTCTTTGCCAACAG-3'	5'-AAGCTGCAGGCCTCAGGAT-3'
Fgfr1	5'-TACAAGGTTCGCTATGCCAC-3'	5'-TGCGGAGATCGTTCCACGAC-3'
Il10	5'-AAGGCAGTGGAGCAGGTGAA-3'	5'-CCAGCAGACTCAATACACAC-3'
Il4	5'-ACAGGAGAAGGGACGCCAT-3'	5'-GAAGCCCTACAGACGAGCTCA-3'
Il6	5'-GAGGATACCACTCCCAACAGACC-3'	5'-AAGTGCATCATCGTTGTTCATACA-3'
Itgax (Cd11c)	5'-ACGTCAGTACAAGGAGATGTTGGA-3'	5'-ATCCTATTGCAGAATGCTTCTTTACC-3'
Klb (β-klotho)	5'-ACGACCCGACGAGGGCTGTT-3'	5'-GGAGGAGACCGTAAACTCGGGCTTA-3'
Lipe (Hsl)	5'-CTGCTTCTCCCTCTCGTCTG-3'	5'-CAAAATGGTCCTCTGCCTCT-3'
Lpl	5'-GCCAAGAGAAGCAGCAAGAT-3'	5'-CCATCCTCAGTCCCAGAAAA-3'
Tlr4	5'-TGGTTGCAGAAAATGCAGG-3'	5'-AGGAACTACCTCTATGCAGGG-3'

Supplementary Table 3. TaqMan Assays-on-Demand oligonucleotides used for qRT-PCR analyses.

Gene symbol	Catalog number
Lep	Mm00434759_m1
Pnpla2 (Atgl)	Mm00503040_m1
Scd1	Mm00772290_m1
Tbx1	Mm00448949_m1
Tfam	Mm00447485_m1
Tmem26	Mm01173641_m1
Tnfrsf9 (Cd137)	Mm00441899_m1

CHAPTER 2

Changes in brown adipose tissue lipid mediator signatures with aging, obesity, and DHA supplementation in female mice

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Abstract

Brown adipose tissue (BAT) dysfunction in aging and obesity has been related to chronic unresolved inflammation, which could be mediated by an impaired production of specialized proresolving lipid mediators (SPMs), such as Lipoxins-LXs, Resolvins-Rvs, Protectins-PDs and Maresins-MaRs. Our aim was to characterize the changes in BAT SPMs signatures and their association with BAT dysfunction during aging, especially under obesogenic conditions, and their modulation by a docosahexaenoic acid (DHA)-rich diet. Lipidomic, functional and molecular studies were performed in BAT of 2- and 18-months old lean (CT) female mice and in 18 months old diet-induced obese (DIO) mice fed a high-fat diet (HFD), or a DHA-enriched HFD. Aging downregulated Prdm16 and UCP1 levels, especially in DIO mice, while DHA partially restored them. Arachidonic acid (AA)-derived LXs and DHA-derived MaRs and PDs were the most abundant SPMs in BAT of young CT mice. Interestingly, the sum of LXs and of PDs were significantly lower in aged DIO mice compared to young CT mice. Some of the SPMs most significantly reduced in obese-aged mice included LXB4, MaR2, 4S,14S-diHDHA, 10S,17S-diHDHA (a.k.a. PDX) and RvD6. In contrast, DHA increased DHA-derived SPMs, without modifying LXs. However, MicroPET studies showed that DHA was not able to counteract the impaired cold-exposure response in BAT of obese-aged mice. Our data suggest that a defective SPMs production could underlie the decrease of BAT activity observed in obese-aged mice and highlight the relevance to further characterize the physiological role and therapeutic potential of specific SPMs on BAT development and function.

Keywords: brown adipose tissue, aging, obesity, DHA, lipidomic, proresolving lipid mediators.

1. Introduction

Brown adipose tissue (BAT) is a thermogenic tissue that dissipates energy as heat under certain stimuli, mainly through the uncoupling protein 1 (UCP1)[1]. Characterized by multilocular adipocytes with a high mitochondrial content, BAT also plays a relevant role in glucose homeostasis and triglyceride clearance[2,3]. In the last years, BAT has also emerged as a secretory organ that produces batokines which can influence the activity of other metabolic organs[4]. Growing evidence supports that BAT mass/activity negatively correlates with BMI, total and visceral adipose tissue, fasting glucose levels and insulin resistance in rodents and humans[5–9]. Therefore, BAT activation has been proposed as a target for the treatment of obesity and related metabolic disorders, including type 2 diabetes and dyslipidaemias[10].

However, BAT activity is gradually lost during the aging process[8,11] and accordingly, the percentage of adults showing detectable BAT decreases with age[12]. The reduced BAT activation that occurs in aging could favor fat accumulation in older individuals[12]. Therefore, one of the current challenges is to characterize the mechanisms leading to the age-induced reduction of BAT activity, and to discover effective strategies to prevent BAT loss or to reactivate existing BAT depots[13].

Inflammation seems to be a key process underlying both physiological and pathological processes of aging and obesity[14]. Indeed, both obesity and aging have been identified as chronic, low-grade inflammatory processes. Normal aging leads to a more proinflammatory profile that is further accentuated by increased adiposity[15]. White adipose tissue (WAT) dysfunction, characterized by increased expression of proinflammatory mediators including interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), and cyclooxygenase-2 (COX-2), and downregulation of the antiinflammatory peroxisome proliferator-activated receptor γ (PPAR-γ) and adiponectin, contributes to the development of the systemic inflammatory state during aging[16] and obesity[17]. In fact, the concept of 'Adipaging' has been proposed, as aging and obesity share inflammation and other biological hallmarks related to a dysfunctional adipose tissue[17].

Interestingly, BAT seems to be less susceptible to develop local inflammation in response to obesity than WAT[18]. However, strong/chronic proinflammatory signals can impair BAT insulin sensitivity and affect its glucose uptake, which is in turn essential for BAT function[1]. Moreover, UCP1-mediated cold-induced thermogenesis is severely impaired in inflamed BAT from diet induced obese (DIO) mice[19], and inflammation seems to inhibit the sympathetic tone in BAT through mechanisms yet to be elucidated[1].

In this context, resolution of inflammation is an active process which involves the production of specialized proresolving lipid mediators (SPMs) such as lipoxins (LXs), resolvins (Rvs), protectins (PDs) and maresins (MaRs). Noteworthy, SPMs are decreased in aging in human studies[20,21], and the time required for the resolution of inflammation is increased in aged mice[22]. On the other hand, it has been shown that WAT of obese mice exhibits an impaired production of some SPMs[23,24], an event that constitutes one of the earliest alterations in diet-induced inflammation[24], Interestingly, treatment with some of these SPMs (RvD1, MaR1) or precursors (17-HDHA) reduces WAT inflammation, improves insulin signaling and systemic insulin sensitivity, as well as reduces fatty liver[24–27].

With this regard, a lipidomic study has revealed a dramatic switch in BAT lipidome towards a WAT-like lipidome after a high-fat feeding period of 20 weeks[28]. Regarding SPMs, a recent study has described Lipoxin A4 (LXA_4) and SPMs precursors 18-HEPE, 17-HDHA and 15-HETE among the main contributors to

differentiate BAT from WAT fatty acid metabolomic phenotype[29]. However, studies characterizing changes in SPMs levels in BAT during aging and obesity are lacking in the current literature. n-3 polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) serve as substrates for SPMs (Rvs, PDs and MaRs). Thus, dietary enrichment with n-3 PUFA leads to an increase in SPMs production in several tissues, including WAT in rodents and humans[24,30–33]. Furthermore, several studies in cultured adipocytes and in animal models have proposed n-3 PUFA as novel inducers of BAT activity through the stimulation of UCP1, PR-domain containing 16 (PRDM16), peroxisome proliferator-activated receptors (PPARs), peroxisome proliferator-activated receptor γ co-activator 1 α (PGC1α), G-coupled protein receptor 120 (GPR120) and fibroblast growth factor 21 (FGF21)[34–37].

Based in these previous observations, our hypothesis is that the decay in BAT activity that occurs during aging and obesity could be the result of an impaired production of SPMs in this thermogenic tissue. Because n-3 PUFA serve as substrates for the synthesis of SPMs, we propose that long-term dietary supplementation with n-3 PUFA could restore SPMs levels and prevent the alterations in BAT function associated to obesity and aging. Therefore, the aim of this study was to characterize the changes in BAT SPMs signatures in young (2 months old) and aged (18 months old) female mice, as well as their potential associations with BAT function markers, especially in obesogenic conditions and under long-term dietary supplementation with a DHA-rich fish oil concentrate.

2. Materials and methods

2.1 Animal study design

Seven weeks-old female C57BL/6J mice were purchased from Harlan Laboratories (Barcelona, Spain) and housed at the animal facilities of the University of Navarra under controlled conditions (22 ± 2 ºC, 12-h light/12-h dark cycle; relative humidity 55% ± 10%). After 10-days acclimation, 15 animals (young CT, 2 months old) were sacrificed. Next, mice were divided into 2 experimental groups: a control group (aged CT, 18 months old, n = 15) fed a standard control diet up to 18 months of age (20% proteins, 67% carbohydrates, and 13% lipids; Harlan Teklad Global Diets, Harlan Laboratories, Indianapolis, IN, USA); and a diet-induced obese (DIO) group fed a high- saturated fat diet (High-fat diet, HFD, 20% proteins, 35% carbohydrates, and 45% lipids; Research Diets Inc., New Brunswick, N.J., USA) for 4 months. Afterwards, the 6 month-old DIO group was divided into 2 experimental groups: one continued with the HFD during 12 months (aged DIO, 18 months old, n = 14); and the DIO+omega-3 (aged DIOMEG, 18 months old, n = 11) that was fed for 12 months with the HFD supplemented with a high DHA fish oil concentrate (SOLUTEX0063TG, containing 683.4 mg DHA/g and 46.7 mg EPA/g, with a total content of 838.9 mg of n-3 PUFA/g as triglycerides, provided by Solutex, Spain), replacing 15% w/w of dietary lipids (Research Diets Inc., New Brunswick, N.J., USA). Because the DHA-rich n-3 PUFA concentrate contained 2 mg/g of mixed tocopherols (Covi-ox® T-70EU) to preserve from n-3 PUFA oxidation, the same amount was added to the HFD of the DIO mice that continued with the standard high-fat feeding during this experimental period (from 6 to 18 months). The different HFDs (prepared by Research Diets Inc) were vacuum sealed in 2.5 kg plastic bags and kept frozen (-20 °C) until used to avoid rancidity. Specific dietary compositions can be found in a recent manuscript of our group[38].

All experimental groups were fed *ad libitum*. Animals were controlled for weight and food intake 3 days/week during the whole experiment. At 2 and 18 months of age, animals underwent body composition analyses. After an overnight fast, fat depots (interscapular BAT, subcutaneous inguinal, mesenteric, gonadal and retroperitoneal WAT) were collected, weighted and frozen at -80 ºC. Total WAT weight was estimated as the weight sum of inguinal, gonadal, retroperitoneal, and mesenteric white fat depots. BAT samples for histological analysis were also obtained, and frozen BAT samples were used for lipidomic, protein and gene expression analyses. Blood samples were collected, and serum was obtained and frozen at -80 ºC for biochemical determinations. At 2 and 18 months of age, a subset of 5 animals per group underwent MicroPET imaging analyses for *in vivo* BAT activity determination. The animal experimental design is available at **Figure 1**.

The study was designed in female mice since sex-dependent inactivation of thermogenesis in BAT has been proposed as one of the mechanisms favoring fat accumulation, and underlying the higher propensity for obesity under hypercaloric conditions in female rats as compared to males[39]. Moreover, BAT thermogenic activity was found to be depressed in female rats during caloric restriction as compared to males.[40] Furthermore, a reduced norepinephrine turnover rate in cold-induced thermogenesis has also been described in older female rats, but not in male[41], suggesting a more impaired thermogenic response to diet and in aging in female than in male rodents.

All experiments were performed according to national animal care guidelines, with the approval of the Ethics Committee for Animal Experimentation of the University of Navarra (protocol no. 113-15) in accordance with the EU Directive 2010/63/EU.

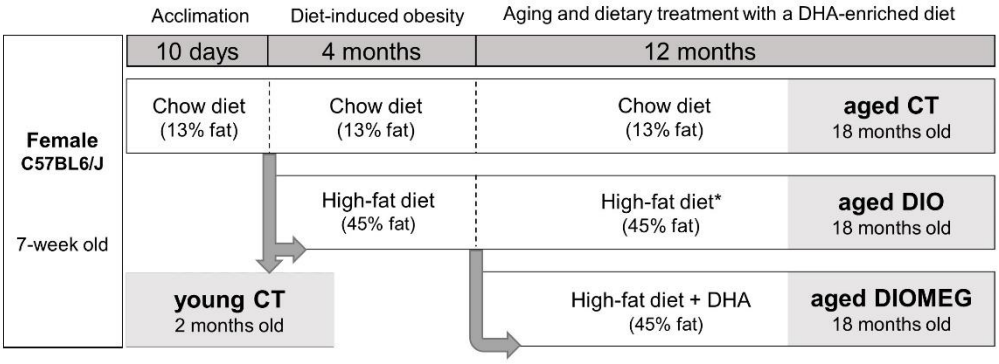


Figure 1. Animal experimental design. After 10 days of acclimation, 2 months old (young CT) mice were sacrificed. The rest of mice were divided into two groups, one fed with a standard diet (CT) and other fed with a high fat diet (HFD) for 4 months to induce obesity (DIO). These 6-month-old DIO mice were divided into two subgroups and aged up to 18 months: the aged DIO group was fed with the HFD* and the aged DIOMEG group was fed with a DHA-enriched HFD. The HFD* (from 6 to 18 months) was formulated with the same amount of tocopherol mix contained in the DHA-enriched HFD to preserve from oxidation. An aged CT group fed with the standard diet up to 18 months was also included. Body composition was determined in young CT and aged (CT, DIO and DIOMEG) groups. Serum and adipose tissue samples were also collected, and BAT was characterized (by morphological, gene and protein expression and lipidomic analysis). BAT activity was evaluated by MicroPET analysis.

2.2 Body composition analyses

Before the sacrifice, whole-animal body composition was measured in live conscious animals with Quantum molecular resonance technology (EchoMRI-100-700; Echo Medical Systems, Houston, TX, USA) as previously described[42].

2.3 Biochemical analysis

Serum biochemical analyses were performed after a 12 h fasting period. Glucose, total cholesterol, HDL-cholesterol, triglycerides, and β-hydroxybutyrate serum levels were determined using a Pentra C200 autoanalyzer (HORIBA ABX, Madrid, Spain), following manufacturer's instructions. LDL-cholesterol (LDL-chol) values were calculated using the Friedewald equation (LDL cholesterol = Total-cholesterol – HDL-cholesterol – triglycerides/5). Insulin levels were determined with a commercially available ELISA kit (Mercodia, Uppsala, Sweden), according to supplier's guidelines.

2.4 *In vivo* BAT activity by Micro-Positron Emission Tomography (MicroPET) assay

In vivo BAT activity was estimated by MicroPET imaging using the glucose analog [^{18}F]Fluoro-2-deoxy-2-D-glucose ([^{18}F]FDG). Studies were performed in a small animal Philips Mosaic tomograph (Cleveland, OH, USA) at the MicroPET Unit of Clínica Universidad de Navarra[43]. On the day of each PET study, mice were pre-exposed to cold stimulation for 1 h. [^{18}F]FDG (10.1 ± 0.9 MBq) was injected through the tail vein 1 h before PET scanning. Mice were under anesthesia throughout the PET acquisition procedure. For the assessment of [^{18}F]FDG uptake, all studies were exported and analyzed using the PMOD software (PMOD Technologies Ltd., Adliswil, Switzerland). Images were expressed in standardized uptake value (SUV) units, using the formula SUV = [tissue activity concentration (Bq/cm^3)/injected dose (Bq)] × body weight (g). For semiquantitative analysis, [^{18}F]FDG uptake by BAT was evaluated drawing volume-of-interest (VOIs) on coronal PET images including the interscapular BAT. From each VOI, maximum voxel intensity within the VOI (SUVmax) was recorded.

Ex vivo quantification of [^{18}F]FDG uptake in BAT depots was also measured. For this purpose, interscapular BAT depots were extracted immediately after PET imaging. Tissues were weighed and measured for [^{18}F]FDG activity on a high-energy γ counter. Uptake levels were expressed as percentage of injected dose per gram of tissue.

2.5 Histological analysis of BAT

BAT samples of the four experimental groups were fixed in 3.7-4.0% neutral formalin (pH 7.4) for 24 h, dehydrated with 70% ethanol, and embedded in paraffin. 5 µm thick sections were deparaffinized and stained with hematoxylin-eosin (H&E). BAT images (magnification 40X) were taken with an Olympus inverted microscope (CKX31SF, Olympus Corp., Tokyo, Japan) coupled to an Olympus C-5060 WIDE ZOOM digital compact camera (Olympus Corp.). Mean lipid droplet surface in BAT H&E-stained samples was quantified with ImageJ 2.0 imaging suite (U.S. National Institutes of Health, Bethesda, MD, USA).

2.6 BAT lipid mediator profiling

All samples were extracted using solid-phase extraction columns as previously described[44,45]. Prior to sample extraction, deuterated internal standards, representing each region in the chromatographic analysis (500 pg each) were added to facilitate quantification in 1 mL of methanol. Samples were kept at −20 °C for a minimum of 45 min to allow protein precipitation. Supernatants were subjected to solid phase extraction, methyl formate and methanol fractions were collected, brought to dryness, and suspended in phase (methanol/water, 1:1, vol/vol) for injection on a Shimadzu LC-20CE HPLC and a Shimadzu SIL-20AC autoinjector (Shimadzu Corp., Kyoto, Japan), paired with a QTrap 6500+ (Sciex, Warrington, UK). For identification and quantitation of products eluted in the methyl formate an Agilent Poroshell 120 EC-C18 column (100 mm × 4.6 mm × 2.7 µm, Agilent Technologies, Santa Clara, CA, USA) was kept at 50 °C and mediators eluted using a mobile phase consisting of methanol-water-acetic acid of 20:80:0.01 (vol/vol/vol) that was ramped to 50:50:0.01 (vol/vol/vol) over 0.5 min and then to 80:20:0.01 (vol/vol/vol) from 2 min to 11 min, maintained till 14.5 min and then rapidly ramped to 98:2:0.01 (vol/vol/vol) for the next 0.1 min. This was subsequently maintained at 98:2:0.01 (vol/vol/vol) for 5.4 min, and the flow rate was maintained at 0.5 ml/min. QTrap 6500+ was operated using a multiple reaction monitoring method coupled with information-dependent acquisition and enhanced production scan. In the analysis of peptide-lipid conjugated mediators eluted in the methanol fraction, Agilent Poroshell 120 EC-C18 column (100 mm × 4.6 mm × 2.7 µm, Agilent Technologies) was kept at 50 °C and mediators were eluted using a mobile phase consisting of methanol-water-acetic acid at 55:45:0.1 (vol:vol:vol) over 5 min, that was ramped to 80:20:0.1 (vol:vol:vol) for 2 min, maintained at 80:20:0.1 (vol:vol:vol) for the next 3 min, and ramped to 98:2:0.1 (vol:vol:vol) over 3 min. This was kept at 98:2:0.1 (vol:vol:vol) for 3 min. A flow rate of 0.60 ml/min was used throughout the experiment. QTrap 6500+ was operated in positive ionization mode using multiple reaction monitoring (MRM) coupled with information-dependent acquisition and enhanced production scan. Each LM was identified using established criteria, including matching retention time to synthetic and authentic materials and at least 6 diagnostic ions. Calibration curves were obtained for each using synthetic compound mixtures at 0.78, 1.56,3.12, 6.25, 12.5, 25, 50, 100, and 200 pg that gave linear calibration curves with an r^2 values of 0.98–0.99[46].

2.7 Protein isolation and Western Blot analyses

BAT samples were homogenized by sonication (SONOPULS Ultrasonic homogenizer HD 3100, Bandelin, Berlin, Germany) two times for 10 s each in 200 µL lysis buffer (8 mmol/L NaH_2PO_4, 42 mmol/L Na_2HPO_4, 1% sodium dodecyl sulfate (SDS), 0.1 mol/L NaCl, 0.1% NP40, 1 mmol/L NaF, 10 mmol/L sodium orthovanadate, 2 mmol/L phenylmethylsulphonylfluoride (PMSF), 10 mM ethylenediaminetetraacetic acid (EDTA) and 1% protease inhibitor cocktail 1 (Millipore Sigma, St. Louis, MO, USA). Then, samples were centrifuged at 13000 rpm for 15 min to obtain the supernatant fraction containing the proteins. The protein extracts were quantified with the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) to determine their concentration[47].

Protein extracts (15–20 µg) were resolved by electrophoresis on 12% SDS-polyacrylamide gels and then electroblotted onto a polyvinylidene difluoride membrane (Amersham™ Hybond™, GE Healthcare Life Science, Freiburg, Germany), which was blocked in TBS-Tween 1X buffer (TBS-T 1X) with 10% of milk (Nestle, Switzerland) for 1 h at room temperature. Then, primary antibody anti-UCP1 (rabbit, Abcam,

Cambridge, UK), was used at 1:1000 overnight at 4 °C. After incubation, goat anti-rabbit IgG peroxidase conjugated (HRP) secondary antibody (Cell Signaling Technology, MA, USA) was used at 1:10000 for 1 h at room temperature. The immunoreactive proteins were detected with enhanced chemiluminescence (Thermo Fisher Scientific) and quantified by densitometry analysis (LI-COR Biosciences, Lincoln, NE, USA). The results are expressed in relation to the young CT group value, which was set to 100%.

2.8 Gene expression by qRT-PCR

Total RNA from BAT depots was extracted with QIAzol Lysis reagent (Qiagen, Hilden, Germany). After quality and concentrations were measured (Nanodrop Spectrophotometer ND1000, Thermo Fisher Scientific), RNA (2 µg) was incubated with DNase I (Life Technologies, Carlsbad, CA, USA) for 30 min at 37 °C. Retrotranscription to cDNA was performed using High-Capacity cDNA Reverse Transcription (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the Touch Real-Time PCR System (C1000 + CFX384, BIO-RAD, Hercules, CA, USA). Gene expression was analyzed using Power SYBR® Green PCR (Bio-Rad, München, Germany). Primer-Blast software (National Center for Biotechnology Information, Bethesda, MD, USA (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the following primers: *Ccl2* (forward: 5'-AGCACCAGCCAACTCTCACT-3'; reverse: 5'-TCATTGGGATCATCTTGCTG-3'), *Fgf21* (forward: 5'-CCTCTAGGTTTCTTTGCCAACAG-3', reverse: 5'-AAGCTGCAGGCCTCAGGAT-3'), *Il10* (forward: 5'-AAGGCAGTGGAGCAGGTGAA-3', reverse: 5'-CCAGCAGACTCAATACACAC-3'), *Il4* (forward: 5'-ACAGGAGAAGGGACGCCAT-3', reverse: 5'-GAAGCCCTACAGACGAGCTCA-3'), *Prdm16* (forward: 5'-CAGCACGGTGAAGCCATTC-3', reverse: 5'-GCGTGCATCCGCTTGTG-3') and *36b4* (forward: 5'-CACTGGTCTAGGACCCGAGAAG-3', reverse: 5'-GGTGCCTCTGGAGATTTTCG-3'). Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method[48] after internal normalization to *36b4*.

2.9 Statistical analysis

Statistical analyses were performed using 1-way ANOVA or Kruskal–Wallis test followed by post-hoc test for multiple groups-comparisons, and unpaired Student's *t* or Mann-Whitney's U test for direct comparisons between 2 groups after testing the normality with the Kolmogorov-Smirnov and Shapiro-Wilk tests. GraphPad Prism 9 (La Jolla, CA, USA) software and Stata 14 (Stata, Collage Station, TX, USA) were used for statistical analysis. Results were expressed as mean ± SEM and differences were considered significant at a P value <0.05.

For lipid mediator analyses by PLSDA, its Scores Plot and VIP scores, as well as the heatmap of lipid mediator's distribution across the four experimental groups and the Pearson's correlations with BAT genes, MetaboAnalyst 4.0 (University of Alberta, Edmonton, AB, Canada) was used.

3. Results

3.1 Body composition, fat depots weight and serum biomarkers of glucose and lipid metabolism

Table 1 shows body composition data and serum glucose and lipid metabolism biomarkers of young (2 months old) and aged (18 months old) mice fed with a control diet (CT) or a HFD without (DIO) or with DHA

(DIOMEG). As expected, the aged CT mice exhibited increased body weight[47], fat mass, as well as hyperglycemia and higher levels of total and LDL-cholesterol as compared to young CT mice. The metabolic changes that occurred during aging were aggravated in the aged DIO mice, which accumulated more fat and exhibited a worsened hyperglycemia, hyperinsulinemia, and dyslipidemia (**Table 1**). Chronic feeding with the DHA-rich diet (aged DIOMEG group) tended to reduce body weight[38,47] and fat accumulation especially in the white adipose depots, in parallel with improved total chol, LDL-chol, and the atherogenic index LDL-cholesterol/HDL-cholesterol compared to the aged DIO group, without significantly affecting fasting glucose and insulin levels (**Table 1**). Thus, long-term feeding with a DHA-enriched HFD improved lipid serum profile as compared to aged DIO animals but had no significant effects on body composition and glucose metabolism biomarkers as compared to aged DIO mice.

Table 1. Effects of aging on body composition, white and brown adipose tissue weight and serum biomarkers of glucose and lipid metabolism in young and aged lean (CT), and aged obese mice fed with a rich-saturated fat diet (DIO) or a diet enriched in DHA (DIOMEG).

	young	aged		
	CT	CT	DIO	DIOMEG
Fat mass (%)	13.19 ± 0.59	29.82 ± 1.89***	53.44 ± 1.69$^{***,\#\#\#}$	50.45 ± 1.35$^{***,\#\#\#}$
BAT (g)	0.06 ± 0.00	0.09 ± 0.01***	0.26 ± 0.03$^{***,\#\#\#}$	0.20 ± 0.01$^{***,\#\#\#}$
WAT (g)	0.40 ± 0.03	1.79 ± 0.19***	8.15 ± 0.81$^{***,\#\#\#}$	5.66 ± 0.16$^{**,\#}$
Triglycerides (mg/dL)	52.50 ± 6.70	58.29 ± 3.26	66.67 ± 3.94	61.50 ± 6.25
Total cholesterol (mg/dL)	66.75 ± 4.85	98.86 ± 7.05*	134.78 ± 7.33$^{**,\#\#}$	90.75 ± 9.72‡
LDL-cholesterol (mg/dL)	21.95 ± 3.42	38.65 ± 3.72*	67.48 ± 5.07$^{**,\#\#}$	27.06 ± 3.76‡‡
HDL-cholesterol (mg/dL)	34.30 ± 2.54	48.55 ± 3.37*	53.93 ± 3.04**	51.39 ± 5.46
LDL-cholesterol/HDL-cholesterol	0.64 ± 0.08	0.79 ± 0.03	1.25 ± 0.07$^{**,\#\#\#}$	0.52 ± 0.03$^{\#\#,‡‡}$
β-Hydroxybutyrate (mmol/L)	1.53 ± 0.34	1.31 ± 0.15	1.32 ± 0.11	1.35 ± 0.31
Glucose (mmol/L)	4.89 ± 0.64	5.84 ± 0.49*	7.61 ± 0.50$^{**,\#}$	7.79 ± 0.68$^{**,\#}$
Insulin (mU/L)	2.99 ± 0.21	2.90 ± 0.06	4.43 ± 0.33$^{*,\#\#\#}$	3.61 ± 0.32$^{\#}$

Data are expressed as mean ± SEM. Body weight and fat mass (%) correspond to body composition analysis performed in non-fasted mice. BAT and WAT weights and biochemical analysis are from overnight fasted mice. (n=5-10).

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* young CT group. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* aged CT group. $^{‡}P<0.05$, $^{‡‡}P<0.01$ *vs.* aged DIO group.

3.2 Aging-induced morphological, transcriptomic, and functional changes in BAT of lean, obese and DHA-supplemented obese mice

In order to study the effects of aging and obesity and the long-term DHA supplementation on BAT biology, the changes on BAT morphology and on the expression of genes/proteins related to BAT development, function and inflammatory status were investigated. Aging induced lipid accumulation also in brown

adipocytes, as revealed by the increased weight and the histological images of BAT depots in young *vs.* aged CT mice. A dramatic BAT hypertrophy accompanied by the appearance of unilocular adipocytes was observed in the aged DIO group, which was partly attenuated in the aged DIOMEG group, although no significant differences were reached neither in BAT weight nor in adipocyte droplets size (**Table 1, Figure 2A, Supplementary Figure 1**).

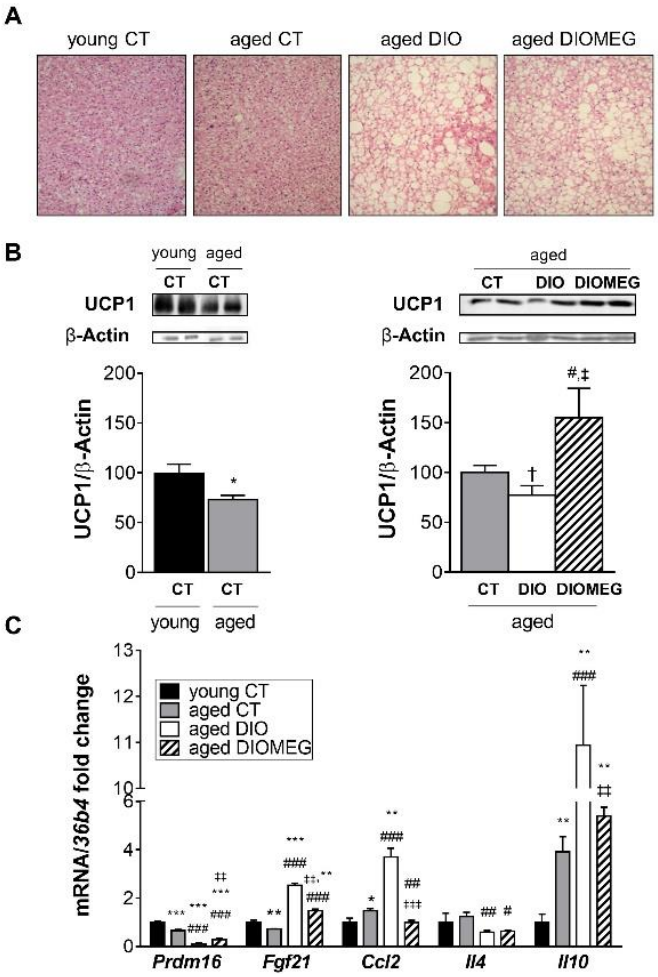


Figure 2. Differential effects of aging on BAT morphology, gene, and protein expression in young and aged lean (CT), as well as in obese female mice chronically fed with a HFD rich in saturated fat (DIO) or with a DHA-enriched HFD (DIOMEG) at 18 months of age. **A**. Representative BAT histology images. **B**. UCP1 protein levels in BAT of young *vs.* aged control mice (CT, left panel) and in BAT of aged CT, DIO and DIOMEG mice (right panel). Representative western blot (top panel) and densitometry analysis (bottom panel) of UCP1. Band densities of UCP1 were normalized to β-actin. **C**. mRNA levels of genes involved in BAT development,function, and inflammation. Data are mean ± SEM. (n=5-10). **P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* young CT group; $^{\#}$*P*<0.05, $^{\#\#}$*P*<0.01, $^{\#\#\#}$*P*<0.001 *vs.* aged CT group; ‡*P*<0.05, ‡‡*P*<0.01, ‡‡‡*P*<0.001 *vs.* aged DIO group; †*P*=0.071 *vs.* aged CT group.

We further evaluated if the hypertrophy of BAT associated to obesity and aging occurred alongside an altered expression of genes and proteins related to BAT development and function. Our data show that the levels of UCP1, the main responsible of BAT thermogenesis, was significantly reduced in aged CT as compared to young CT mice. Such reduction tended to be more pronounced in aged DIO mice. Noteworthy,

the HFD supplementation with DHA restored UCP1 expression in BAT of aged obese mice (**Figure 2B**). A similar pattern was observed for *Prdm16*, which is required for the maintenance and function of brown adipocytes in adult mice[49]. Indeed, *Prdm16* mRNA expression was reduced with aging and worsened by obesity, but partially recovered in the aged DIOMEG group (**Figure 2C**). Moreover, *fibroblast growth factor 21* (*Fgf21)*, which has been also related to brown fat activation[36], was decreased with aging in BAT of aged CT mice but increased in BAT of aged DIO mice. Increased FGF21 has been related to resistance to its action and as a mechanism to counteract the increased inflammatory/oxidative stress state associated to obesity[50].

Interestingly, *Fgf21* mRNA levels were normalized in the aged DIO mice fed with the DHA-enriched diet (**Figure 2C**). In line with this result, the mRNA levels of the proinflammatory marker *Ccl2* (encoding for macrophage chemoattractant protein 1, MCP-1) were increased by aging (aged CT) and further increased by obesity (aged DIO), but normalized to those of the aged CT mice with the DHA treatment in the aged DIOMEG group. By contrast, the levels of *Il4*, which induces M2 macrophages polarization[51], were decreased in the aged DIO group as compared with age-matched CT mice, but dietary DHA was not able to rescue this drop in *Il4* mRNA levels (**Figure 2C**). Strikingly, an upregulation of the antiinflammatory cytokine *Il10* was observed during aging and especially in obese mice, which was reversed in the obese mice receiving the DHA-supplemented diet (**Figure 2C**). Taken together, all these data suggest that DHA was able to reverse the detrimental gene and protein expression pattern in BAT induced by aging and further worsened by obesity, even in the absence of major morphological changes.

3.3 Effects of aging, obesity, and DHA-supplementation on BAT lipid mediators' signature

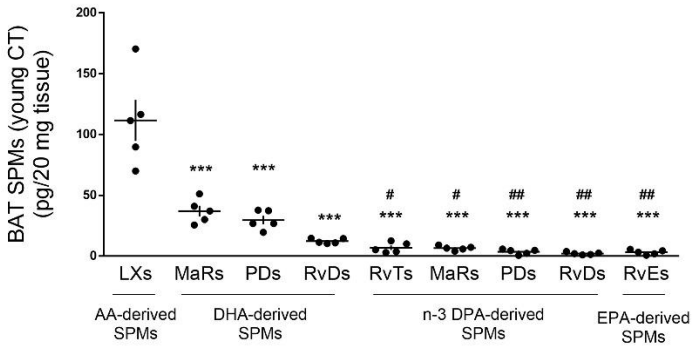


Figure 3. Abundance of AA, DHA, n-3 DPA and EPA-derived specialized proresolving lipid mediators (SPMs) in BAT from young female mice (2 months of age). (n=5). ***P<0.001, *vs.* LXs; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* MaRs.

Because lipid mediators have been considered as key regulators of BAT function[52,53], we next aimed to characterize the lipid mediator signature of BAT in young and aged mice as well as the changes induced by long term feeding with saturated fat or n-3 PUFA-enriched fat. Lipid mediator profiling of the n-3 Docosapentaenoic Acid (DPA), EPA, DHA, and Arachidonic acid (AA)-derived bioactive metabolomes demonstrated that the AA-derived LXs are the most abundant SPMs, followed by DHA-derived MaRs and PDs, in BAT of young female mice housed at 22 ºC (**Figure 3**). **Supplementary Figure 2** shows a heatmap of all lipid mediators analyzed, as well as the sum of each lipid mediator's classes in the young, the aged lean (CT), aged obese (DIO) and aged obese+DHA (DIOMEG) groups. **Figure 4A** shows the PLSDA

analysis of lipid mediators' concentrations in BAT of the four experimental groups. Colored ellipse areas display the 95% confidence region. As shown by the Scores Plot, the young and aged CT groups shared more similarities in lipid mediators' distribution than the aged DIO group, suggesting that the differences in lipid mediators induced by aging were aggravated by the diet-induced obesity. On the other hand, the aged DIOMEG group was clearly differentiated from the rest of the groups, despite being fed with a diet providing the same fat amount, but partially replaced by a high DHA n-3 PUFA concentrate. Moreover, the PLSDA VIP Scores revealed that mediators from the Rvs and MaRs families were predominant among the main 15 mediators contributing to such differences between groups (**Figure 4B**). Furthermore, the analyses of the total SPMs abundance between the four experimental groups showed that the aged DIO group displayed significantly lower SPMs levels when compared to the young CT group. Noteworthy, n-3 PUFA supplementation was able to significantly increase SPMs levels in the DIOMEG group compared not only to the aged CT and DIO groups, but also to the young CT group (**Figure 4C**).

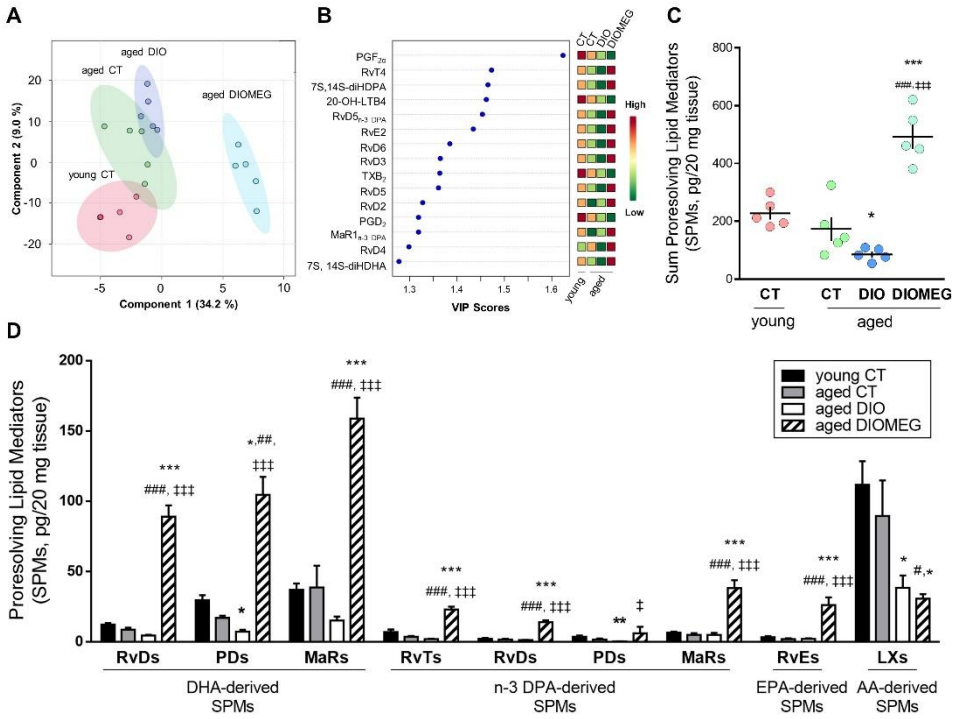


Figure 4. Changes in BAT lipid mediators profile induced by aging, obesity and dietary DHA supplementation. **A**. PLSDA analysis of lipid mediator concentrations in BAT of the four experimental groups. Colored spherical areas display the 95% confidence region. **B**. Vip Scores of the 15 lipid mediators contributing more to the separation between groups in the PLSDA model. **C**. Sum of specialized proresolving lipid mediators (SPMs) showing reduced levels in aged (18 months old) DIO mice but not in DIOMEG mice. **D**. Graph bar showing the differential distribution of the different types of SPMs (DHA, n-3 DPA, EPA and AA-derived) in the four groups of study. Data are mean ± SEM. (n=5). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001 *vs.* young CT group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 *vs.* aged CT group; $^{‡}P$<0.05, $^{‡‡‡}P$<0.001 *vs.* aged DIO group.

We further aimed to characterize the contribution of the n-3 DPA, DHA, EPA and AA-derived SPMs to the observed effects in BAT of the obese-aged mice and after the long-term feeding with the DHA-enriched young CT group (**Figure 4D**). The reduction in LXs levels found in the DIO-aged mice compared to CT young mice did not improve with n-3 PUFA supplementation, likely because LXs are produced from AA. In

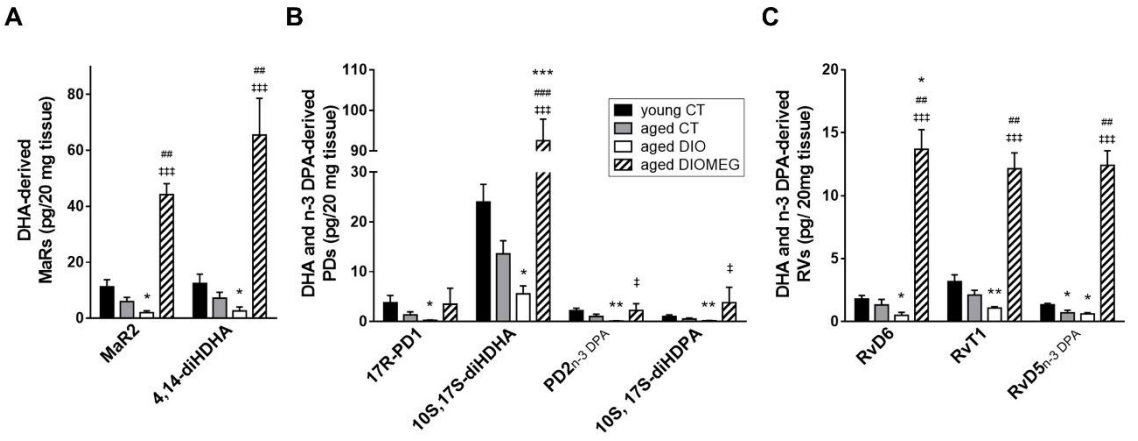


Figure 5. Quantification of principal changes induced by aging, obesity and dietary DHA supplementation on BAT specific SPMs and lipid intermediates of the DHA and/or n-3 DPA-derived Maresins (MaRs, **A**), Protectins (PDs, **B**) and Resolvins (Rvs, **C**) pathways. Data are mean ± SEM. (n=5). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* young CT group; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* aged CT group; $^{\ddagger}P<0.05$, $^{\ddagger\ddagger\ddagger}P<0.001$ *vs.* aged DIO group.

contrast, the sum of each n-3 DPA, DHA and EPA-derived SPMs classes was significantly elevated in those mice fed with the HFD DHA-rich HFD, reaching levels significantly higher than those found even in young CT mice (**Figure 4D**). Our next step was to analyze the changes in individual n-3 DPA, DHA and EPA-derived lipid mediators, to identify those with most relevance/contribution to the reduced levels of SPMs observed in aged obese animals, and those that were most markedly stimulated by n-3 PUFA supplementation. Among them, MaR2 and 4S,14S-diHDHA (**Figure 5A**), 10S,17S-diHDHA, 17R-PD1, $PD2_{n-3\ DPA}$ and 10S, 17S-diHDPA (**Figure 5B**), and RvD6, RvT1 and $RvD5_{n-3\ DPA}$ (**Figure 5C**) were the most decreased by the combination of chronic high-fat feeding and aging, as revealed by the comparison

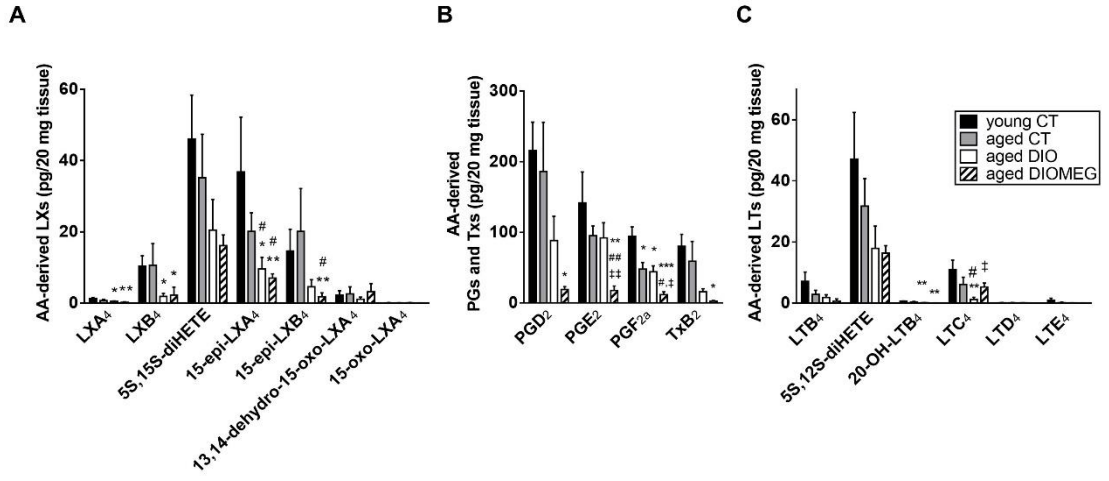


Figure 6. Characterization of changes in specific AA-derived lipid mediators involved in the Lipoxins (LXs, **A**) Prostaglandins and Thromboxanes (PGs and Txs, **B**) and Leukotrienes (LTs, **C**) pathways in BAT of female mice induced by aging, obesity and dietary DHA supplementation. Data are mean ± SEM. (n=5). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* young CT group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* aged CT group; $^{\ddagger}P<0.05$, $^{\ddagger\ddagger}P<0.01$ *vs.* aged DIO group.

of the aged DIO to the young CT group. More information concerning the changes in other SPMs and lipid intermediates of the DHA and n-3 DPA-derived MaRs, PDs and Rvs pathways can be found in **Supplementary Figure 3A-C**. It is worth mentioning that E-series Rvs concentrations were not affected by either age or obesity (**Supplementary Figure 3D**). Concerning the changes induced by long-term supplementation with the DHA-enriched diet on specific SPMs, our data revealed that almost all DHA and n-3 DPA derived MaRs (MaR1, MaR2, 4S,14S-diHDHA, 7S,14S-diHDHA, $MaR2_{n\text{-}3\ DPA}$, 7S,14S-diHDPA), PDs (PD1, 10S,17S-diHDHA, 17R-PD1, $PD2_{n\text{-}3\ DPA}$, 10S,17S-diHDPA) and Rvs (RvD1-6, 17R-RvD3, RvT1 and RvT4, and $RvD1_{n\text{-}3\ DPA}$, $RvD2_{n\text{-}3\ DPA}$, $RvD5_{n\text{-}3\ DPA}$) (**Supplementary Figure 3A-C**). Regarding EPA-derived Rvs, RvE2 and RvE3, but not RvE1, were markedly increased in the aged DIOMEG group (**Supplementary Figure 3D**). A moderate increase in some EPA-derived Rvs could be expected, as the DHA-rich n-3 PUFA concentrate also contained a small amount of EPA.

The analyzed AA-derived pro and antiinflammatory lipid mediators are shown in **Figure 6**. Our data revealed a significant decrease in LXA_4, LXB_4 and 15-epi-LXA_4 in BAT of aged DIO mice compared to young CT mice (**Figure 6A**). Another relevant finding was the decrease in $PGF_{2\alpha}$ observed in BAT of aged mice (both CT and DIO) when compared with young mice (**Figure 6B**). A reduction in LTC_4 and 20-OH-LTB_4 was also observed in aged DIO mice as compared to the young CT mice (**Figure 6C**). No other significant changes were observed in AA-derived lipid mediators because of aging and/or obesity. Regarding the effects of the DHA-rich HFD, PGE_2 and $PGF_{2\alpha}$ were decreased (**Figure 6B**), while LTC_4 was increased (**Figure 6C**), in the aged DIOMEG group as compared to the aged DIO group.

In summary, aging and obesity rather than aging alone had lowering effects on SPMs abundance, while few proinflammatory lipid mediators were significantly affected by these conditions. The DHA-rich HFD reverted this decrease in several of the DHA, n-3 DPA and EPA-derived SPMs, without restoring the AA-derived SPMs.

3.4. Correlations between BAT genes and lipidomics

With the aim to characterize the potential relationship between the levels of lipid mediators and the mRNA levels of genes involved in BAT development, function and inflammatory status, Pearson's correlations were carried out. **Figure 7** shows the heatmap of correlations between BAT genes (*Prdm16, Fgf21, Ccl2, Il4, Il10)* and each lipid mediator/pathway marker identified, or with sums of the different lipid mediator's families, considering all experimental groups of the study. *Prdm16* gene expression positively correlated with the sum of LXs, as well as with some individual lipid mediators from the LXs family (LXB_4, 15-epi-LXA_4 and 15-epi-LXB_4). A positive correlation was also found with LTC_4 and 5S,12S-diHETE (LTB_4 pathway marker), and with the sums of LTBs and cysLTs. These apparently contradictory results could be explained by the fact that these lipid mediators are all derived from the lipoxygenase (LOX) AA-pathway. Moreover, *Prdm16* correlated with 4S,14S-diHDHA, pathway marker of the DHA-derived MaRs.

Regarding *Fgf21,* its mRNA levels negatively correlated with some individual AA-derived proinflammatory lipid mediators (LTC_4, PGD_2, TxB_2), and with the sum of LTBs, cysLTs, TXs and the total of proinflammatory lipid mediators (Pro-LMs). Nevertheless, a negative correlation was observed for 15-epi-LXA_4, 15-epi-LXB_4 and for the sum of LXs, suggesting a role for both proinflammatory and proresolving AA-derived lipid mediators in the regulation of *Fgf21* in BAT.

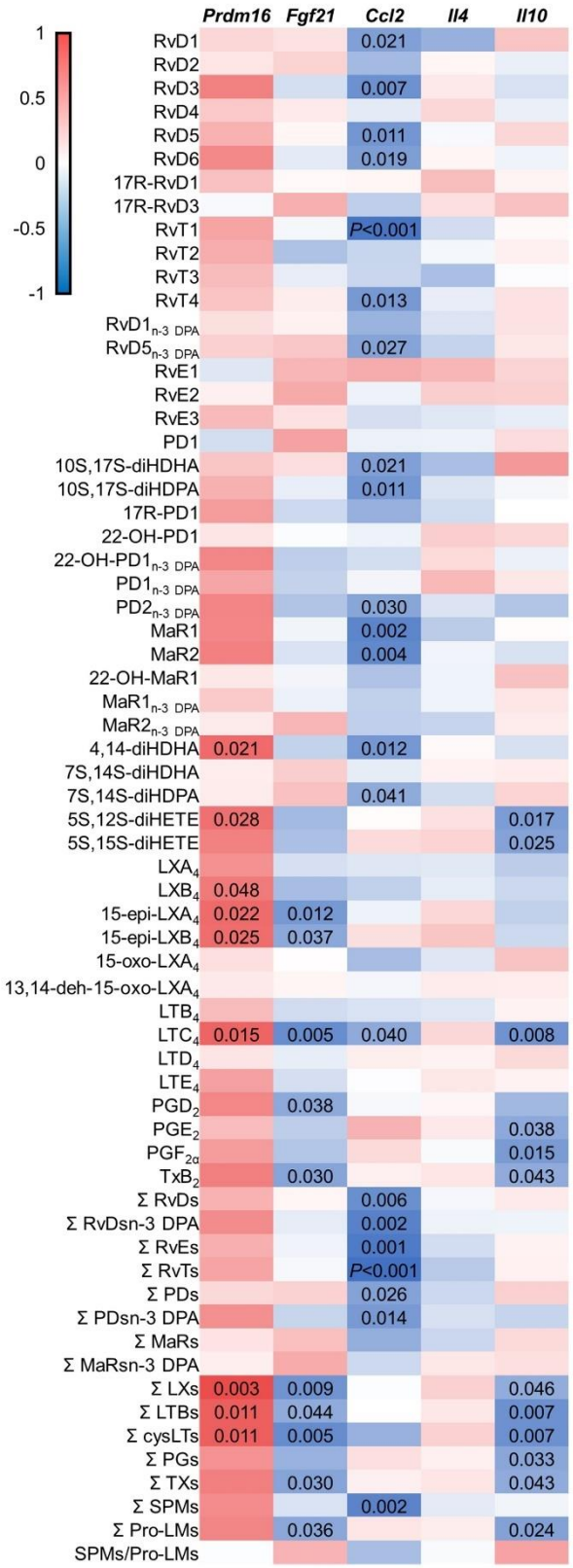


Figure 7. Heatmap showing Pearson's correlations of individualized lipid mediators and the sums of lipid mediator's classes with the expression of genes involved in BAT development, function, and inflammatory status (*Prdm6, Fgf21, Ccl2, Il10, Il4*). Negative correlations are shown in blue and positive correlations are in red (see color scale). *P* value is indicated for significant correlations ($P<0.05$).

Interestingly, the gene expression levels of the proinflammatory chemokine *Ccl2* (MCP-1) negatively correlated with the sum of SPMs and with the sum of all Rvs and PDs classes (RvDs, RvEs, RvTs, $RvDs_{n\text{-}3DPA}$, PDs and $PDs_{n\text{-}3DPA}$). Individual SPMs that negatively correlated with *Ccl2* included DHA and/or n-3 DPA derived RvD1, RvD3, RvD5, RvD6, RvT1, RvT4, $RvD5_{n\text{-}3DPA}$, 10S,17S-diHDHA, 10S,17S-diHDPA, $PD2_{n\text{-}3DPA}$, MaR1, MaR2, 4,14-diHDHA, and 7S,14S-diHDPA. Despite a negative correlation was also found with the proinflammatory LTC_4, all these observations suggest that the expression of this chemokine, which promotes the recruitment of M1 proinflammatory macrophages to adipose tissue, was clearly related to the levels of SPMs.

On the other hand, the mRNA expression of the anti-inflammatory cytokine *Il10* was negatively correlated with the sum of Pro-LMs and, accordingly, with the sum of LTBs, cysLTs, PGs, and TXs. Individual negative correlations were found for 5S,15S-diHETE, 5S,12S-diHETE, LTC_4, PGE_2, $PGF_{2\alpha}$ and TXB_2. Although no correlations were found between *Il10* and any of the individual LXs measured, a negative association was observed for the sum of LXs, suggesting a potential dual role for LOX-derived lipid mediators in the modulation of *Il10* mRNA levels, and *vice versa*. Despite IL-4 functions as an antiinflammatory interleukin are well established, no associations were found between any SPM and this interleukin. Altogether, these data suggest strong associations of the analyzed lipid mediators with BAT function and inflammatory status, which were more significant for *Prdm16*, *Ccl2* and *Il10* mRNA levels.

3.5. Effects of aging, obesity, and long-term DHA supplementation on cold-induced BAT activation

Finally, we evaluated the changes in BAT activity in response to cold activation during aging and under obesogenic conditions, as well as the potential ability of DHA to prevent the decline in BAT activation. BAT activity in response to cold exposure was estimated by in vivo [^{18}F]FDG uptake MicroPET (**Figure 8**). PET analysis (coronal images, SUVmax value and *ex vivo* quantification of [^{18}F]FDG uptake in BAT depots)[54] revealed a clear decrease in BAT activation with aging, which was dramatically exacerbated in aged, obese groups. Besides the beneficial effects of DHA on genes and proteins regulating BAT development and function (*Prdm16* and UCP1), chronic dietary supplementation with this fatty acid was not able to reverse the impaired BAT response to cold observed in aged DIO mice (**Figure 8A-C**).

4. Discussion

Here, we describe for the first time BAT composition in n-3 PUFA-derived and AA-derived proresolving lipid mediators in young control mice, along with their changes during aging, especially in obese mice and after chronic feeding with a DHA-enriched HFD.

Lipid mediator profiling studies of BAT revealed that the most abundant SPMs in CT mice at 2 months of age housed at room temperature were AA-derived LXs followed by DHA-derived MaRs and PDs. The most abundant AA-derived LXs pathway included 5S, 15S-diHETE, 15-epi-LXA_4 and LXB_4, suggesting a potential role of these lipid mediators in the active BAT. A recent study has shown that LXA_4 and its precursor 15-HETE are amongst the major contributors differentiating BAT from WAT under thermoneutrality conditions[29]. Moreover, a study has suggested the relevance of the LXs pathway in BAT activity. Indeed, the overexpression of the arachidonate 5-lipoxygenase-activating protein (ALOX5AP), which is involved in the

biosynthesis of LXs, increased LXA_4 production, upregulated UCP1 and thermogenesis, resulting in protection against diet-induced obesity and insulin resistance in mice[55]. In our study, LXA_4 was also identified in BAT of young mice, but the levels were lower than those of LXB_4 or 15-epi-LXA_4, suggesting an undescribed role for these two AA-derived lipid mediators in BAT function. Interestingly, the levels of 15-epi-LXA_4, LXB_4 and LXA_4 were significantly decreased in BAT of aged DIO mice, in parallel with the dramatic drop in BAT activity as revealed by the lower UCP1 levels and especially the [^{18}F]FDG uptake by this tissue. LXs were shown to be decreased in WAT from aging animals, which also showed decreased resolution of inflammation[22]. On the other hand, a study has shown that aging is accompanied by a profound decrease in LXA_4 urine levels, especially from middle-aged adulthood to older adults[21].

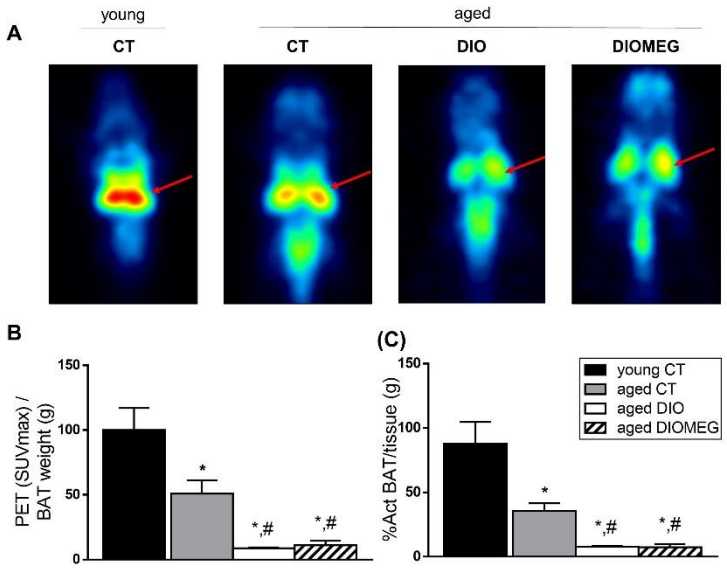


Figure 8. Cold-stimulated BAT activity is decreased with aging especially in obese mice, and it is not preserved by chronic DHA supplementation. **A**. BAT activity assessed by [^{18}F]FDG MicroPET in the four experimental groups. Mice were exposed for 1 h at 4 °C, prior injection of [^{18}F]FDG: coronal sections of mice. Red arrow: interscapular BAT pads. **B**. Maximum standardized uptake value (SUVmax/BAT weight (g)). **C**. *Ex vivo* quantification of [^{18}F]FDG uptake in BAT depots. (n=4-5). Data are mean ± SEM. $^{*}P<0.05$ *vs.* young CT group; $^{\#}P<0.05$ *vs.* aged CT group.

Concerning AA-derived lipid mediators, our study has also found that AA-derived prostaglandins (PGs: PGD_2, PGE_2 and $PGF_{2\alpha}$) constitute the most abundant analyzed lipid mediators in BAT of young mice. In addition to their role in inflammation, AA-derived PGs are also involved in the regulation of cell proliferation and differentiation and energy metabolism[56]. Indeed, a study has shown that the COX-2-derived PGs pathway seems to play a relevant role in controlling the differentiation of defined mesenchymal progenitors towards the phenotype of brown adipocytes[57]. Moreover, PGE_2 has been proposed to promote white-to-brown adipogenic differentiation[58]. In this way, the study of Garcia-Alonso *et al.*[59] has shown that PGE_2 reduced inflammatory genes (IL-6 and MCP-1) and induced the expression of brown markers (UCP1 and PRDM16) in WAT and adipocytes. Although PGE_2 levels were moderately reduced during aging in BAT, no significant changes were observed. In contrast, our study has revealed that the levels of $PGF_{2\alpha}$ were significantly inhibited in aged CT and DIO animals, suggesting that the drop in this eicosanoid could be

involved in BAT reduced activity during aging. A study in humans has shown that serum 15-keto-$PGF_{2\alpha}$ is decreased, and correlates negatively with aging in nonobese humans.[20] However, the role for PGE_2 and $PGF_{2\alpha}$ on thermogenic adipocytes is controversial, since other study has shown that AA inhibits the browning process in human adipose tissue-derived mesenchymal stem cells (hMADS) adipocytes through the secretion of PGE_2 and $PGF_{2\alpha}$[60]. Moreover, PGE_2 and $PGF_{2\alpha}$ as well as 10S,17S-diHDHA (PDX) were also recently unraveled as one of the main lipid mediators that contribute to differentiate BAT from WAT under thermoneutrality conditions[29].

Our study has also identified PDX as the most abundant DHA-derived PD in BAT of young mice that is reduced by aging specially in obese mice. PDX administration to obese diabetic mice improves insulin sensitivity by raising IL-6 skeletal muscle, without any impact on adipose tissue inflammation.[61] However, the same authors found that PDX is present in WAT and promotes PPAR-γ transcriptional activity in *fat1* transgenic mice[62]. Our current data suggest that PDX could play an uncharacterized role in BAT maintenance during aging.

DHA-derived MaR2 and 4S,14S-diHDHA are the most abundant molecules of the MaRs pathways, and both are significantly reduced during aging mainly in obese-aged mice. A similar trend was observed for MaR1 which was less abundant in BAT of young mice. MaRs, biosynthetized through the 12-LOX pathway, are macrophage mediators with potent antiinflamatory and proresolving actions[63,64]. Previous studies from our group have demonstrated that MaR1 attenuates inflammation, improve insulin resistance and restore metabolic pathways in adipocytes and in WAT of obese mice[26,65]. MaR1 has been also shown to resolve aged-associated macrophage inflammation to improve bone regeneration[66]. However, the actions of MaR2 in brown/white adipose tissue are still unknown. Clària *et al.*[23] found that 14-HDHA, a precursor of MaRs, was reduced in WAT of obese mice. Interestingly, Leiria *et al.*[53] described that 14-HDHA was increased in BAT from female mice after 7 days of cold exposure compared to thermoneutrality conditions, and that this change occurred along with improved glucose uptake in BAT. This observation clearly suggests a role of the MaRs pathway in the regulation of BAT thermogenesis.

Although the abundance of Rvs in BAT was significantly lower than LXs, PDs and MaRs, some of them showed relevant changes during obesity and aging. Thus, the levels of RvD6 and RvT1 were significantly lower in BAT of aged DIO mice. To our knowledge there is no previous data about the identification of these Rvs in adipose tissue or with changes in obesity. However, a metabolome study in human plasma identified RvD6 as a biomarker decreased in the aging process[20]. Although the effects of some Rvs such as RvD1 and RvD2 have been well characterized on the obese WAT[23,25,67], there is few information available about their effects on BAT. Interestingly, Pascoal *et al.*[68] found that the intracerebroventricular administration of RvD2 promotes UCP1 and PGC1α expression in BAT at doses of 3 ng, but not at 50 ng, which did not have any significant effect.

Few studies have analyzed the changes in BAT lipidomics during aging. A recent study using integrated metabolomics has found an altered lipid profile in BAT of an animal model of extreme longevity, the Ames dwarf mice, identifying increased levels of 5-HEPE, an n-3 PUFA metabolite, in BAT of these animals which exhibit increased thermogenesis[69].This study reinforces that changes in BAT lipid signatures might account for the changes in BAT activity during aging.

In this sense, our study provides novel observations supporting that the decrease in BAT activity that occurs with aging, and especially in obesogenic conditions, could be related with a decrease in the abundance of

proresolving lipid mediators in this tissue. However, the observations that the decrease in SPMs was much more pronounced in aged-obese than in aged-lean mice, suggests that the HFD feeding or obesity *per se* could be major determinants of SPMs reduction in BAT as compared to aging alone. Indeed, shorter periods of HFD-feeding or genetic obesity have been shown to reduce SPMs content in WAT[24]. Therefore, to better discriminate the effects of obesity and aging on BAT SPMs signature, it would be of interest to determine if high-fat feeding in younger animals is able to alter the SPMs profile of BAT in future studies.

In this context, a recent study in human adipose tissue has revealed that the presence of active BAT is positively associated with an antiinflammatory oxylipins/eicosanoids profile[70]. Similarly, EPA and DHA have been recently associated with the levels of BAT activity in humans[71]. A lipidomic analysis of BAT in young mice showed that phospholipids and free fatty acids were more abundant in BAT than in WAT, and that phospholipids in BAT were mostly composed of PUFA, especially DHA[72].

Our study shows that chronic dietary supplementation with DHA was able to reverse the drop in the sum of SPMs observed in BAT of aged DIO mice, reaching even values significantly higher than those found in BAT of young mice. This occurs in parallel with the upregulation of UCP1 and *Prdm16* and with a drop of proinflammatory genes such as *Ccl2* in BAT of DHA-supplemented aged DIOMEG mice. Several studies in cultured brown adipocytes have demonstrated the ability of n-3 PUFA, mainly EPA to induce brown development and thermogenic functions[36,73,74]. Moreover, several studies in mice have shown that dietary supplementation with n-3 PUFA-rich fish oil (8-12 weeks) induce UCP1 expression in BAT[35,74,75].

Our current data suggest that DHA supplementation could be an approach to restore the deleterious effects of aging and obesity on key genes and proteins involved in BAT development and function, and that these DHA's actions are mediated through the increase in the production of all types of n-3 PUFA-derived SPMs (PDs, MaRs, and Rvs). Previous studies have shown that DHA administration led to an increase in SPMs and oxylipins in WAT both in animal models of obesity[24,26,32] and obese subjects[33]. Such increase in SPMs occurred along with decreased time for resolution of inflammation, decreased macrophages infiltration and induction of an antiinflammatory phenotype. Our current data also show a relationship between the changes in SPMs and the expression of proinflammatory and antiinflammatory genes in BAT, suggesting a causative correlation between both facts. Only two recent investigations analyzed the effects of n-3 PUFA supplementation on BAT activity and its relation to SPMs. Colson *et al*.[76] recently compared the effects of 12 weeks feeding with a isocaloric diet enriched with n-6 PUFA or n-3 PUFA on oxylipin synthesis and adipose tissue inflammatory markers, observing that diet-enrichment with n-3 PUFA induced an increase in oxylipins derived from EPA and DHA, without affecting the n-6 AA-derived metabolites. However, the levels of the final metabolites of oxylipins-pathway (PDX, MaR1, RvD1, RvD2, RvE1) were barely or non-detected in this study as compared to our current data. However, DHA-derived LOX pathway 17-HDHA and 14-HDHA were detectable and increased with the n-3-PUFA enriched diet. In parallel, an increase in M2 macrophage markers was observed without changes in proinflammatory (*Il1β, Il2, Tnfa*) or antiinflammatory (*Il4, Il10*) gene markers in BAT. Nevertheless, mice were younger (22 weeks) and were housed in thermoneutrality conditions (28 ± 2 ºC), without a high-fat dietary challenge and with a different dietary content of n-3 PUFA. Interestingly, Ghandour *et al.*[77] found that dietary n-3 PUFA supplementation increased UCP1 in BAT and reduced the AA-derived TXB_2. Our current data also show that DHA-supplementation increased UCP1 in BAT and reduce TXB_2 levels in aged DIO mice.

We also tested if the increase in UCP1 and *Prdm16* along with the rise in n-3 PUFA-derived SPMs was associated with an improved BAT activity in response to cold-stimuli. Intriguingly, the MicroPET studies

revealed that the drop in BAT [^{18}F]FDG uptake observed in aged DIO mice was not recovered by chronic DHA supplementation, suggesting that other mechanisms involved in the cold-induced BAT activation impaired by obesity and aging are not restored by DHA supplementation. Sympathetic tone plays a key role in cold-induced BAT thermogenesis. Indeed, denervation of BAT in cold-exposed animals highlights the importance of its sympathetic innervation for thermogenic responses[78]. Moreover, an altered sympathetic tone has been hypothesized as a possible cause for the decrease in BAT recruitment in aging[79]. Importantly, a study has found that reduced cold-induced thermogenesis correlate with norepinephrine turnover rates that were reduced in older female rats, but not in male[41]. Our study has been carried out in female mice and therefore we hypothesized that the defective cold response in aged DIO mice could be secondary to altered sympathetic tone. In this way, a study showed that fish oil intake was not able to induce UCP1 expression in transient receptor potential vanilloid 1 (TRPV1) knockout mice, suggesting that n-3 PUFA could induce UCP1 upregulation in BAT via the sympathetic nervous system[35].

Another hypothesis for lack of relevant effects on BAT activity beside the increase in SPMs after chronic DHA supplementation could be that obesity and aging might also impair the expression/function SPMs receptors in BAT. No study has investigated this issue, but others have found decreased expression of these receptors (ALX/FPR2, Chemerin Receptor 23-ChemR23- and G-protein coupled receptor 32 -GPR32-) in WAT depots from obese mice and humans compared to lean WAT[23]. Moreover, the knockout mice for *ChemR23* and *ALX/FPR2* developed obesity and, over the course of 12 months, cardiometabolic dysfunctions typical of aging[80,81]. Future studies should focus in characterizing the changes in the expression and function of the SPMs receptors and SPMs signaling in BAT during aging and obesity, as they could be also relevant for the changes in BAT activation and for the efficient response to these metabolically active proresolving molecules.

In contrast to the rise observed for n-3 PUFA-derived SPMs, DHA supplementation was not able to reverse the dramatic drop in LXs observed in BAT of obese-aged mice. Taken into account the previously described thermogenic properties of the LXs pathway activation in BAT[55], our data suggest that LXs could be important in mediating the loss of BAT activity during aging and the cold-response capacity in this tissue. Indeed, we have observed a correlation between *Prdm16* and the sum of LXs in BAT. However, further studies are needed to test this possibility.

On the other hand, previous studies have suggested sex differences in BAT lipidomic profile[72]. Moreover, differential responses in BAT proteome and function have been observed between male and female after dietary interventions with both high-fat feeding or caloric restriction[40,82,83]. A limitation of our current study is that it has been conducted only in female mice. Therefore, it would be of high relevance to carry out further comparative studies of SPMs changes in aged obese male and female mice in future studies. Another interesting issue to be addressed in future trials is the characterization of the changes in SPMs in BAT under thermoneutrality and cold-exposure conditions.

In summary, our lipid mediator profiling analysis revealed for the first time that obesity promotes the decay in n-3 PUFA and AA-derived SPMs that occurs during aging in BAT, which relates with reduced markers of BAT function. Although long-term DHA supplementation prevented the drop in n-3 PUFA-derived SPMs in BAT, and improved BAT biomarkers, it was not able to restore the impaired response to cold exposure observed in obese-aged mice. Our study highlights the relevance to carry out further research to better characterize the physiological role of specific SPMs on BAT development and function as they could be potential therapeutic targets to modulate this thermogenic tissue.

5. Conclusions

In summary, our lipid mediator profiling analysis revealed for the first time that obesity promotes the decay in n-3 PUFA and AA-derived SPMs that occurs during aging in BAT, which relates with reduced markers of BAT function. Although long-term DHA supplementation prevented the drop in n-3 PUFA-derived SPMs in BAT, and improved BAT biomarkers, it was not able to restore the impaired response to cold exposure observed in obese-aged mice. Our study highlights the relevance to carry out further research to better characterize the physiological role of specific SPMs on BAT development and function as they could be potential therapeutic targets to modulate this thermogenic tissue.

References

1. Villarroya F, Cereijo R, Gavaldà-Navarro A, Villarroya J, Giralt M. Inflammation of brown/beige adipose tissues in obesity and metabolic disease. *J Intern Med*. 2018;284(5):492-504. doi:10.1111/joim.12803.

2. Chondronikola M, Volpi E, Børsheim E, et al. Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. *Diabetes*. 2014;63(12):4089-4099. doi:10.2337/db14-0746.

3. Bartelt A, Bruns OT, Reimer R, et al. Brown adipose tissue activity controls triglyceride clearance. *Nat Med*. 2011;17(2):200-206. doi:10.1038/nm.2297.

4. Villarroya F, Cereijo R, Villarroya J, Giralt M. Brown adipose tissue as a secretory organ. *Nat Rev Endocrinol*. 2017;13(1):26-35. doi:10.1038/nrendo.2016.136.

5. Fuse S, Sugimoto M, Kurosawa Y, et al. Relationships between plasma lipidomic profiles and brown adipose tissue density in humans. *Int J Obes*. 2020;44(6):1387-1396. doi:10.1038/s41366-020-0558-y.

6. Hanssen MJW, Wierts R, Hoeks J, et al. Glucose uptake in human brown adipose tissue is impaired upon fasting-induced insulin resistance. *Diabetologia*. 2015;58(3):586-595. doi:10.1007/s00125-014-3465-8.

7. Matsushita M, Yoneshiro T, Aita S, Kameya T, Sugie H, Saito M. Impact of brown adipose tissue on body fatness and glucose metabolism in healthy humans. *Int J Obes*. 2014;38(6):812-817. doi:10.1038/ijo.2013.206.

8. Saito M, Okamatsu-Ogura Y, Matsushita M, et al. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes*. 2009;58(7):1526-1531. doi:10.2337/db09-0530.

9. Soundarrajan M, Deng J, Kwasny M, et al. Activated brown adipose tissue and its relationship to adiposity and metabolic markers: an exploratory study. *Adipocyte*. 2020;9(1):87-95. doi:10.1080/21623945.2020.1724740.

10. Loh RKC, Kingwell BA, Carey AL. Human brown adipose tissue as a target for obesity management; beyond cold-induced thermogenesis. *Obes Rev*. 2017;18(11):1227-1242. doi:10.1111/obr.12584.

11. Fuse S, Nirengi S, Amagasa S, et al. Brown adipose tissue density measured by near-infrared time-resolved spectroscopy in Japanese, across a wide age range. *J Biomed Opt*. 2018;23(6):1-9. doi:10.1117/1.jbo.23.6.065002.

12. Cypess AM, Lehman S, Williams G, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med*. 2009;360(15):1509-1517. doi:10.1056/NEJMoa0810780.

13. Moreno-Aliaga MJ, Villarroya F. Nutritional and metabolic regulation of brown and beige adipose tissues. *J Physiol Biochem*. 2020;76(2):181-184. doi:10.1007/s13105-020-00745-1.

14. Serhan CN, Chiang N, Dalli J. New pro-resolving n-3 mediators bridge resolution of infectious inflammation to tissue regeneration. *Mol Aspects Med*. 2018;64:1-17. doi:10.1016/j.mam.2017.08.002.

15. Livshits G, Kalinkovich A. Inflammaging as a common ground for the development and maintenance of sarcopenia, obesity, cardiomyopathy and dysbiosis. *Ageing Res Rev*. 2019;56:100980. doi:10.1016/j.arr.2019.100980.

16. Wu D, Ren Z, Pae M, et al. Aging Up-Regulates Expression of Inflammatory Mediators in Mouse Adipose Tissue. *J Immunol*. 2007;179(7):4829-4839. doi:10.4049/jimmunol.179.7.4829.

17. Pérez LM, Pareja-Galeano H, Sanchis-Gomar F, Emanuele E, Lucia A, Gálvez BG. 'Adipaging': ageing and obesity share biological hallmarks related to a dysfunctional adipose tissue. *J Physiol*. 2016;594(12):3187-3207. doi:10.1113/JP271691.

18. Fitzgibbons TP, Kogan S, Aouadi M, Hendricks GM, Straubhaar J, Czech MP. Similarity of mouse perivascular and brown adipose tissues and their resistance to diet-induced inflammation. *Am J Physiol - Hear Circ Physiol*. 2011;301(4):1425-1437. doi:10.1152/ajpheart.00376.2011.

19. Sakamoto T, Nitta T, Maruno K, et al. Macrophage infiltration into obese adipose tissues suppresses the induction of UCP1 level in mice. *Am J Physiol - Endocrinol Metab*. 2016;310(8):E676-E687. doi:10.1152/ajpendo.00028.2015.

20. Jové M, Maté I, Naudí A, et al. Human Aging Is a Metabolome-related Matter of Gender. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2016;71(5):578-585. doi:10.1093/gerona/glv074.

21. Gangemi S, Pescara L, D'Urbano E, et al. Aging is characterized by a profound reduction in anti-inflammatory lipoxin A4 levels. *Exp Gerontol*. 2005;40(7):612-614. doi:10.1016/j.exger.2005.04.004.

22. Arnardottir HH, Dalli J, Colas RA, Shinohara M, Serhan CN. Aging Delays Resolution of Acute Inflammation in Mice: Reprogramming the Host Response with Novel Nano-Proresolving Medicines. *J Immunol*. 2014;193(8):4235-4244. doi:10.4049/jimmunol.1401313.

23. Clària J, Dalli J, Yacoubian S, Gao F, Serhan CN. Resolvin D1 and Resolvin D2 govern local inflammatory tone in obese fat. *J Immunol*. 2012;189(5):2597-2605. doi:10.4049/jimmunol.1201272.

24. Neuhofer A, Zeyda M, Mascher D, et al. Impaired local production of proresolving lipid mediators in obesity and 17-HDHA as a potential treatment for obesity-associated inflammation. *Diabetes*. 2013;62(6):1945-1956. doi:10.2337/db12-0828.

25. Hellmann J, Tang Y, Kosuri M, Bhatnagar A, Spite M. Resolvin D1 decreases adipose tissue macrophage accumulation and improves insulin sensitivity in obese-diabetic mice. *FASEB J*. 2011;25(7):2399-2407. doi:10.1096/fj.10-178657.

26. Martínez-Fernández L, González-Muniesa P, Laiglesia LM, et al. Maresin 1 improves insulin sensitivity and attenuates adipose tissue inflammation in ob/ob and diet-induced obese mice. *FASEB J*. 2017;31(5):2135-2145. doi:10.1096/fj.201600859R.

27. Laiglesia LM, Lorente-Cebrián S, Martínez-Fernández L, et al. Maresin 1 mitigates liver steatosis in ob/ob and diet-induced obese mice. *Int J Obes*. 2018;42(3):572-579. doi:10.1038/ijo.2017.226.

28. Grzybek M, Palladini A, Alexaki VI, et al. Comprehensive and quantitative analysis of white and brown adipose tissue by shotgun lipidomics. *Mol Metab*. 2019;22:12-20. doi:10.1016/j.molmet.2019.01.009.

29. Dieckmann S, Maurer S, Fromme T, et al. Fatty Acid Metabolite Profiling Reveals Oxylipins as Markers of Brown but Not Brite Adipose Tissue. *Front Endocrinol (Lausanne)*. 2020;11:73. doi:10.3389/fendo.2020.00073.

30. Calder PC. Eicosapentaenoic and docosahexaenoic acid derived specialised pro-resolving mediators : Concentrations in humans and the effects of age , sex , disease and increased omega-3 fatty acid intake. *Biochimie*. 2020;178:105-123. doi:10.1016/j.biochi.2020.08.015.

31. Ostermann AI, West AL, Schoenfeld K, et al. Plasma oxylipins respond in a linear dose-response manner with increased intake of EPA and DHA: Results from a randomized controlled trial in healthy humans. *Am J Clin Nutr*. 2019;109(5):1251-1263. doi:10.1093/ajcn/nqz016.

32. González-Périz A, Horrillo R, Ferré N, et al. Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB J*. 2009;23(6):1946-1957. doi:10.1096/fj.08-125674.

33. Itariu BK, Zeyda M, Hochbrugger EE, et al. Long-chain n-3 PUFAs reduce adipose tissue and systemic inflammation in severely obese nondiabetic patients: A randomized controlled trial. Am J Clin Nutr. 2012;96:1137-1149. doi:10.3945/ajcn.112.037432.

34. Bargut TCL, Martins FF, Santos LP, Aguila MB, Mandarim-de-Lacerda CA. Administration of eicosapentaenoic and docosahexaenoic acids may improve the remodeling and browning in subcutaneous white adipose tissue and thermogenic markers in brown adipose tissue in mice. *Mol Cell Endocrinol*. 2019;482:18-27. doi:10.1016/j.mce.2018.12.003.

35. Kim M, Goto T, Yu R, et al. Fish oil intake induces UCP1 upregulation in brown and white adipose tissue via the sympathetic nervous system. *Sci Rep*. 2015;5:18013. doi:10.1038/srep18013.

36. Quesada-López T, Cereijo R, Turatsinze JV, et al. The lipid sensor GPR120 promotes brown fat activation and FGF21 release from adipocytes. *Nat Commun*. 2016;17:13479. doi:10.1038/ncomms13479.

37. Fernández-Galilea M, Félix-Soriano E, Colón-Mesa I, Escoté X, Moreno-Aliaga MJ. Omega-3 fatty acids as regulators of brown/beige adipose tissue: from mechanisms to therapeutic potential. *J Physiol Biochem*. 2019;76(2):251-257. doi:10.1007/s13105-019-00720-5.

38. Yang J, Sáinz N, Félix-soriano E, et al. Effects of long-term DHA supplementation and physical exercise on non-alcoholic fatty liver development in obese aged female mice. *Nutrients*. 2021;13(2):501. doi:10.3390/nu13020501.

39. Roca P, Rodriguez AM, Oliver P, et al. Brown adipose tissue response to cafeteria diet-feeding involves induction of the UCP2 gene and is impaired in female rats as compared to males. *Pflugers Arch Eur J Physiol*. 1999;438(5):628-634. doi:10.1007/s004249900107.

40. Valle A, García-palmer FJ, Oliver J. Sex differences in brown adipose tissue thermogenic features during caloric restriction. *Cell Physiol Biochem*. 2007;19(1-4):195-204. doi:10.1159/000099207.

41. McDonald RB, Horwitz BA. Brown adipose tissue thermogenesis during aging and senescence. *J Bioenerg Biomembr*. 1999;31(5):507-516. doi:10.1023/A:1005404708710.

42. Moreno-Aliaga MJ, Pérez-Echarri N, Marcos-Gómez B, et al. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab*. 2011;14(2):242-253. doi:10.1016/j.cmet.2011.05.013.

43. Becerril S, Rodríguez A, Catalán V, et al. Deletion of inducible nitric-oxide synthase in leptin-deficient mice improves brown adipose tissue function. *PLoS One*. 2010;5(6):e10962. doi:10.1371/journal.pone.0010962.

44. Gomez EA, Colas RA, Souza PR, et al. Blood pro-resolving mediators are linked with synovial pathology and are predictive of DMARD responsiveness in rheumatoid arthritis. *Nat Commun*. 2020;11(1):5420. doi:10.1038/s41467-020-19176-z.

45. Colas RA, Gomez EA, Dalli J. Methodologies and Procedures Employed in the Identification and Quantitation of Lipid Mediators via LC-MS/MS. *Res Sq*. Published online 2020:1-16. doi:10.21203/RS.3.PEX-1147/V1.

46. Dalli J, Colas RA, Walker ME, Serhan CN. Lipid mediator metabolomics via LC-MS/MS profiling and analysis. *Methods Mol Biol*. 2018;1730:59-72. doi:10.1007/978-1-4939-7592-1_4.

47. Gil-Iturbe E, Félix-Soriano E, Sáinz N, et al. Effect of aging and obesity on GLUT12 expression in small intestine, adipose tissue, muscle and kidney, and its regulation by docosahexaenoic acid and exercise in mice. *Appl Physiol Nutr Metab*. 2020;45(9):957-967. 10.1139/apnm-2019-0721.

48. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262.

49. Harms MJ, Ishibashi J, Wang W, et al. Prdm16 is required for the maintenance of brown adipocyte identity and function in adult mice. *Cell Metab*. 2014;19(4):593-604. doi:10.1016/j.cmet.2014.03.007.

50. Fisher FM, Chui PC, Antonellis PJ, et al. Obesity is a fibroblast growth factor 21 (FGF21)-resistant state. *Diabetes*. 2010;59(11):2781-2789. doi:10.2337/db10-0193.

51. Villarroya F, Cereijo R, Villarroya J, Gavaldà-Navarro A, Giralt M. Toward an understanding of how immune cells control brown and beige adipobiology. *Cell Metab*. 2018;27(5):954-961. doi:10.1016/j.cmet.2018.04.006.

52. Lynes MD, Leiria LO, Lundh M, et al. The cold-induced lipokine 12,13-diHOME promotes fatty acid transport into brown adipose tissue. *Nat Med*. 2017;23(5):631-637. doi:10.1038/nm.4297.

53. Leiria LO, Wang CH, Lynes MD, et al. 12-Lipoxygenase regulates cold adaptation and glucose metabolism by producing the omega-3 lipid 12-HEPE from brown fat. *Cell Metab*. 2019;30(4):768-783. doi:10.1016/j.cmet.2019.07.001.

54. Corrales P, Vivas Y, Izquierdo-Lahuerta A, et al. Long-term caloric restriction ameliorates deleterious effects of aging on white and brown adipose tissue plasticity. *Aging Cell*. 2019;18(3):e12948. doi:10.1111/acel.12948.

55. Elias I, Ferré T, Vilà L, et al. ALOX5AP overexpression in adipose tissue leads to LXA4 production and protection against diet-induced obesity and insulin resistance. *Diabetes*. 2016;65(8):2139-2150. doi:10.2337/db16-0040.

56. Chan PC, Liao MT, Hsieh PS. The dualistic effect of COX-2-mediated signaling in obesity and insulin resistance. *Int J Mol Sci*. 2019;20(13):3115. doi:10.3390/ijms20133115.

57. Madsen L, Pedersen LM, Lillefosse HH, et al. UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PLoS One*. 2010;5(6):e11391. doi:10.1371/journal.pone.0011391.

58. García-Alonso V, López-Vicario C, Titos E, et al. Coordinate functional regulation between microsomal prostaglandin e synthase-1 (mPGES-1) and peroxisome proliferator-activated receptor γ (PPARγ) in the conversion of white-to-brown adipocytes. *J Biol Chem*. 2013;288(39):28230-28242. doi:10.1074/jbc.M113.468603.

59. García-Alonso V, Titos E, Alcaraz-Quiles J, et al. Prostaglandin E2 exerts multiple regulatory actions on human obese adipose tissue remodeling, inflammation, adaptive thermogenesis and lipolysis. *PLoS One*. 2016;11(4):e0153751. doi:10.1371/journal.pone.0153751.

60. Pisani DF, Ghandour RA, Beranger GE, et al. The ω6-fatty acid, arachidonic acid, regulates the conversion of white to brite adipocyte through a prostaglandin/calcium mediated pathway. *Mol Metab*. 2014;3(9):834-847. doi:10.1016/j.molmet.2014.09.003.

61. White PJ, St-Pierre P, Charbonneau A, et al. Protectin DX alleviates insulin resistance by activating a myokine-liver glucoregulatory axis. *Nat Med*. 2014;20(6):664-669. doi:10.1038/nm.3549.

62. White PJ, Mitchell PL, Schwab M, et al. Transgenic ω-3 PUFA enrichment alters morphology and gene expression profile in adipose tissue of obese mice: Potential role for protectins. *Metabolism*. 2015;64(6):666-676. doi:10.1016/j.metabol.2015.01.017.

63. Serhan CN, Yang R, Martinod K, et al. Maresins: Novel macrophage mediators with potent antiinflammatory and proresolving actions. *J Exp Med*. 2009;206(1):15-23. doi:10.1084/jem.20081880.

64. Deng B, Wang CW, Arnardottir HH, et al. Maresin biosynthesis and identification of maresin 2, a new anti-inflammatory and pro-resolving mediator from human macrophages. *PLoS One*. 2014;9(7):e102362. doi:10.1371/journal.pone.0102362.

65. Laiglesia LM, Lorente-Cebrián S, López-Yoldi M, et al. Maresin 1 inhibits TNF-alpha-induced lipolysis and autophagy in 3T3-L1 adipocytes. *J Cell Physiol*. 2018;233(3):2238-2246. doi:10.1002/jcp.26096.

66. Huang R, Vi L, Zong X, Baht GS. Maresin 1 resolves aged-associated macrophage inflammation to improve bone regeneration. *FASEB J*. 2020;34(10):13521-13532. doi:10.1096/fj.202001145R.

67. Titos E, Rius B, López-Vicario C, et al. Signaling and Immunoresolving Actions of Resolvin D1 in Inflamed Human Visceral Adipose Tissue. *Physiol Behav*. 2016;176(1):139-148. doi:10.1016/j.physbeh.2017.03.040.

68. Pascoal LB, Bombassaro B, Ramalho AF, et al. Resolvin RvD2 reduces hypothalamic inflammation and rescues mice from diet-induced obesity. *J Neuroinflammation*. 2017;14(1):5. doi:10.1186/s12974-016-0777-2.

69. Darcy J, Fang Y, McFadden S, et al. Integrated metabolomics reveals altered lipid metabolism in adipose tissue in a model of extreme longevity. *GeroScience*. 2020;42(6):1527-1546. doi:10.1007/s11357-020-00221-0.

70. Kulterer OC, Niederstaetter L, Herz CT, et al. The presence of active brown adipose tissue determines cold-induced energy expenditure and oxylipin profiles in humans. *J Clin Endocrinol Metab*. 2020;105(7):2203-2216. doi:10.1080/2331186X.2019.1662162.

71. Xiang AS, Giles C, Loh RKC, et al. Plasma Docosahexaenoic Acid and Eicosapentaenoic Acid Concentrations Are Positively Associated with Brown Adipose Tissue Activity in Humans. *Metabolites*. 2020;10(10):388. doi:10.3390/metabo10100388.

72. Hoene M, Li J, Häring HU, Weigert C, Xu G, Lehmann R. The lipid profile of brown adipose tissue is sex-specific in mice. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2014;1841(10):1563-1570. doi:10.1016/j.bbalip.2014.08.003.

73. Fleckenstein-Elsen M, Dinnies D, Jelenik T, Roden M, Romacho T, Eckel J. Eicosapentaenoic acid and arachidonic acid differentially regulate adipogenesis, acquisition of a brite phenotype and mitochondrial function in primary human adipocytes. *Mol Nutr Food Res*. 2016;60(9):2065-2075. doi:10.1002/mnfr.201500892.

74. Kim J, Okla M, Erickson A, Carr T, Natarajan SK, Chung S. Eicosapentaenoic acid potentiates brown thermogenesis through FFAR4-dependent up-regulation of miR-30b and miR-378. *J Biol Chem*. 2016;291(39):20551-20562. doi:10.1074/jbc.M116.721480.

75. Bargut TCL, Silva-e-Silva ACAG, Souza-Mello V, Mandarim-de-Lacerda CA, Aguila MB. Mice fed fish oil diet and upregulation of brown adipose tissue thermogenic markers. *Eur J Nutr*. 2016;55(1):159-169. doi:10.1007/s00394-015-0834-0.

76. Colson C, Ghandour RA, Dufies O, et al. Diet supplementation in ω3 polyunsaturated fatty acid favors an anti-inflammatory basal environment in mouse adipose tissue. *Nutrients*. 2019;11(2):438. doi:10.3390/nu11020438.

77. Ghandour RA, Colson C, Giroud M, et al. Impact of dietary ω3 polyunsaturated fatty acid supplementation on brown and brite adipocyte function. *J Lipid Res*. 2018;59(3):452-461. doi:10.1194/jlr.M081091.

78. Bartness TJ, Vaughan CH, Song CK. Sympathetic and sensory innervation of brown adipose tissue. *Int J Obes*. 2010;34 Suppl 1(0 1):S36-S42. doi:10.1038/ijo.2010.182.

79. Graja A, Schulz TJ. Mechanisms of aging-related impairment of brown adipocyte development and function. *Gerontology*. 2015;61(3):211-217. doi:10.1159/000366557.

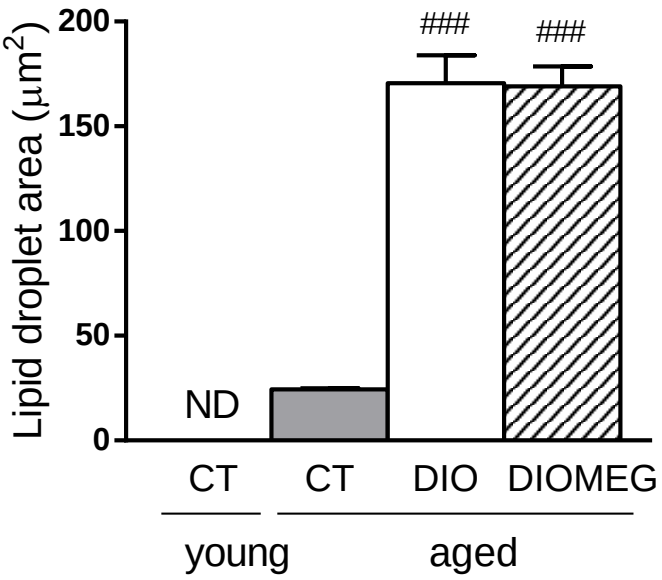
80. Rouger L, Denis GR, Luangsay S, Parmentier M. ChemR23 knockout mice display mild obesity but no deficit in adipocyte differentiation. *J Endocrinol*. 2013;219(3):279-289. doi:10.1530/JOE-13-0106.

81. Tourki B, Kain V, Pullen AB, et al. Lack of resolution sensor drives age-related cardiometabolic and cardiorenal defects and impedes inflammation-resolution in heart failure. *Mol Metab*. 2020;31:138-149. doi:10.1016/j.molmet.2019.10.008.

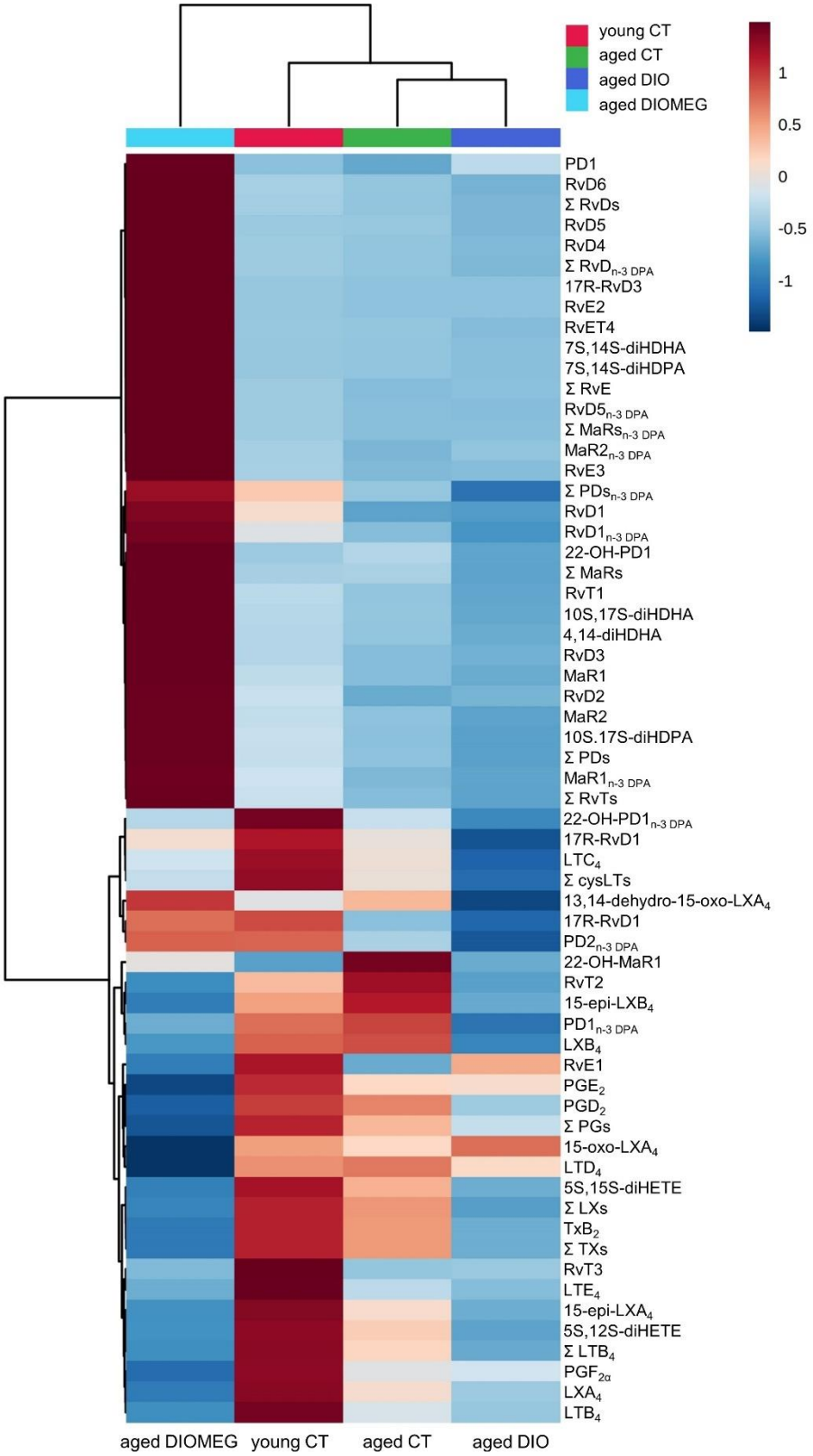
82. Choi DK, Oh TS, Choi JW, et al. Gender difference in proteome of brown adipose tissues between male and female rats exposed to a high fat diet. *Cell Physiol Biochem*. 2011;28(5):933-948. doi:10.1159/000335807.

83. Valencak TG, Osterrieder A, Schulz TJ. Sex matters: The effects of biological sex on adipose tissue biology and energy metabolism. *Redox Biol*. 2017;12:806-813. doi:10.1016/j.redox.2017.04.012.

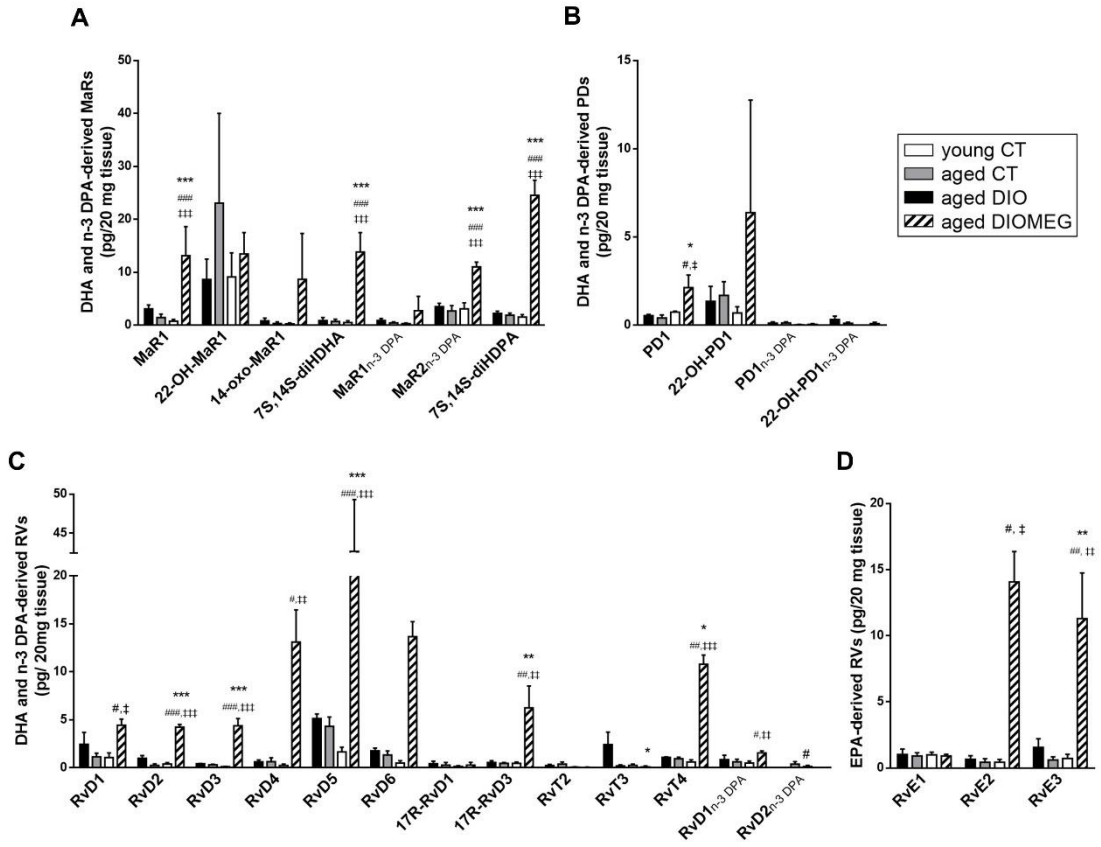
Supporting information



Supplementary Figure 1. Brown adipose tissue lipid droplet size in 18 months old female lean (CT) and diet-induced obese (DIO) mice and in DIO mice fed with a diet enriched in DHA (DIOMEG). Data are shown as mean ± SEM. $^{###}P<0.001$ *vs.* CT 18m group.



Supplementary Figure 2. Heatmap representing the results of the lipidomic analysis in BAT of young and aged lean control (CT) mice, as well as in BAT of aged diet-induced obese (DIO) mice with or without a DHA-enriched diet (DIOMEG). Colours represent concentrations, which are normalized to 1 (see colour scale).



Supplementary Figure 3. Quantification of changes induced by obesity and dietary DHA supplementation on BAT specific SPMs and lipid intermediates of the DHA and n-3 DPA-derived Maresins (MaRs, **A**), Protectins (PDs, **B**) and D-series Resolvins (RvDs, **C**) pathways, and of EPA-derived E-series Resolvins (RvEs, **D**). Data are mean ± SEM. (n=5). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* young CT; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs.* aged CT; $^{\ddagger}P<0.05$, $^{\ddagger\ddagger}P<0.01$, $^{\ddagger\ddagger\ddagger}P<0.001$ *vs.* aged DIO.

CHAPTER 3

Differential remodeling of subcutaneous white and interscapular brown adipose tissue by long-term exercise training in aged obese female mice

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Abstract

Obesity exacerbates aging-induced adipose tissue dysfunction. This study aimed to investigate the effects of long-term exercise on inguinal white adipose tissue (iWAT) and interscapular brown adipose tissue (iBAT) of aged obese mice. Two-month-old female mice received a high-fat diet for 4 months. Then, 6-month-old diet-induced obese animals were allocated to sedentarism (DIO) or to a long-term treadmill training (DIOEX) up to 18 months. In exercised mice, the iWAT depot revealed a high adaptability, with an increase in the expression of fatty acid oxidation (*Cpt1a*, *Acox1*) genes, and an amelioration of the inflammatory status with a favorable modulation of pro/antiinflammatory genes and lower macrophage infiltration. Additionally, the iWAT of trained animals showed an increased expression of genes involved in mitochondrial biogenesis (*Pgc1a, Tfam, Nrf1*), thermogenesis (*Ucp1*), and beige adipocytes selective genes (*Cd137, Tbx1*). In contrast, iBAT of aged obese mice was less responsive to exercise. Indeed, although an increase in functional brown adipocytes genes and proteins (*Pgc1a*, *Prdm16* and UCP1) was observed, few changes were found on inflammation-related and fatty acid metabolism genes. The remodeling of iWAT and iBAT occurred along with an improvement in the HOMA insulin resistance (HOMA-IR) index and in glucose tolerance. In conclusion, long-term exercise effectively prevented the loss of iWAT and iBAT thermogenic markers during aging and obesity. Exercise could be also beneficial for reducing inflammatory status in iWAT. These exercise-induced adipose tissue adaptations could contribute to the beneficial effects on glucose homeostasis.

Keywords: aging, obesity, physical exercise, white adipose tissue, brown adipose tissue.

1. Introduction

In the past years, the existence of three major types of adipose tissue has been evidenced (white, brown and beige)[1]. The 95% of adipose mass is constituted by white adipose tissue (WAT), which main function is energy storage as triglycerides and also participates in whole-body metabolic regulation through the production of adipokines[1]. WAT is organized into visceral and subcutaneous depots, which are heterogeneous and show marked metabolic differences. While visceral WAT accumulation is associated to insulin resistance and whole-body metabolic disturbances, the accumulation of triglycerides in subcutaneous depots does not seem to have deleterious effects and could be beneficial to prevent metabolic syndrome[2]. On the other hand, brown adipose tissue (BAT) is a thermogenic tissue that dissipates energy as heat mainly through the uncoupling protein 1 (UCP1). Although BAT represents only 1-2% of body fat[2], BAT is relevant for the regulation of energy homeostasis and the prevention of obesity[3]. Moreover, BAT activity has also been involved in the regulation of glucose homeostasis and triglycerides clearance[2,4]. Finally, there is a third type of adipose tissue constituted by beige or brite (brown-in white) adipocytes, a distinct type of thermogenic adipocytes[5] that can be found within white fat depots, mainly the subcutaneous WAT[6]. Hence, activation of classical brown or beige adipocytes has been proposed as an anti-obesity and related metabolic disorders target[7].

During aging, fat distribution shifts from subcutaneous to visceral fat depots, while BAT activity and WAT beiging are decreased[7,8]. These alterations can be accelerated by obesity[9,10]. On the other hand, the dysfunctional WAT and BAT that develops during aging and obesity has been associated to the local chronic, low grade inflammation that accompanied both processes[11–14].

In this context, physical exercise has been recognized in the last years as a therapeutic and preventive approach for cardiovascular disease, diabetes, obesity, certain types of cancer, functional and cognitive decline, dementia, and indeed, for a healthy aging[15–20]. These beneficial actions are mediated by the exercise-induced modulation of physiological functions in many organs[21]. Certainly, physical exercise is able to prevent age-related disorders, including visceral fat accumulation, skeletal muscle loss and age-related inflammation in animal experimental models [22]. In young adult mice, short-term exercise is sufficient to directly improve the metabolism of the different adipose depots[23]. In WAT, exercise improves glucose uptake and insulin sensitivity, induces smaller adipocytes, regulates lipolysis and increases mitochondrial activity, promotes adipose tissue beiging, reduces inflammation, modulates adipokine secretion and slows down cellular senescence[24–30]. Moreover, subcutaneous WAT plays a key role in exercise-induced improvements in whole-body glucose homeostasis, since the transplantation of subcutaneous WAT from exercise-trained mice to high-fat diet (HFD)-induced obese (DIO) mice reversed the deleterious effects of high-fat feeding on glucose tolerance and insulin sensitivity[24]. However, the effects of exercise on BAT thermogenic activation are unclear, and controversial results showing its activation, no effects, or even an exercise-induced inhibition have been observed[23,31–33].

Despite the growing evidence, the beneficial effects of exercise training on WAT and BAT metabolism and inflammation have been mainly described in young adult, and not in older, animal models of obesity. Few studies have analyzed the beneficial effects of long-term exercise started at midlife[34,35], showing efficacy on preventing body composition and/or resting energy expenditure disturbances in old mice, a phenotype that, interestingly, was more pronounced in female animals[35].

Therefore, and based in all these previous data, we aim to characterize if a long-term exercise program started at late adulthood can prevent the alterations induced by a HFD and aging on glucose homeostasis and adipose tissue lipid metabolism and inflammatory status in white and brown adipose tissue, together with the maintenance/stimulation of BAT activity and browning of WAT markers in aged, obese female mice.

2. Materials and Methods

2.1. Animal experimental design

Female C57BL6/J mice were purchased at 7 weeks of age from Harlan Laboratory (Barcelona, Spain) and housed at the animal facilities of the University of Navarra under controlled conditions (22 ± 2°C, 12-h light/12-h dark cycle; relative humidity 55% ± 10%). After 10 days acclimation, animals started consuming a HFD providing 20% kcal as proteins, 35% as carbohydrates, and 45% as lipids (Research Diets Inc., New Brunswick, N.J., USA) for 4 months to induce obesity. Then, these 6-month-old diet-induced obese (DIO) animals were maintained on the HFD and divided into two experimental groups: a DIO sedentary group (n=10) and a DIO + exercise (DIOEX group, n=8), performing a long-term treadmill running protocol up to 18 months old.

All experimental groups were fed *ad libitum* and controlled for weight 3 days/week during the whole experiment. Before sacrifice, animals underwent *in vivo* body composition and glucose tolerance test (GTT). At the end of the experiment, animals were overnight fasted before sacrifice and then fat depots, including subcutaneous-inguinal (iWAT) and visceral WAT (mesenteric, gonadal, retroperitoneal), as well as interscapular BAT (iBAT) were collected, weighted and frozen at -80 ºC. Prior to freezing, an aliquot of iWAT tissue samples were selected for stroma vascular fraction (SVF) isolation. Blood samples were collected, and serum samples obtained and frozen at -80ºC for biochemical determinations. All experiments were performed according to National Animal Care guidelines, and with the approval of the Ethics Committee for Animal Experimentation of the University of Navarra (protocol no. 113-15) in accordance with the EU Directive 2010/63/EU.

2.2. Treadmill running protocol

The DIOEX group was subjected to a treadmill (LE8710M; Panlab, Barcelona, Spain) training program from 6 until 18 months old as previously described[36]. Prior to the beginning of the treadmill protocol, mice were adapted to treadmill by running two consecutive days for 10 min (first day at 3 m min^{-1}, second day at 4.8 m min^{-1}). Then, 6 months old DIOEX animals started the treadmill running protocol (3 m min^{-1} for 5 min, increased to 4.8 m min^{-1} for 5 min, and then reaching a maximum of 7.2 m min^{-1} for 20 min; 0% slope) 3 alternate days/week. At 10 months of age, sessions were increased to 5 days/week for 5 weeks and the protocol was intensified (5 m min^{-1} for 5 min, followed by 8 m min^{-1} for 5 min and 12 m min^{-1} for 20 min; 0% slope). During the next 7 months, sessions were decreased to 3 days/week, and the program was maintained, according to previous studies[37]. Untrained DIO mice were left on the treadmill for the same time (30 min) without running.

2.3. Body composition

Before the sacrifice, whole-animal body composition was measured by magnetic resonance (EchoMRI-100-700; Echo Medical Systems, Houston, TX, USA), as previously described[38].

2.4. Glucose tolerance test

The week before sacrifice, mice were challenged a GTT by intraperitoneal injections of glucose (1.5 g kg^{-1}) to fasted animals[39]. Tail vein blood was obtained for glucose determinations prior and post injection (at 30, 60, 90 and 120 min). Glucose levels were measured with a standard glucometer (Accu-Check Advantage blood glucose meter, Roche Diagnostic, Basel, Switzerland).

2.5. Biochemical analyses

Fasting serum circulating levels of glucose, total cholesterol, and HDL-cholesterol were determined on a Pentra C200 autoanalyzer (HORIBA ABX, Madrid, Spain), following supplier's instructions. Insulin levels were determined with a commercially available ELISA kit (Mercodia, Uppsala, Sweden), according to the supplier's guidelines. LDL-cholesterol was calculated using the Friedewald equation[40], and the HOMA-IR index was calculated as described by Matthews *et al.*[41].

2.6. Flow Cytometry in iWAT stroma vascular fraction

iWAT SVF cells were isolated to analyze cell surface markers by flow cytometry. iWAT samples were cut into small pieces and digested with collagenase buffer (Sigma-Aldrich; St. Louis, MO, USA) during 45 min. Then, blood cells were lysed by the addition of ACK buffer (Gibco, Invitrogen Corporation; Carlsbad, CA, USA). For SVF isolation, cells were centrifuged at 300 *g* for 5 min and washed 3 times with DMEM-F12 medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Invitrogen). The obtained SVF was disaggregated mechanically and filtered in 70 µm cell strainers (Falcon™, #352350, Thermo Fisher Scientific, Waltham, MA, USA). Then, 100 µl of the obtained cells were incubated with the respective antibodies for 15 min at 4 ºC and FcBlock to prevent non-specific binding of Fc receptor. The quantified populations included B cells ($CD19^+$), T cells ($CD3^+$), granulocytes ($Ly6G/Ly6C^+$) and macrophages ($F4/80^{High/Low}$, $CD11b^+$). Antibodies were purchased from Biolegend (San Diego, CA, USA), including CD11b FITC (Clone M1/70), CD19-APC-cy7 (Clone 6D5), CD3-PE-cy7 (Clone 17A2) F4/80-PE (Clone BM8) and Ly6G/Ly6C-APC (Clone RB6-8C5). After that, cells were washed with PBS and centrifuged at 1500 rpm (5 min, 4 ºC). After discarding the supernatant, cells were stained with 7-AAD (#A1310, Invitrogen) to assess cell viability (1/100 dilution in PBS, 5 min, room temperature). Flow cytometry was performed in a FACSCantoII device (Becton Dickinson) and analyzed using the FlowJo software (TreeStar).

2.7. Protein expression

iWAT and iBAT samples were homogenized in 200 µl lysis buffer (Pierce ® RIPA Buffer, Thermo Fisher Scientific) with 10 mM ethylenediaminetetraacetic acid and 100x phosphatase inhibitor cocktail (Halt™, Thermo Fisher Scientific) by sonication (SONOPULS Ultrasonic homogenizer HD 3100, Bandelin, Berlin, Germany) two times for 10 s each. Samples were centrifuged (20000 *g*, 15 min, 4 ºC) to obtain the supernatant fraction, and protein concentrations were obtained by quantification of the extracts with the BCA

protein assay kit (Thermo Fisher Scientific). Protein extracts (40-60 µg) were resolved by electrophoresis on 12% SDS-polyacrylamide electrophoresis gels and electroblotted onto a polyvinylidene difluoride membrane (Amersham™ Hybond™, GE Healthcare Life Science, Freiburg, Germany), which was blocked with 1% bovine serum albumin (BSA) in TBS (Sigma-Aldrich). Then, membranes were incubated overnight (4 ºC) with primary antibodies for uncoupling protein 1 (UCP1, rabbit, #23841 Abcam, Cambridge, UK) and β-Actin (rabbit, #SAB5500001, Sigma-Aldrich). Secondary goat anti-rabbit IgG HRP (#1705046, Bio-Rad, Munich, Germany) was used (1 h, room temperature). Thereafter, immunoreactivity was detected with enhanced chemiluminescence (Thermo Fisher Scientific) and quantified by densitometry analysis (Imagen Studio Lite; LI-COR Biosciences, Lincoln, Ne., USA).

2.8. RNA isolation and qRT-PCR

iWAT and iBAT total RNA was extracted with QIAzol lysis reagent® protocol (Qiagen; Venlo, Limburg, The Netherlands) and eluted in Rnase-free DEPC-treated water (Thermo Fisher Scientific). RNA quality and quantity were measured on the Nanodrop Spectrophotometer ND1000 (Thermo Fisher Scientific) and then 2 µg were incubated (30 min, 37 °C) with Dnase I (Life Technologies, Carlsbad, CA, USA) and reverse transcribed into cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following the suppliers' instructions.

Real-time PCR assays were performed using the Touch Real-Time PCR System (C1000 + CFX384, Bio-Rad, Hercules, CA, USA). Gene expression was analyzed using Taqman Universal Master Mix (Applied Biosystems) methodology for predesigned Taqman Assays-on-Demand, and Power SYBR® Green PCR (Bio-Rad) methodology was used for primers designed with Primer-Blast software (NCBI, MD, USA, https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

For oligonucleotides sequences and references of genes assessed, refer to **Supplementary Tables 1 and 2**: *Fatty acid synthase* (*Fasn), Diacylglycerol O-acyltransferase 1* (*Dgat1), Stearoyl-Coenzyme A desaturase 1 (Scd1), Lipoprotein lipase (Lpl*), *Patatin-like phospholipase domain containing 2* (*Pnpla2*, symbol *Atgl), Lipase, hormone sensitive (Lipe*, symbol *Hsl*), *Carnitine palmitoyltransferase 1a, liver (Cpt1a), Acyl-Coenzyme A oxidase 1, palmitoyl (Acox1), tumor necrosis factor (Tnf), Toll-like receptor 4 (Tlr4), Interleukin 4 (Il4), Interleukin 6 (Il6), Interleukin 10 (Il10), Chemokine (C-C motif) ligand 2 (Ccl2), Integrin alpha X (Itgax*, symbol *Cd11c), mannose receptor, C type 1 (Mrc1,* symbol *Cd206), Adiponectin (Adipoq), Leptin (Lep), Peroxisome proliferative activated receptor, gamma, coactivator 1 α (Ppargc1a,* symbol *Pgc1a), PR domain-containing protein 16 (Prdm16), Uncoupling protein 1 (Ucp1), Transcription factor A, mitochondrial (Tfam), Nuclear respiratory factor 1 (Nrf1), Tumor necrosis factor receptor superfamily, member 9 (Tnfrsf9*, symbol *Cd137), T-box 1 (Tbx1), Transmembrane protein 26 (Tmem26), Fibroblast growth factor 21 (Fgf21), klotho beta (Klb,* symbol *β-Klotho), Fibroblast growth factor receptor 1 (Fgfr1), Early growth response 1 (Egr1)* were assayed and relative expression was determined by the $2^{-\Delta\Delta Ct}$ method[42] after normalization to *36b4* gene expression.

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.0 (Graph Pad software, La Jolla, CA, USA). Comparisons between groups were analyzed with Student's *t* or Mann-Whitney's *U* test after testing the normality with Shapiro-Wilk tests. Differences were considered significant at *P* value <0.05.

3. Results

3.1. Body composition and serum metabolic biomarkers of aged, obese exercised mice

Table 1 shows the effects of long-term exercise training on body composition and serum biomarkers of lipid metabolism in 18 months old DIO mice. No changes were observed on food intake between the untrained and trained groups. As observed, the trained mice (DIOEX group) tended to reduce body weight and fat mass (g) although no significant differences were reached. Accordingly, the weight of visceral and subcutaneous WAT depots was slightly but not significantly reduced, and no changes were either observed in iBAT mass. Exercise did not promote significant changes in fasting serum levels of triglycerides nor in total, LDL- and HDL-cholesterol (**Table 1**).

Table 1. Effects of long-term treadmill exercise on body weight, fat mass, and fat depots weights, as well as on serum lipid profile in 18 months old DIO female mice.

	DIO	DIOEX
Body weight (g)	51.19 ± 2.53	48.11 ± 2.41
Body weight gain (g)	25.48 ± 3.15	23.51 ± 1.88
Food intake (g)	2.57 ± 0.05	2.50 ± 0.07
Fat mass (g)	27.69 ± 2.16	25.91 ± 1.92
Interscapular BAT (g)	0.26 ± 0.03	0.24 ± 0.03
Subcutaneous WAT (g)	2.41 ± 0.24	2.21 ± 0.28
Visceral WAT (g)	5.75 ± 0.59	5.28 ± 0.38
Triglycerides (mg/dl)	66.67 ± 3.94	74.00 ± 7.87
Total chol (mg/dl)	134.78 ± 7.33	137.10 ± 6.11
LDL-cholesterol (mg/dl)	67.48 ± 5.07	64.96 ± 4.79
HDL-cholesterol (mg/dl)	53.96 ± 3.04	57.38 ± 2.41

DIO: diet-induced obese; DIOEX: diet-induced obese + exercise; BAT: brown adipose tissue; WAT: white adipose tissue. Data are mean ± SEM. (n=6-10**).**

Regarding glucose homeostasis, fasting glucose and insulin levels were moderately but not significantly reduced in the aged, exercised DIO mice *vs*. the non-exercised group (**Figure 1A**). However, the trained mice showed a significant reduction in the HOMA-IR, an index for insulin resistance (**Figure 1A**), which agrees with the lower peak of glucose at 30 min observed in the GTT excursion curve (**Figure 1B**).

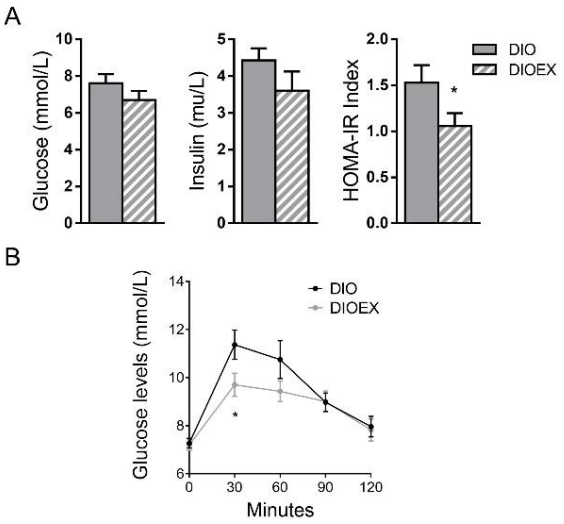

Figure 1. Effects of long-term exercise training on glucose homeostasis and insulin resistance in 18 months old DIO female mice. **A**. Fasting glucose and insulin, as well as the derived HOMA-IR index. **B**. Glucose excursion curves after the glucose tolerance test. Data are mean ± SEM. (n=6-9). $^{*}P<0.05$.

3.2. Exercise promotes fatty acid oxidation genes in iWAT, but not in iBAT of aged, obese mice

The balance between lipid anabolic (lipogenesis) and catabolic pathways (lipolysis and fatty acid oxidation) is determining of adipocyte and adipose tissue size[43]. The regulation of these processes seems to be adipose depot dependent[44,45]. A comparative study of the effects of exercise on the expression of key lipolytic, lipogenic and fatty acid oxidation genes was performed between iWAT (**Figure 2A**) and iBAT (**Figure 2B**). As observed, iWAT was more responsive than iBAT to the training program.

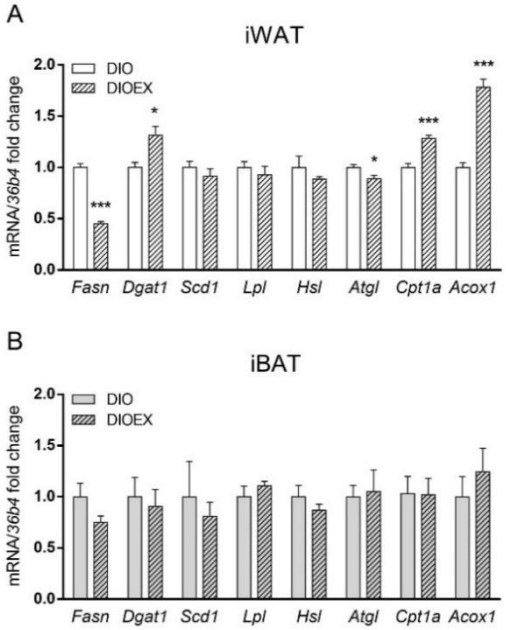

Figure 2. Effects of long-term exercise training on genes controlling fat accumulation and deposition in iWAT (**A**) and iBAT (**B**) of 18 months old DIO female mice. Data are mean ± SEM. (n=6-8). $^{*}P<0.05$, $^{***}P<0.001$.

In iWAT, the effects of long-term exercise on genes promoting fat accumulation in adipocytes were contradictory. Thus, a reduction was observed on *Fasn,* which could suggest a decreased *de novo* synthesis of fatty acids. However, *Dgat1,* involved in triglycerides esterification, was upregulated, and no changes were observed on *Lpl* in the DIOEX group. The treadmill running program had marginal effects on lipolytic genes, showing no effects on *Hsl* and a minor but significant decrease in *Atgl.* However, fatty acid oxidation genes *Acox1* and *Cpt1a* were markedly increased in the iWAT of the DIOEX group (**Figure 2A**). On the contrary, the exercise program did not induce any significant change on the expression levels of any of these genes in iBAT (**Figure 2B**).

3.3 Effects of long-term exercise training on local inflammation in iWAT and iBAT

To characterize if treadmill training started at late adulthood could attenuate the inflammatory microenvironment associated to obesity and aging in iWAT and iBAT, the gene expression of pro- and antiinflammatory adipocytokines, chemokines and macrophages markers was studied (**Figure 3**). Importantly, several proinflammatory cytokines (*Tnf*, *Il6*) were decreased by exercise in iWAT from trained mice. By contrast, the expression of antiinflammatory adipocytokines (*Adipoq* and *Il4*) was significantly increased (**Figure 3A**). Unexpectedly, *leptin* mRNA levels, which are usually positively associated with adiposity and inflammation[46], were moderately but significantly upregulated by exercise training (**Figure 3A**), despite iWAT mass tended to be reduced (**Table 1**). However, other proinflammatory (*Ccl2*, *Tlr4*) and anti-inflammatory (*Il10*) genes showed similar expression levels in both experimental groups (**Figure 3A**).

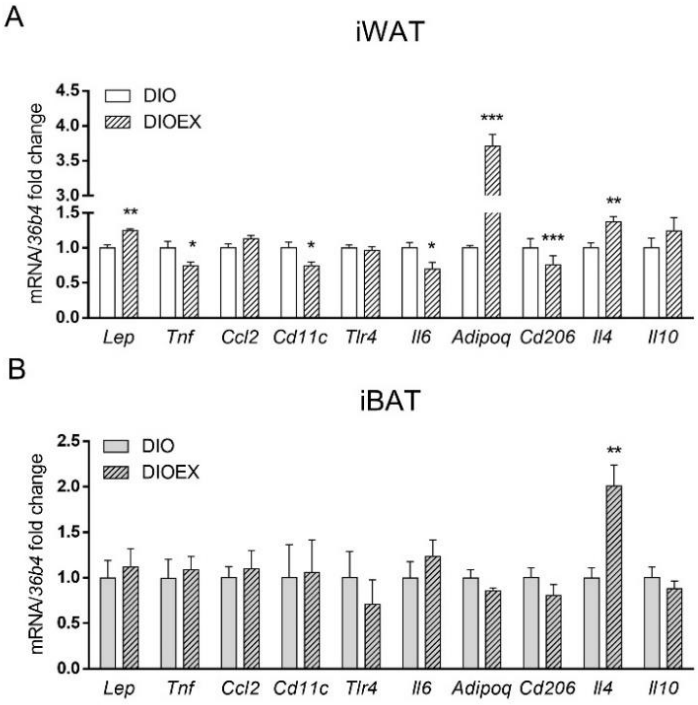

Figure 3. Effects of long-term exercise training on pro and anti-inflammatory genes in iWAT (**A**) and iBAT (**B**) in 18 months old DIO female mice. Data are mean ± SEM. (n=6-8). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Furthermore, the levels of markers of M1 proinflammatory macrophages (*Cd11c*) and M2 antiinflammatory macrophages (*Cd206*) were both decreased in DIOEX mice, suggesting a reduced infiltration of macrophages in this fat depot in response to exercise. Indeed, the characterization of the immune cell populations in iWAT by flow cytometry revealed a decrease in total macrophages (F4/80$^+$/CD11b$^+$) and an increase in B lymphocytes (CD19$^+$) in obese exercise-trained mice. However, no changes were observed for T lymphocytes (CD3$^+$) or granulocytes (Gr1$^+$) in the DIOEX group as compared to the DIO group (**Figure 4**).

On the other hand, iBAT depot was also less responsive to the exercise program concerning to inflammation-related genes, as only one of the studied genes revealed significant changes. The results showed an upregulation of the antiinflammatory *Il4* mRNA levels in DIOEX group as compared to untrained DIO mice (**Figure 3B**).

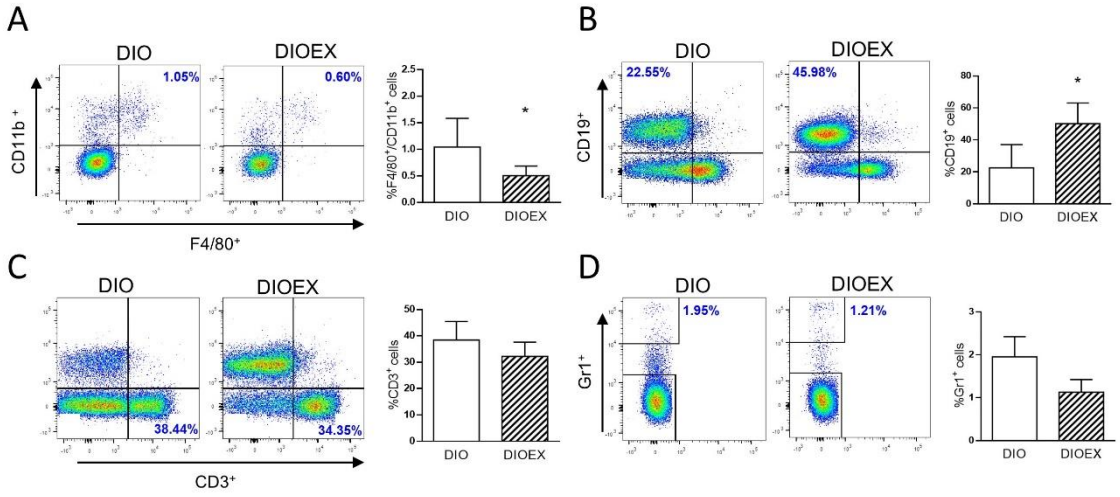

Figure 4. Effects of long-term exercise training on immune cell populations in iWAT. A-D: Analysis of adipose tissue SVF by flow cytometry. Representative dot plots and quantification of **A**. total macrophages (F4/80$^+$/CD11b$^+$), **B**. B lymphocytes (CD19$^+$), **C**. T lymphocytes (CD3$^+$) and **D**. granulocytes (Gr1$^+$). Data are mean ± SEM. (n= 5-8). $^*P$<0.05.

3.4. Exercise-induced thermogenic and mitochondrial adaptations in iWAT and iBAT of aged obese mice

We next aimed to characterize if long-term exercise could induce browning markers in iWAT of obese aged mice, which are known to be reduced by both obesity and aging[8,11]. Our data show that the iWAT of trained DIOEX mice exhibited increased expression of genes related to mitochondrial biogenesis (*Pgc1a*, *Tfam*, *Nrf1*), thermogenic function (*Ucp1*), and beige-specific genes (*Cd137*, *Tbx1*) as compared to the DIO group. By contrast, *Prdm16*, a regulator of the thermogenic program in subcutaneous WAT[6], was moderately decreased in DIOEX *vs*. DIO mice (**Figure 5A**, left panel). However, the levels of the thermogenic protein UCP1 tended to be increased (P=0.057) in DIOEX mice (**Figure 5A**, middle panel). Moreover, the mRNA levels of *Fgf21, Fgfr1* and *β-klotho* were unaltered, while *Egr1*, a downstream effector of FGF21 signaling, was upregulated in iWAT from DIOEX animals as compared to the DIO group (**Figure 5A**, right panel).

Exercise training induced some common and some differential effects on iBAT as compared to those observed on iWAT. Thus, iBAT of DIOEX mice showed increased gene expression of *Pgc1a* and *Prdm16* as well as higher UCP1, both at gene and protein levels, while *Tfam* and *Nrf1* were unaltered (**Figure 5B**, left and middle panels). Moreover, *Fgf21* levels were significantly upregulated in the exercise-trained group, while no changes were observed in its receptors *Fgfr1* and *β-klotho*. However, the levels of *Egr1* were decreased in the DIOEX group as compared to the DIO group (**Figure 5B**, right panel).

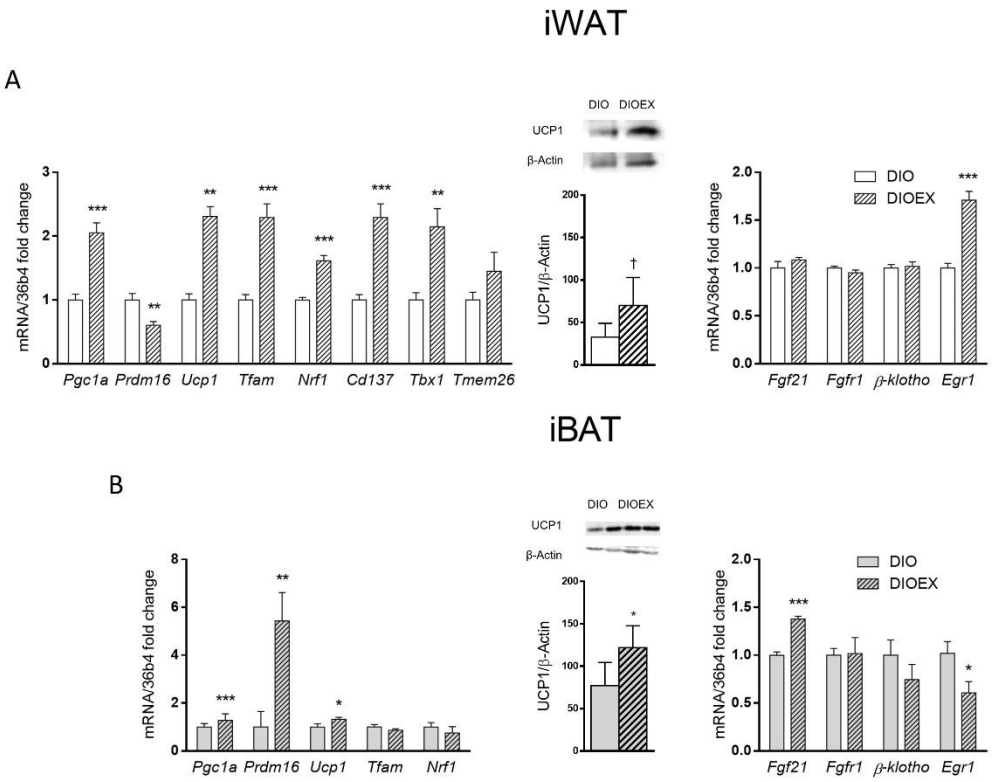

Figure 5. Effects of long-term exercise training on thermogenic function markers in iWAT and iBAT of 18 months old DIO female mice. **A**. mRNA levels of genes involved in mitochondrial biogenesis (*Pgc1a, Tfam, Nrf1*) and thermogenic function (*Prdm16, Ucp1*) and beige adipocytes selective genes (*Cd137, Tbx1 and Tmem26*) (Left panel) in iWAT, as well as protein levels of the thermogenic UCP1 (middle panel) in iWAT. The right panel shows mRNA levels of *Fgf21* and signaling pathway genes in iWAT of DIO and DIOEX mice. **B**. iBAT genes involved in mitochondrial biogenesis and thermogenic function (left panel) and UCP1 protein levels (middle panel). The right panel shows mRNA levels of *Fgf21* and signaling pathway genes in iBAT of DIO and DIOEX mice. Data are mean ± SEM. (n=6-8). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; $^{\dagger}P=0.057$.

4. Discussion

We here show for the first time how a long-term treadmill running protocol (12 months) induce a differential remodeling of iWAT and iBAT to a more thermogenic and antiinflammatory phenotype in aged obese female mice.

It is interesting to note that these actions took place even in the absence of significant changes either in body weight or in fat mass, as only moderate trends to reduce both parameters were found. This apparently contrast with other studies observing significant reductions of body weight and WAT depots or even

increases in BAT mass in response to different types of exercise training in younger animals (6-10 months old)[30,47–51]. Few studies have investigated the effects of exercise at older ages. Among them, a study in 23 months old male fed a chow diet and exercised for 10 weeks revealed no significant changes in body weight, but a lower epididymal fat mass compared to their sedentary counterparts[52]. Other studies analyzing lifelong exercise or exercise started at 12 months of age, have shown to reduce body weight and body fat mass at 24 and 28 months of age. However, the experimental conditions were different to those of our study since they were performed in aged non-obese mice that followed a voluntary wheel-running training[22,35]. Other study, in which 48 weeks-old mice were fed a HFD for 16 weeks and then started an 8-week treadmill protocol, showed a significant reduction in body weight and fat mass[34]. Although our exercise program was prolonged for 12 months, it was not able to totally counteract the increased adiposity induced by a HFD feeding that started much earlier (at 2 months of age) and maintained lifelong.

However, the exercise training protocol seemed to be beneficial on glucose homeostasis since a reduction in the HOMA-IR and an improvement in the glucose tolerance test could be observed. This is in agreement with previous studies reporting beneficial effects of exercise on glucose metabolism biomarkers after different exercise periods in younger DIO animals[33,43,59] . Our data suggest that the improvements observed in glucose homeostasis could be secondary to the remodeling induced by the long-term exercise training on iWAT and iBAT. In this way, the study of Stanford *et al.*[24] described a novel role for subcutaneous WAT in mediating the improvements on glucose homeostasis induced by exercise. Thus, our current data show that, even in the background of obesity and aging, long-term exercise could induce relevant adaptations in iWAT that may have accounted for the beneficial effects on glucose homeostasis and insulin resistance. These adaptations included an increase in fatty acid oxidation and mitochondrial biogenesis genes, the induction of thermogenic beige adipocytes markers, and reduction of inflammation in the exercised 18 months old DIO animals.

An intriguing finding was the upregulation induced by exercise on *Dgat1*, which suggest a promotion of fatty acid esterification and accumulation as triglycerides in iWAT. In the context of a chronic HFD, the promotion of fat deposition in subcutaneous WAT, which is known to be reduced by aging, could be beneficial as it could buffer the excess of fat and prevent the ectopic accumulation in other metabolic tissues like the liver, as we have recently reported in these animals[56]. However, exercise caused a significant reduction in *Fasn*, a master regulator of *de novo* lipogenesis, suggesting an inhibition of novel fatty acid formation, which is in agreement with previous studies in visceral WAT depots of exercised young DIO animals[60]. On the other hand, the exercise program did not modify the expression of *Hsl,* and even induced a small downregulation in *Atgl*, suggesting that the long-term exercise training did not stimulate iWAT lipolysis in aged mice in the context of a HFD. However, we have only measured gene expression levels of these lipases, which activity is known to be post-translationally regulated[59]. Therefore, the changes in lipases activity induced by exercise cannot be completely ruled out. Moreover, the increases in hormone-mediated lipolysis in response to exercise have been demonstrated in several studies in younger DIO animals[32,50,60,61]. However, depot-dependent effects, with increases in visceral WAT, but not in iWAT, have been described[44]. By contrast, other studies have shown decreased circulating non-esterified fatty acids and glycerol after exercising DIO animals[53,62,63], and unchanged HSL[34]. These data suggest that the classic role of WAT as a supplier of energy fuel as fatty acids by lipolysis in exercise[64] might not make sense in the context of chronic overfeeding. Moreover, it has been shown that exercise increases whole body lipolysis and fatty acid oxidation even in the absence of adipose tissue lipolysis[65].

Concerning fatty acid oxidation, our study showed that in iWAT long-term exercise significantly increased genes involved in peroxisomal and mitochondrial fatty acid oxidation (*Acox1*, *Cpt1a*) as described by previous studies in visceral and/or iWAT depots of exercised young DIO animals[53,57,58] and in aged DIO mice[34]. This increment in fatty acid oxidation genes occurred along with significant increases in genes involved in mitochondrial biogenesis (*Pgc1a, Nrf1, Tfam*), specific beige adipocytes genes (*Cd137*, *Tbx1*) and thermogenic response (*Ucp1*), suggesting that the long-term moderate exercise program promotes WAT browning in obese aged mice. Although growing evidence has supported the effects of exercise in promoting WAT browning in young and adult DIO animals[58,66–70], our current data are relevant since they suggest that exercise started in the late adulthood can be also effective to prevent the well-established loss of beige adipocytes induced by aging[71], especially in an obesogenic environment.

A moderate increase in the expression of leptin (*Lep*) mRNA was observed in iWAT in response to exercise. This upregulation of leptin is independent of changes in adiposity. Although most of the studies have described a reduction in leptin secondary to the fat mass loss after moderate to intense exercise programs[33,55], other studies have found controversial results describing increases in *Lep* mRNA in retroperitoneal fat after an endurance training in rats[69]. Interestingly, some studies have reported that administration of leptin induces UCP1 in WAT[72]. This suggests that the upregulation of *Lep* could also contribute to the thermogenic genes' induction observed in iWAT of exercise-trained mice. However, on the other hand, as a cytokine, leptin regulates immune cells and promotes inflammatory responses[73].

Inflammation of WAT has also been described to impair beige adipogenesis and browning of white adipocytes[7]. Our current data show that long-term exercise was able to counteract the iWAT inflammation that accompanies obesity and aging, as revealed by: i) the lower macrophages ($F4/80^+/Cd11b^+$) content in iWAT SVF; ii) the decrease observed in inflammatory cytokines (*Tnf*, *Il6)*; and iii) the increase in antiinflammatory adipocytokines *Adiponectin* (*Adipoq)* and *Il4* mRNA levels. Similar anti-inflammatory effects have been widely described in the current literature for different types of exercise in iWAT and visceral WAT of young DIO animals[28,68,75–77]. Concerning the mechanisms by which exercise could promote beige adipocytes markers in iWAT, it could be hypothesized the involvement of local increases in adiponectin and IL-4, which have been shown to promote browning of subcutaneous WAT by inducing M2 macrophage proliferation[78,79]. However, the reduced macrophage infiltration in the DIOEX group occurred together with a reduction in markers of both M1 proinflammatory (*Cd11c)* and M2 antiinflammatory (*Cd206*) macrophages, suggesting that long-term exercise reduced total macrophage content without a polarization of M1 to M2 macrophages, as observed in others studies in iWAT and epididymal WAT from adult exercised DIO animals[39,63]. Nevertheless, other studies have reported increased CD206 levels in subcutaneous WAT from 6 months old rats exercised for only 10 weeks[30], suggesting a switch from M1 to M2 macrophage phenotype as a consequence of exercise.

Importantly, the flow cytometry analysis of iWAT SVF also found higher markers of B lymphocytes ($CD19^+$) in DIOEX mice. Studies have established that the accumulation of B cells in epididymal WAT is one of the earliest responses to HFD, mediating a worsening of glucose tolerance, insulin resistance and secretion of proinflammatory cytokines, activating both T cells and macrophages to mediate inflammation (reviewed by Srikakulapu and McNamara[80]). However, it is worth of mention that the role of B lymphocytes in inflammation is, like that of macrophages, subset-dependent (B1-antiinflammatory or B2- proinflammatory), and the subset abundance in WAT has not been well established yet[80]. Despite these interrelations between macrophages, B and T lymphocytes, we could not find any significant change in T cells ($CD3^+$) in SVF from

DIOEX mice. Significant decreases in T cells have been found in previous studies, with other surface markers ($CD8^+$, $CD4^+$)[29,81], in younger exercised DIO animals. Hence, it seems that exercise promoted an antiinflammatory local environment in iWAT of obese aged mice mainly by the promotion of an antiinflammatory signaling and the reduction of macrophages markers, while the role of B lymphocytes remains to be elucidated.

On the other hand, our current study has also revealed that iBAT was, in general, less responsive than iWAT to the training protocol. In turn, no changes were observed on the studied genes related to lipid metabolism nor in most of those associated with iBAT inflammatory status. Among the genes that were similarly regulated by exercise in iWAT and iBAT, *Pgc1a* and *Ucp1* were included, suggesting an activation of the thermogenic response in both fat depots. Another common response to exercise between iWAT and iBAT was the upregulation of the anti-inflammatory cytokine *Il4*. Interestingly, IL-4 signaling has been described to play a major role in development of functional beige fat[79]. Indeed, genetic loss of IL-4/13 signaling impairs biogenesis of beige fat in response to cold. On the contrary, administration of IL-4 increases beige fat mass and thermogenic capacity in obese mice[79]. Therefore, the upregulation observed on *Il4* could be also underlying the browning properties of long-term exercise both in iWAT and iBAT.

Our data also show that *Prdm16* was differentially upregulated in iWAT and iBAT. A marked upregulation of *Prdm16* induced by exercise was observed only in iBAT. PRDM16 is higher in BAT cells compared to WAT cells and is in charge of brown fat cell identity development and maintenance in adulthood[82,83]. Therefore, the upregulation of *Prdm16* induced by exercise could help to promote BAT recruitment and to prevent the loss of the characteristics of brown fat that occurs during obesity and aging, as recently reported by our group[84].

The regulation of *Fgf21* in response to chronic exercise was also differential between iWAT and iBAT. The increase observed in *Fgf21* in iBAT of aged DIOEX group has been previously reported in younger obese exercised mice[85]. Stimulation of FGF21 in BAT has been related to an increased thermogenesis[86]. Therefore, increased *Fgf21* could be also related to the higher expression of UCP1 observed in the trained mice. On the other hand, FGF21 has been also identified as an inducer of browning of WAT[87,88], and exercise has been shown to restore FGF21 signaling in WAT of young (4-5 months old) DIO mice[89]. Importantly, the beneficial effects of exercise on WAT metabolism have been related with an increased FGF21 sensitivity mediated by enhanced levels of its receptors, FGFR1 and β-klotho[89]. However, our current data show that long-term exercise did not have any effect either on iWAT *Fgf21* mRNA levels or on the expression of both components of the *Fgf21* receptor (*Fgfr1* and *β-klotho*), which could suggest that *Fgf21* signaling activation was not mediating the induction of iWAT browning observed in DIOEX mice. However, the increased expression of *Erg1*, a canonical downstream effector of FGF21 pathway, seems to argue against this possibility. Based on this, the stimulation induced by long-term exercise on *Egr1* could suggest an increased sensitivity to FGF21 proposed to occur after exercise, according to Geng *et al.*[89]. On the other hand, EGR1 has been identified as a key transcription factor induced by acute exercise in muscle[90]. However, to our knowledge there is no studies analyzing the changes in *Egr1* in response to exercise in adipose tissue. We found that exercise seems to regulate *Egr1* in an adipose depot-dependent manner. In contrast to what was observed in iWAT, *Egr1* was downregulated in iBAT of exercised mice. The role of *Egr1* in BAT has not been yet described, but the observation that *Egr1* deficiency promoted WAT browning highlight the relevance to carry out future studies to uncover the potential involvement of Egr1 on BAT thermogenic function.

5. Conclusions

Long-term exercise promotes a beneficial remodeling of iWAT in aged obese mice, characterized by the modulation of pro and antiinflammatory signaling genes towards an antiinflammatory profile, lower macrophage infiltration, and the upregulation of genes involved in fatty acid oxidation, mitochondrial biogenesis, and adipose tissue beiging. Although the upregulation of thermogenic markers was also observed in iBAT, this depot seems to be less responsive to exercise than iWAT. The remodeling of iWAT and iBAT could contribute to the improvement in glucose tolerance and insulin resistance observed in aged exercise-trained mice. These data suggest that long-term moderate exercise started in the adulthood could ameliorate the deleterious effects of obesity and aging on beige and brown fat, thus helping to prevent metabolic disturbances even in an obesogenic environment.

References

1. Peirce V, Carobbio S, Vidal-Puig A. The different shades of fat. *Nature*. 2014;510:76-83. doi:10.1038/nature13477
2. Kahn CR, Wang G, Lee KY. Altered adipose tissue and adipocyte function in the pathogenesis of metabolic syndrome. *J Clin Invest*. 2019;129:3990-4000. doi:10.1172/JCI129187
3. Cypess AM, Kahn CR. Brown fat as a therapy for obesity and diabetes. *Curr Opin Endocrinol Diabetes Obes*. 2010;17:143-149. doi:10.1097/MED.0b013e328337a81f
4. Moreno-Aliaga MJ, Villarroya F. Nutritional and metabolic regulation of brown and beige adipose tissues. *J Physiol Biochem*. 2020;76:181-184. doi:10.1007/s13105-020-00745-1
5. Wu J, Boström P, Sparks LM, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell*. 2012;150:366-376. doi:10.1016/j.cell.2012.05.016.Beige
6. Bargut TCL, Souza-Mello V, Aguila MB, Mandarim-De-Lacerda CA. Browning of white adipose tissue: Lessons from experimental models. *Horm Mol Biol Clin Investig*. 2017;31. doi:10.1515/hmbci-2016-0051
7. Zoico E, Rubele S, De Caro A, et al. Brown and beige adipose tissue and aging. *Front Endocrinol (Lausanne)*. 2019;10:368. doi:10.3389/fendo.2019.00368
8. Berry DC, Jiang Y, Arpke RW, et al. Cellular aging contributes to failure of cold-induced beige adipocyte formation in old mice and humans differential anti-adipogenic effects of eicosapentaenoic and docosahexaenoic acids in obesity. *Cell Metab*. 2017;25:166-181. doi:10.1016/j.cmet.2016.10.023
9. Li H, Shen L, Zhang L, et al. Reduced beige adipogenic potential in subcutaneous adipocytes derived from obese Chinese individuals. *Diabetes, Metab Syndr Obes Targets Ther*. 2020;13:2551-2562. doi:10.2147/DMSO.S248112
10. Pérez LM, Pareja-Galeano H, Sanchis-Gomar F, Emanuele E, Lucia A, Gálvez BG. 'Adipaging': ageing and obesity share biological hallmarks related to a dysfunctional adipose tissue. *J Physiol*. 2016;594:3187-3207. doi:10.1113/JP271691
11. Villarroya F, Cereijo R, Gavaldà-Navarro A, Villarroya J, Giralt M. Inflammation of brown/beige adipose tissues in obesity and metabolic disease. *J Intern Med*. 2018;284:492-504. doi:10.1111/joim.12803
12. Rogers NH, Landa A, Park S, Smith RG. Aging leads to a programmed loss of brown adipocytes in murine subcutaneous white adipose tissue. *Aging Cell*. 2012;11:1074-1083. doi:10.1111/acel.12010
13. Rodríguez A, Ezquerro S, Méndez-Giménez L, Becerril S, Frühbeck G. Revisiting the adipocyte: a model for integration of cytokine signaling in the regulation of energy metabolism. *Am J Physiol - Endocrinol Metab*. 2015;309:E691-E714. doi:10.1152/ajpendo.00297.2015
14. Rojas JXS, Frutos MGS, Horrillo D, et al. Differential development of inflammation and insulin resistance in different adipose tissue depots along aging in wistar rats: effects of caloric restriction.

Journals Gerontol - Ser A Biol Sci Med Sci. 2016;71:310-322. doi:10.1093/gerona/glv117

15. Thompson PD, Buchner D, Piña IL, et al. Exercise and physical activity in the prevention and treatment of atherosclerotic cardiovascular disease: A statement from the council on clinical cardiology (subcommittee on exercise, rehabilitation, and prevention) and the council on nutrition, physical. *Circulation*. 2003;107:3109-3116. doi:10.1161/01.CIR.0000075572.40158.77

16. Colberg SR, Sigal RJ, Yardley JE, et al. Physical activity/exercise and diabetes: a position statement of the American Diabetes Association. *Diabetes Care*. 2016;39:2065-2079. doi:10.2337/dc16-1728

17. Lee I, Sesso HD, Wang L. Physical activity and weight gain prevention. *JAMA - J Am Med Assoc*. 2010;303:1173-1179. doi:10.1001/jama.2010.312.

18. Physical activity and cancer fact sheet - National Cancer Institute. Accessed January 19, 2021. https://www.cancer.gov/about-cancer/causes-prevention/risk/obesity/physical-activity-fact-sheet

19. Siemonsma PC, Blom JW, Hofstetter H, et al. The effectiveness of functional task exercise and physical therapy as prevention of functional decline in community dwelling older people with complex health problems. *BMC Geriatr*. 2018;18:1-8. doi:10.1186/s12877-018-0859-3

20. Ahlskog JE, Geda YE, Graff-Radford NR, Petersen RC. Physical exercise as a preventive or disease-modifying treatment of dementia and brain aging. *Mayo Clin Proc*. 2011;86:876-884. doi:10.4065/mcp.2011.0252

21. Heinonen I, Kalliokoski KK, Hannukainen JC, Duncker DJ, Nuutila P, Knuuti J. Organ-specific physiological responses to acute physical exercise and long-term training in humans. *Physiology*. 2014;29:421-436. doi:10.1152/physiol.00067.2013

22. Nilsson MI, Bourgeois JM, Nederveen JP, et al. Lifelong aerobic exercise protects against inflammaging and cancer. *PLoS One*. 2019;14:e0210863. doi:10.1371/journal.pone.0210863

23. Lehnig AC, Dewal RS, Baer LA, et al. Exercise training induces depot-specific adaptations to white and brown adipose tissue. *iScience*. 2019;11:425-439. doi:10.1016/j.isci.2018.12.033

24. Stanford KI, Middelbeek RJW, Townsend KL, et al. A novel role for subcutaneous adipose tissue in exercise-induced improvements in glucose homeostasis. *Diabetes*. 2015;64:2002-2014. doi:10.2337/db14-0704

25. Stephenson EJ, Lessard SJ, Rivas DA, et al. Exercise training enhances white adipose tissue metabolism in rats selectively bred for low- or high-endurance running capacity. *Am J Physiol - Endocrinol Metab*. 2013;305:E429-438. doi:10.1152/ajpendo.00544.2012

26. Knuth CM, Peppler WT, Townsend LK, Miotto PM, Gudiksen A, Wright DC. Prior exercise training improves cold tolerance independent of indices associated with non-shivering thermogenesis. *J Physiol*. 2018;596:4375-4391. doi:10.1113/JP276228

27. Gollisch KSC, Brandauer J, Jessen N, et al. Effects of exercise training on subcutaneous and visceral adipose tissue in normal- and high-fat diet-fed rats. *Am J Physiol - Endocrinol Metab*. 2009;297:495-504. doi:10.1152/ajpendo.90424.2008

28. Schafer MJ, White TA, Evans G, et al. Exercise prevents diet-induced cellular senescence in adipose tissue. *Diabetes*. 2016;65:1606-1615. doi:10.2337/db15-0291

29. Wasinski F, Bacurau RFP, Moraes MR, et al. Exercise and caloric restriction alter the immune system of mice submitted to a high-fat diet. *Mediators Inflamm*. 2013;2013:395672. doi:10.1155/2013/395672

30. Kolahdouzi S, Talebi-Garakani E, Hamidian G, Safarzade A. Exercise training prevents high-fat diet-induced adipose tissue remodeling by promoting capillary density and macrophage polarization. *Life Sci*. 2019;220:32-43. doi:10.1016/j.lfs.2019.01.037

31. De Matteis R, Lucertini F, Guescini M, et al. Exercise as a new physiological stimulus for brown adipose tissue activity. *Nutr Metab Cardiovasc Dis*. 2013;23:582-590. doi:10.1016/j.numecd.2012.01.013

32. Wu M V., Bikopoulos G, Hung S, Ceddia RB. Thermogenic capacity is antagonistically regulated in classical brown and white subcutaneous fat depots by high fat diet and endurance training in rats: Impact on whole-body energy expenditure. *J Biol Chem*. 2014;289:34129-34140. doi:10.1074/jbc.M114.591008

33. Dewal RS, Stanford KI. Effects of exercise on brown and beige adipocytes. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2019;1864:71-78. doi:10.1016/j.bbalip.2018.04.013

34. Bae JY. Aerobic exercise increases meteorin-like protein in muscle and adipose tissue of chronic high-fat diet-induced obese mice. *Biomed Res Int*. 2018;2018:6283932. doi:10.1155/2018/6283932

35. McMullan RC, Kelly SA, Hua K, et al. Long-term exercise in mice has sex-dependent benefits on body composition and metabolism during aging. *Physiol Rep*. 2016;4:e13011. doi:10.14814/phy2.13011

36. Gil-Iturbe E, Félix-Soriano E, Sáinz N, et al. Effect of aging and obesity on GLUT12 expression in small intestine, adipose tissue, muscle and kidney, and its regulation by docosahexaenoic acid and exercise in mice. *Appl Physiol Nutr Metab*. 2020;45:957-967. doi:10.1139/apnm-2019-0721

37. Zhao G, Liu HL, Zhang H, Tong XJ. Treadmill exercise enhances synaptic plasticity, but does not alter β-amyloid deposition in hippocampi of aged APP/PS1 transgenic mice. *Neuroscience*. 2015;298:357-366. doi:10.1016/j.neuroscience.2015.04.038

38. Moreno-Aliaga MJ, Pérez-Echarri N, Marcos-Gómez B, et al. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab*. 2011;14:242-253. doi:10.1016/j.cmet.2011.05.013

39. Sun D, Zhang L, Chen H, Feng R, Cao P, Liu Y. Effects of Antarctic krill oil on lipid and glucose metabolism in C57BL/6J mice fed with high fat diet. *Lipids Health Dis*. 2017;16:218. doi:10.1186/s12944-017-0601-8

40. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18:499-502. doi:10.1093/clinchem/18.6.499

41. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28:412-419. doi:10.1007/BF00280883

42. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402-408. doi:10.1006/meth.2001.1262

43. Jo J, Shreif Z, Periwal V. Quantitative dynamics of adipose cells. *Adipocyte*. 2012;1:80-88. doi:10.4161/adip.19705

44. Chen N, Cheng J, Zhou L, et al. Effects of treadmill running and rutin on lipolytic signaling pathways and TRPV4 protein expression in the adipose tissue of diet-induced obese mice. *J Physiol Biochem*. 2015;71:733-742. doi:10.1007/s13105-015-0437-5

45. Song Z, Xiaoli AM, Yang F. Regulation and metabolic significance of De Novo lipogenesis in adipose tissues. *Nutrients*. 2018;10:1-22. doi:10.3390/nu10101383

46. Pérez-Pérez A, Sánchez-Jiménez F, Vilariño-García T, Sánchez-Margalet V. Role of leptin in inflammation and vice versa. *Int J Mol Sci*. 2020;21:1-24. doi:10.3390/ijms21165887

47. Slocum N, Durrant JR, Bailey D, et al. Responses of brown adipose tissue to diet-induced obesity, exercise, dietary restriction and ephedrine treatment. *Exp Toxicol Pathol*. 2013;65:549-557. doi:10.1016/j.etp.2012.04.001

48. Xu X, Liu C, Xu Z, et al. Altered adipocyte progenitor population and adipose-related gene profile in adipose tissue by long-term high-fat diet in mice. *Life Sci*. 2012;90:1001-1009. doi:10.1016/j.lfs.2012.05.016

49. Rocha-Rodrigues S, Rodríguez A, Gonçalves IO, et al. Impact of physical exercise on visceral adipose tissue fatty acid profile and inflammation in response to a high-fat diet regimen. *Int J Biochem Cell Biol*. 2017;87:114-124. doi:10.1016/j.biocel.2017.04.008

50. Liu Y, Dong G, Zhao X, Huang Z, Li P, Zhang H. Post-exercise effects and long-term training adaptations of hormone sensitive lipase lipolysis induced by high-intensity interval training in adipose tissue of mice. *Front Physiol*. 2020;11:535722. doi:10.3389/fphys.2020.535722

51. Vieira VJ, Valentine RJ, Wilund KR, Antao N, Baynard T, Woods JA. Effects of exercise and low-fat diet on adipose tissue inflammation and metabolic complications in obese mice. *Am J Physiol - Endocrinol Metab*. 2009;296:1164-1171. doi:10.1152/ajpendo.00054.2009

52. Ziegler AK, Damgaard A, Mackey AL, et al. An anti-inflammatory phenotype in visceral adipose tissue of old lean mice, augmented by exercise. *Sci Rep*. 2019;9:12069. doi:10.1038/s41598-019-48587-2

53. Rocha-Rodrigues S, Rodríguez A, Becerril S, et al. Physical exercise remodels visceral adipose tissue and mitochondrial lipid metabolism in rats fed a high-fat diet. *Clin Exp Pharmacol Physiol*.

2017;44:386-394. doi:10.1111/1440-1681.12706

54. Xu X, Ying Z, Cai M, et al. Exercise ameliorates high-fat diet-induced metabolic and vascular dysfunction, and increases adipocyte progenitor cell population in brown adipose tissue. *Am J Physiol - Regul Integr Comp Physiol*. 2011;300:1115-1125. doi:10.1152/ajpregu.00806.2010

55. Gopalan V, Yaligar J, Michael N, et al. A 12-week aerobic exercise intervention results in improved metabolic function and lower adipose tissue and ectopic fat in high fat diet fed rats. *Biosci Rep*. 2020;Jan 12:BSR20201707. doi:10.1042/BSR20201707

56. Yang J, Sáinz N, Félix-soriano E, et al. Effects of long-term DHA supplementation and physical exercise on non-alcoholic fatty liver development in obese aged female mice. *Nutrients*. 2021;13:501. doi:10.3390/nu13020501

57. De Farias JM, Bom KF, Tromm CB, et al. Effect of physical training on the adipose tissue of diet-induced obesity mice: interaction between reactive oxygen species and lipolysis. *Horm Metab Res*. 2013;45:190-196. doi:10.1055/s-0032-1323740

58. Khalafi M, Mohebbi H, Symonds ME, et al. The impact of moderate-intensity continuous or high-intensity interval training on adipogenesis and browning of subcutaneous adipose tissue in obese male rats. *Nutrients*. 2020;12:925. doi:10.3390/nu12040925

59. Frühbeck G, Méndez-Giménez L, Fernández-Formoso JA, Fernández S, Rodríguez A. *Regulation of Adipocyte Lipolysis*. Vol 27.; 2014. doi:10.1017/S095442241400002X

60. Bae JY, Woo J, Roh HT, et al. The effects of detraining and training on adipose tissue lipid droplet in obese mice after chronic high-fat diet. *Lipids Health Dis*. 2017;16:13. doi:10.1186/s12944-016-0398-x

61. Woo J, Kang S. Diet change and exercise enhance protein expression of CREB, CRTC 2 and lipolitic enzymes in adipocytes of obese mice. *Lipids Health Dis*. 2016;15:147. doi:10.1186/s12944-016-0316-2

62. Chapados N, Collin P, Imbeault P, Corriveau P, Lavoie JM. Exercise training decreases in vitro stimulated lipolysis in a visceral (mesenteric) but not in the retroperitoneal fat depot of high-fat-fed rats. *Br J Nutr*. 2008;100:518-525. doi:10.1017/S0007114508921735

63. De Queiroz KB, Guimarães JB, Coimbra CC, et al. Endurance training increases leptin expression in the retroperitoneal adipose tissue of rats fed with a high-sugar diet. *Lipids*. 2014;49:85-96. doi:10.1007/s11745-013-3854-7

64. Jones NL, Heigenhauser GJF, Kuksis A, Matsos CG, Sutton JR, Toews CJ. Fat metabolism in heavy exercise. *Clin Sci*. 1980;59:469-478. doi:10.1042/cs0590469

65. Zderic TW, Davidson CJ, Schenk S, Byerley LO, Coyle EF. High-fat diet elevates resting intramuscular triglyceride concentration and whole body lipolysis during exercise. *Am J Physiol - Endocrinol Metab*. 2004;286:217-225. doi:10.1152/ajpendo.00159.2003

66. Rocha-Rodrigues S, Rodríguez A, Gouveia AM, et al. Effects of physical exercise on myokines expression and brown adipose-like phenotype modulation in rats fed a high-fat diet. *Life Sci*. 2016;165:100-108. doi:10.1016/j.lfs.2016.09.023

67. Giori IG, Magliano DC, Alexandre-Santos B, et al. Enalapril and treadmill running reduce adiposity, but only the latter causes adipose tissue browning in mice. *J Cell Physiol*. 2021;236:900-910. doi:10.1002/jcp.29900

68. Wang N, Liu Y, Ma Y, Wen D. High-intensity interval versus moderate-intensity continuous training: Superior metabolic benefits in diet-induced obesity mice. *Life Sci*. 2017;191:122-131. doi:10.1016/j.lfs.2017.08.023

69. Ohyama K, Nogusa Y, Suzuki K, Shinoda K, Kajimura S, Bannai M. A combination of exercise and capsinoid supplementation additively suppresses diet-induced obesity by increasing energy expenditure in mice. *Am J Physiol - Endocrinol Metab*. 2015;308:E315-E323. doi:10.1152/ajpendo.00354.2014

70. Barbosa MA, Guerra-Sá R, De Castro UGM, et al. Physical training improves thermogenesis and insulin pathway, and induces remodeling in white and brown adipose tissues. *J Physiol Biochem*. 2018;74:441-454. doi:10.1007/s13105-018-0637-x

71. Graja A, Gohlke S, Schulz TJ. Aging of brown and beige/brite adipose tissue. *Handb Exp Pharmacol*. 2019;251:55-72. doi:10.1007/164_2018_151.

72. Commins SP, Watson PM, Padgett MA, Dudley A, Argyropoulos G, Gettys TW. Induction of uncoupling protein expression in brown and white adipose tissue by leptin. *Endocrinology*. 1999;140:292-300. doi:10.1210/endo.140.1.6399

73. La Cava A. Leptin in inflammation and autoimmunity. *Cytokine*. 2017;98:51-58. doi:10.1016/j.cyto.2016.10.011

74. Villarroya F, Cereijo R, Villarroya J, Gavaldà-Navarro A, Giralt M. Toward an understanding of how immune cells control brown and beige adipobiology. *Cell Metab*. 2018;27:954-961. doi:10.1016/j.cmet.2018.04.006

75. Macpherson REK, Huber JS, Frendo-Cumbo S, Simpson JA, Wright DC. Adipose tissue insulin action and IL-6 signaling after exercise in obese mice. *Med Sci Sports Exerc*. 2015;47:2034-2042. doi:10.1249/MSS.0000000000000660

76. Rodrigues AC, Leal TF, Costa AJLD, et al. Effects of aerobic exercise on the inflammatory cytokine profile and expression of lipolytic and thermogenic genes in β 1 -AR −/− mice adipose tissue. *Life Sci*. 2019;221:224-232. doi:10.1016/j.lfs.2019.02.031

77. Baek KW, Lee DI, Jeong MJ, et al. Effects of lifelong spontaneous exercise on the M1/M2 macrophage polarization ratio and gene expression in adipose tissue of super-aged mice. *Exp Gerontol*. 2020;141:111091. doi:10.1016/j.exger.2020.111091

78. Hui X, Gu P, Zhang J, et al. Adiponectin enhances cold-induced browning of subcutaneous adipose tissue via promoting M2 macrophage proliferation. *Cell Metab*. 2015;22:279-290. doi:10.1016/j.cmet.2015.06.004

79. Qiu Y, Nguyen KD, Odegaard JI, et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell*. 2014;157:1292-1308. doi:10.1016/j.cell.2014.03.066

80. Srikakulapu P, Mcnamara CA. B lymphocytes and adipose tissue inflammation. *Arterioscler Thromb Vasc Biol*. 2020;40:1110-1122. doi:10.1161/ATVBAHA.119.312467

81. Kawanishi N, Mizokami T, Yano H, Suzuki K. Exercise attenuates M1 macrophages and CD8+ T cells in the adipose tissue of obese mice. *Med Sci Sports Exerc*. 2013;45:1684-1693. doi:10.1249/MSS.0b013e31828ff9c6

82. Seale P, Kajimura S, Yang W, et al. Transcriptional control of brown fat determination by PRDM16. *Cell Metab*. 2007;6:38-54. doi:10.1016/j.cmet.2007.06.001

83. Harms MJ, Ishibashi J, Wang W, et al. Prdm16 is required for the maintenance of brown adipocyte identity and function in adult mice. *Cell Metab*. 2014;19:593-604. doi:10.1016/j.cmet.2014.03.007

84. Félix-Soriano E, Sáinz N, Gil-Iturbe E, et al. Changes in brown adipose tissue lipid mediator signatures with aging, obesity, and DHA supplementation in female mice. *FASEB J.* 2021;00:e21592. doi:10.1096/fj.202002531R.

85. Xiong Y, Chen Y, Liu Y, Zhang B. Moderate-intensity continuous training improves FGF21 and KLB expression in obese mice. *Biochem*. 2020;85:938-946. doi:10.1134/S000629792008009X

86. Hondares E, Iglesias R, Giralt A, et al. Thermogenic activation induces FGF21 expression and release in brown adipose tissue. *J Biol Chem*. 2011;286:12983-12990. doi:10.1074/jbc.M110.215889

87. Fisher FF, Kleiner S, Douris N, et al. FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev*. 2012;26:271-281. doi:10.1101/gad.177857.111

88. Quesada-López T, Cereijo R, Turatsinze JV, et al. The lipid sensor GPR120 promotes brown fat activation and FGF21 release from adipocytes. *Nat Commun*. 2016;17:13479. doi:10.1038/ncomms13479

89. Geng L, Liao B, Jin L, et al. Exercise alleviates obesity-induced metabolic dysfunction via enhancing FGF21 sensitivity in adipose tissues. *Cell Rep*. 2019;26:2738-2752.e4. doi:10.1016/j.celrep.2019.02.014

90. Sabaratnam R, Pedersen AJ, Eskildsen T V., Kristensen JM, Wojtaszewski JFP, Højlund K. Exercise induction of key transcriptional regulators of metabolic adaptation in muscle is preserved in type 2 diabetes. *J Clin Endocrinol Metab*. 2019;104:4909-4920. doi:10.1210/jc.2018-02679

Supporting information

Supplementary Table 1. NCBI primer blast-designed oligonucleotides used for qRT-PCR analyses.

Gene symbol	Forward sequence	Reverse sequence
Acox1	5'-CTATGGGATCAGCCAGAAAG-3'	5'-AGTCAAAGGCATCCACCAA-3'
Adipoq	5'-AAGGGAGAGAAAGGAGATGC-3'	5'-TACACATAAGCGGCTTCTCC-3'
Ccl2	5'-AGCACCAGCCAACTCTCACT-3'	5'-TCATTGGGATCATCTTGCTG-3'
Cd206	5'-CAAGGAAGGTTGGCATTTGT-3'	5'-CCTTTCAGTCCTTTGCAAGC-3'
Cpt1a	5'-CACCAACGGGCTCATCTTCTA-3'	5'-CAAAATGACCTAGCCTTCTATCGAA-3'
Dgat1	5'-GAGGCCTCTCTGCCCCTATG-3'	5'-GCCCCTGGACAACACAGACT-3'
Fasn	5'-GCTGCGGAAACTTCAGGAAAT-3'	5'-AGAGACGTGTCACTCCTGGACTT-3'
Egr1	5'-GTCCTTTTCTGACATCGCTCTGA-3'	5'-CGAGTCGTTTGGCTGGGATA-3'
Fgf21	5'-CCTCTAGGTTTCTTTGCCAACAG-3'	5'-AAGCTGCAGGCCTCAGGAT-3'
Fgfr1	5'-TACAAGGTTCGCTATGCCAC-3'	5'-TGCGGAGATCGTTCCACGAC-3'
Il10	5'-AAGGCAGTGGAGCAGGTGAA-3'	5'-CCAGCAGACTCAATACACAC-3'
Il4	5'-ACAGGAGAAGGGACGCCAT-3'	5'-GAAGCCCTACAGACGAGCTCA-3'
Il6	5'-GAGGATACCACTCCCAACAGACC-3'	5'-AAGTGCATCATCGTTGTTCATACA-3'
Itgax (Cd11c)	5'-ACGTCAGTACAAGGAGATGTTGGA-3'	5'-ATCCTATTGCAGAATGCTTCTTTACC-3'
Klb (β-klotho)	5'-ACGACCCGACGAGGGCTGTT-3'	5'-GGAGGAGACCGTAAACTCGGGCTTA-3'
Lipe (Hsl)	5'-CTGCTTCTCCCTCTCGTCTG-3'	5'-CAAAATGGTCCTCTGCCTCT-3'
Lpl	5'-GCCAAGAGAAGCAGCAAGAT-3'	5'-CCATCCTCAGTCCCAGAAAA-3'
Nrf1	5'-GCTCACTTCCTCCGGTCCTTTG-3'	5'-GACAAGATCATCAACCTGCCTGTAG-3'
Ppargc1a (Pgc1a)	5'-CTAGCCATGGATGGCCTATTT-3'	5'-GTCTCGACACGGAGAGTTAAAG-3'
Prdm16	5'-CAGCCATACAGGTGCAAGTA-3'	5'-GAACGGCTTCTCTTTGTTGTG-3'
Tlr4	5'-TGGTTGCAGAAAATGCAGG-3'	5'-AGGAACTACCTCTATGCAGGG-3'
Tnf	5'-CATCTTCTCAAAATTCGAGTGACAA-3'	5'-TGGGAGTAGACAAGGTACAACCC-3'
Ucp1	5'-ACTGCCACACCTCCAGTCATT-3'	5'-CTTTGCCTCACTCAGGATTGG-3'

Supplementary Table 2. TaqMan Assays-on-Demand oligonucleotides used for qRT-PCR analyses.

Gene symbol	Catalog number
Lep	Mm00434759_m1
Pnpla2 (Atgl)	Mm00503040_m1
Scd1	Mm00772290_m1
Tbx1	Mm00448949_m1
Tfam	Mm00447485_m1
Tmem26	Mm01173641_m1
Tnfrsf9 (Cd137)	Mm00441899_m1

CHAPTER 4

Effects of DHA-rich n-3 fatty acid supplementation and/or resistance training on body composition and cardiometabolic biomarkers in overweight and obese post-menopausal women

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Abstract

Resistance training (RT) and n-3 polyunsaturated fatty acids (n-3 PUFA) supplementation have emerged as a strategy to improve muscle function in older adults. Overweight/obese postmenopausal women (55-70 years) were randomly allocated to one of four experimental groups, receiving placebo (olive oil) or docosahexaenoic acid (DHA)-rich n-3 PUFA supplementation alone or in combination with a supervised RT-program for 16 weeks. At baseline and end of the trial, body composition, anthropometrical measures, blood pressure and serum glucose and lipid biomarkers were analyzed. Oral glucose tolerance tests (OGTT) and strength tests were also performed. All groups exhibit a similar moderate reduction in body weight and fat mass, but the RT-groups maintained bone mineral content, increased upper limbs lean mass, decreased lower limbs fat mass, and increased muscle strength and quality compared to untrained-groups. The RT-program also improved glucose tolerance (lowering the OGTT incremental area under the curve). The DHA-rich supplementation lowered diastolic blood pressure and circulating triglycerides and increased muscle quality in lower limbs. In conclusion, 16-weeks RT-program improved segmented body composition, mineral content, and glucose tolerance, while the DHA-rich supplement had beneficial effects on cardiovascular health markers in overweight/obese postmenopausal women. No synergistic effects were observed for DHA supplementation and RT-program combination.

Keywords: postmenopause; obesity; DHA; resistance training; glucose tolerance, body composition; lipid metabolism.

1. Introduction

Menopause is a critical stage in the physiological process of aging among women, with final menstrual period being a marker of aging and health[1], and age at menopause influencing the risk for all-cause mortality[2]. During menopause, redistribution of fat mass from gluteofemoral depots towards the visceral cavity, alongside with muscle and bone mass loss, give rise to a constellation of unfavorable metabolic conditions such as insulin resistance, unhealthy lipid profiles, abnormal glucose metabolism and decreased metabolic rate[3]. Altogether, these circumstances mimic those of aging in a short period of time and increase the risk of developing sarcopenic obesity, metabolic syndrome, type 2 diabetes mellitus, coronary heart disease and osteoporosis, which are more prevalent diseases among post- than pre-menopausal women, and in older women than men[3–5].

On the other hand, obesity can also have a negative impact in the menopausal transition, and thus in the process of aging, as obesity itself increases the risk for such metabolic diseases and also for frailty[6]. Although interrelationships between obesity, menopause and aging are not established yet, several interventions have been developed with the aim to improve health and well-being among the older, obese population. Interestingly, some authors have described sedentary lifestyles as the main factor to affect health and well-being in older subjects[7]. Hence, exercise training interventions have been developed, with resistance training (RT) as a novel approach to increase muscle strength and lean mass, with the consequent improvement in physical function and metabolic profile, together with preventing future frailty and disability in older adults[8,9].

RT can elicit a potent neuromuscular stimulus that, when maintained on a regular basis, is able to improve lean mass, muscle strength, bone mineral density, and physical function also among postmenopausal women[8,10–12]. Such improvements in muscle metabolism are the main cause for RT ability to improve glucose homeostasis in older women[13]. However, effects in insulin resistance are yet to be elucidated, and RT has been established to be effective on insulin resistant, but not healthy, older subjects[13,14]. Likewise, hypotensive effects have been limited to normotensive older subjects in some studies[15], while heterogenous effects have been highlighted for both hypertensive and normotensive older women depending on their response to RT[16]. Concerning lipid metabolism, some studies have revealed an effect on lowering total cholesterol (total chol) and LDL-cholesterol while increasing HDL-cholesterol[17,18], and others have shown neutral effects[15] or pointed out the high variability in individuals responses to RT[19]. Body composition results are also inconclusive, and it seems that only long periods of RT can elicit changes in fat mass and muscle mass[20], and so well designed interventions studying RT programs have found no effects on body composition, even when strength improvements were found[11,12,21].

Among dietary interventions, n-3 polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been recently discovered to play a role in muscle protein synthesis[22]. This finding has prompted the development of interventions with both RT and n-3 PUFA in postmenopausal women[23–26]. Nevertheless, such studies have failed to investigate n-3 PUFA combined with RT effects further than strength gains or muscle protein synthesis. Noteworthy, DHA has been suggested to have more beneficial effects on obesity than EPA[27,28]. Interestingly, higher DHA levels in plasma lipids but not EPA or alpha-linolenic acid are related with lower progression of coronary artery disease in postmenopausal women[29]. Moreover, DHA levels are higher in pre than post-menopausal women[30] and have been demonstrated to have higher antithrombotic effects than EPA[31]. DHA supplementation has been proved to

lower triglycerides in a dose-dependent manner in healthy postmenopausal women[32]. Although DHA effects on lowering LDL-cholesterol have not been demonstrated in this population, effects on lowering small, dense LDL-cholesterol percentage have been reported[33], and also on increasing HDL-cholesterol[31]. Moreover, meta-analyses have highlighted the role of DHA and EPA on lowering blood pressure in the general population[34]. Remarkably, DHA levels are lower also in postmenopausal type 2 diabetes mellitus patients than in their healthy counterparts[30], and it has been highlighted that n-3 PUFA might be effective in improving insulin sensitivity in individuals under metabolic risk[35]. Yet, DHA effects in post-menopausal women are still controversial regarding insulin sensitivity, as well-designed trials have shown no effects[36]. Regarding body composition they seem to have no effects except for bone mineral density, which has been found to be inversely related with the n-3 PUFA content in erythrocytes in osteoporotic, postmenopausal women[37].

Thus, the objective of this study was to examine if supplementation with a DHA-rich fish oil concentrate and a progressive RT program, alone or in combination for 16 weeks, could have beneficial effects on improving body composition, lipid, and glucose metabolism biomarkers, as well as muscle strength and quality in overweight and obese postmenopausal women.

2. Materials and Methods

2.1. Participants

124 postmenopausal women were recruited by advertisement in local newspapers and by phone calls to volunteers from the database of the Metabolic Unit (MU) of the University of Navarra. The inclusion criteria were being 55-70 years old and overweight II/obese type I (BMI of 27.5-35 kg/m^2), with a stable weight in the last 3 months (± 3 kg) and an overall physical and physiological condition in accordance with the aim of the study. Exclusion criteria for enrolment were use of some regular prescription medication, including hormonal therapy, oral antidiabetic drugs, hypolipidemic drugs, and proton pump inhibitors. Antihypertensive therapy, thyroid hormones, anxiolytic, and antidepressant therapies were also included as exclusion criteria if dosage had been modified in the 3 months prior to the screening visit and/or the start of the trial; as well as to suffer from any severe metabolic, hepatic, renal, cardiovascular, neuromuscular, arthritic, pulmonary or other debilitating diseases; or to follow any special diets in the 3 months prior to the start of the trial. Volunteers were also excluded if they had suffered from eating disorders, surgically treated obesity, or had a history of alcohol or drug abuse.

Before inclusion in the study, all candidates were thoroughly screened using an extensive medical history (including blood biochemical data), resting electrocardiogram and blood pressure measurements, at the MU of the University of Navarra. Participants were informed in detail about the possible risks and benefits of the study and gave their written informed consent prior to being enrolled in the study. The intervention was approved by the Research Ethics Committee of the University of Navarra (140/2015mod2) and was performed in compliance with the Helsinki Declaration guidelines[38]. The study was registered at clinicaltrials.gov as NCT03300388.

2.2. Study design

The study was designed as a randomized double-blind placebo-controlled trial (RCT), in which participants were allocated into four parallel intervention groups for 16 weeks: 1) Placebo group (P) received placebo capsules containing olive oil (6 capsules of 0.5 g), 2) Omega-3 group (n-3) received DHA-rich capsules providing 1650 mg/day of DHA and 150 mg/day of EPA as ethyl esters, distributed in 6 capsules of 0.5 g of fish oil concentrate each, 3) Placebo+Resistance Training group (P+RT) received 6 placebo capsules and followed a progressive RT program of 2 sessions/week, and 4) Omega-3+Resistance Training group (n-3+RT) received the 6 DHA-rich fish oil capsules containing 1650 mg/day of DHA and 150 mg/day of EPA, and followed a progressive RT program of 2 sessions/week.

2.3. Nutritional intervention

Once the screening was completed, volunteers were randomly allocated to one of the four groups using the software platform MATLAB® (The Mathworks™, Natick, USA). Randomization criteria were age and BMI according to World Health Organization classification. Thus, the volunteers were randomized to create similar groups depending on whether they belonged to a group of age classified as adult or older adult (55-59 and 60-70 years old, respectively)[39]; and a BMI of overweight grade II or obesity type I (27.5-29.9 and 30-35 kg/m^2, respectively)[40].

At baseline and at the end of the trial, participants attended the MU at the University of Navarra in 8-12 h fasting conditions, where anthropometric measurements, body composition data and blood pressure determinations were collected by a dietitian and a nurse. Basal fasting blood samples were then extracted in order to obtain serum/plasma to measure biochemical parameters, and an Oral Glucose Tolerance Test (OGTT) was carried out as described previously[41]: 75 g of anhydrous glucose (GlycoSull®, Química Clínica Aplicada, Tarragona, Spain) were given to the volunteer and blood samples were extracted at 30', 60', 90' and 120'.

At the end of the baseline visit, volunteers were given written dietary recommendations based on the guidelines from the Spanish Society for Communitarian Nutrition (SENC, 2016)[42]. Follow up dietary consultations were scheduled every two weeks, and dietary patterns were evaluated with a validated questionnaire of 14 items to assess Adherence to the Mediterranean Diet (p14)[43] at baseline and at the end of the study, in order to evaluate potential changes along the intervention.

When baseline visit was completed, volunteers were also given the corresponding supplements. Subjects were asked to report any secondary effect to evaluate its possible association with capsules consumption. Thus, once the baseline visit of the trial was completed and in every follow-up visit, all intervention groups received two boxes containing 6 blisters with 10 capsules each, for a total of 120 capsules. Participants were asked to return boxes in every follow-up visit to evaluate adherence to supplementation by leftover pill count.

Physical activity (PA) was also controlled with a validated PA questionnaire[44] filled by participants at baseline and endpoint study visits. To compare PA between the four study groups also with a direct measure, participants were asked to wear an accelerometer (ActiGraph GT3X, Actigraph Corporation, Pensacola, FL, US), during a random and complete week of the study. The accelerometer was programmed for the subject's gender, age, weight, height, race and worn position in the body. The participants were instructed to not change their habitual physical activity habits during the 16 weeks of the trial.

2.4. Supplements information

Participants consumed two capsules with each meal (breakfast, lunch, and dinner). Both placebo and DHA-rich fish oil concentrate (DHA 55%) capsules (DHA^{scc} premium) were provided by Solutex® (Madrid, Spain). The DHA capsules contained tocopherol extracts as antioxidants to protect the highly unsaturated fatty acids from oxidation and small amounts of silicon dioxide as stabilizer. The same quantity of tocopherols was added to the olive oil capsules, although the monounsaturated fatty acids in olive oil are expected to be more resistant to oxidation. The low amount of the other stabilizer included was not expected to have any significant effect or modify the actions of fish oil concentrate on health benefits. To guarantee that the DHA-rich fish oil-derived supplements were not oxidized, peroxide and anisidine values were tested during the study and were below maximum. Olive and fish oils were provided in hard gelatin transparent liquid fill capsules and were similar in shape and size. Only a small difference in the thickness/color of the oils could be appreciated.

The dose of DHA-rich fish oil-derived supplement was selected based in previous studies[45–47], and in accordance to the of the U.S. Food and Drug Administration (FDA) recommendations of not exceeding 3 g/day EPA and DHA, with up to 2 g/day from dietary supplements[48]. To fulfill these criteria, the consumption of fish was controlled depending on their n-3 PUFA's composition according to the European Food Safety Authority (EFSA) recommendations for normal cardiac function (250 mg/day), based on food composition tables from Mataix-Verdú *et al.*[49] and online food composition databases (Easydiet® and Odimet® softwares). Consumption of n-3-PUFA enriched food and dietary supplements was not allowed during the study. Although the EFSA considers safe long-term consumption of EPA and DHA supplements at combined doses of up to about 5 g/day[50]. more restrictive FDA criteria were applied and therefore, not exceeding 3 g/daily intake of EPA and DHA were allowed/considered for this trial.

2.5. Resistance training program

After the baseline visit was completed, subjects allocated in the RT groups were asked to assist to the Research, Studies and Sports Medicine Center training facilities (CEIMD), twice a week during 16 weeks of intervention, to perform dynamic resistance exercise[51,52]. Eight exercises for upper and lower main muscular groups were included in the training program. Two routines were designed with six exercises each: leg press, chest press, knee extension and lat pulldown were maintained along the RT program, while shoulder press and hip extension (routine 1) and chest fly and leg curl (routine 2) were selected to complete each routine, changing every two weeks. Before testing and training, subjects attended three sessions for familiarization with the procedure of voluntary force production.

Strength tests were performed at the beginning, midst, and at the end of the trial to obtain strength gains/losses data and to adjust training loads to each volunteer's strength. In this study, the 1-repetition maximum (1-RM) approach was used for testing[53]. Training progression was established using the pyramidal training approach, so as 50% of intensity was selected to start the training program, and a maximum intensity of 80% was reached at week 10[54]. Three to four series were performed in each training session with 8-15 repetitions adapting to training loads. In each session, one of the researchers was present to direct and assist each subject towards ensuring adequate performance in each exercise (work rates, loads and ranges of motion) following American College of Sports Medicine (ACSM) guidelines for older adults.

To control for strength gains/losses also in untrained groups, first and last follow-up visits were scheduled at the training facilities for subjects allocated to these groups in order to perform 1-RM tests with its corresponding familiarization session. Strength results were calculated by dividing the maximum weight lifted in the 1-RM test (kg) to the subjects' body weight (kg) for leg press and chest press exercises. Muscle quality was expressed according to Pina *et al.*[55] as the ratio of the maximum weight lifted in the 1-RM tests (kg) to lean soft tissue (kg) of the lower and upper limbs.

2.6. Evaluation of weight loss and body composition

The main outcome of the study was the reduction of fat mass. Body composition was analyzed at baseline and at the end of trial by total and segmented Dual X-ray Absorptiometry (Lunar iDXA, encore 14.5, Madison, WI, USA), as previously reported[56]. In addition, anthropometric measurements were obtained including arm, waist, hip, thigh, and calf circumference, as well as arm, thigh, and calf skinfolds, following the ISAK guidelines at baseline and end of the trial[57].

2.7. Evaluation of lipid and glucose metabolism and other biomarkers

Once basal blood samples were extracted, they were centrifuged at 1500 *g* for 15 min at 4 ºC and aliquots of serum/plasma were frozen at -80 ºC until analysis. Fasting serum lipid and glucose metabolism biomarkers, total cholesterol, HDL-cholesterol, triglycerides, glucose, and OGTT timepoints' glucose levels were determined on an autoanalyzer (Pentra C200, HORIBA ABX, Madrid, Spain) following manufacturer's instructions at baseline and at the end of the trial. LDL-cholesterol was calculated using the Friedewald equation. Fasting insulin was determined with an ELISA kit (#10-1132-01, Mercodia, Uppsala, Sweden) following the manufacturer's instructions on an autoanalyzer (Triturus ELISA Instrument, Grifols, Barcelona, Spain). Indexes for insulin resistance HOMA-IR and triglycerides to Glucose (TyG) index for insulin resistance were calculated as described previously[41].

2.8. Statistical analysis

Considering fat mass losses as the primary outcome, based on the results reported by previous studies on the placebo untrained group and the n-3 trained group[58,59], the estimated effect size was 1.185. Taking a bilateral alpha of 95% and a power calculation of 90%, the number of volunteers per group was 16. Considering a 25% of drop-out rate, the number of subjects per group was 20, with a total of 80 subjects for the study.

Statistical analyses were performed using STATA, version 14. Data were expressed as mean ± SD, and differences were significant at 2-sided *P* value <0.05. Possible confounding variables were used for adjustment, and values were expressed as Mean(SEM). To select the appropriate test, normal distribution was assessed using Shapiro-Wilk test and Breush-Pagan/Cook-Weisberg heteroscedasticity test. Comparisons between groups at baseline were evaluated by 1-factor ANOVA test or Kruskal-Wallis test. The comparison between baseline and endpoint within each group were assessed by paired Student's *t*-test or Wilcoxon signed-rank test as appropriate. To evaluate factor effects between groups, two-way ANOVA test was performed to identify interactions. When differences were statistically significant at the interaction level, contrasts were performed to differentiate groups effects.

3. Results

3.1. Basal characteristics and flowchart of the participants

Of the 124 volunteers screened for the intervention, 85 were allocated to one of the four study groups, 85 initiated the trial and 71 finished the study (**Figure 1**). Baseline characteristics of the study subjects were similar between the four experimental groups, except for the basal glycemia, that was moderately lower in the P group than in the P+RT and n-3+RT groups (**Supplementary Table 1**). All the groups also exhibited a similar adherence to the Mediterranean diet pattern and similar PA levels, as estimated by validated questionnaires and measured by accelerometry. Moreover, the intervention groups did not significantly change their PA pattern during the trial, rather than the RT expected in the allocated groups (**Supplementary Table 2**). Finally, the mean adherence to the RT program and supplementation (capsules intake) was above 95% at the end of the intervention in all groups (**Supplementary Figure 1**).

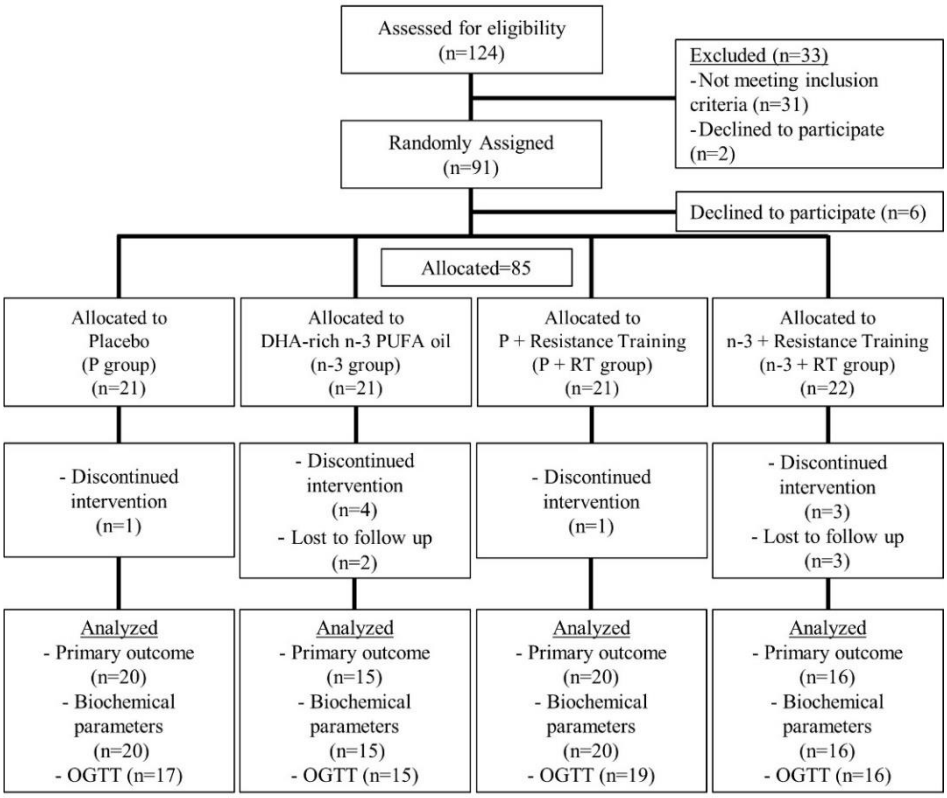


Figure 1. Flowchart of participants from the screening to the endpoint visit of the study (16 weeks). 85 out of the 91 women who met the inclusion criteria started the intervention. 14 participants did not complete the study (16.5% drop out), as they either discontinued follow-up due to unexpected health problems (n=5, 3 unrelated to the study and 2 related to capsules consumption), time incompatibilities (n=2), withdrew from the study (n=3), or were not compliant with the training sessions (n=4). There were two dropouts in the n-3 group probably related to capsules consumption, one of them was related gastroesophageal reflux and the other one related to itch in the hands. Dropout rates were 4.8% for P and P+RT groups, 28.6% for n-3 group and 27.3% for n-3+RT group. For OGTT analyses, 3 subjects were excluded due to problems with venous insertion of the catheter in the P group, and 1 subject was excluded due to lack of measure at one timepoint of the glucose excursion curve in the P+RT group.

Table 1. Effects of 16 weeks of DHA-rich n-3 PUFA (n-3) supplementation and/or resistance training (RT) on whole body composition and anthropometric measures in overweight/obese post-menopausal women.

	P	**n-3**	**P+RT**	**n-3+RT**	**Two-way ANOVA[c]**		
N	20	15	20	16	n-3	RT	n-3xRT
Age (years)	58.75 ± 3.39	58.00 ± 2.78	58.95 ± 3.46	58.13 ± 3.14			
Weight (kg)							
Baseline	76.75 ± 4.99	80.34 ± 8.51	77.76 ± 7.92	80.57 ± 6.60			
Change	-2.66 ± 2.95[a,***]	-2.65 ± 2.47[a,**]	-2.21 ± 2.39[a,***]	-2.70 ± 3.49[a,**]	*ns*	*ns*	*ns*
BMI (kg/m^2)							
Baseline	30.25 ± 2.30	30.39 ± 1.94	30.79 ± 2.34	31.07 ± 1.82			
Change	-1.07 ± 1.16[b,**]	-1.03 ± 0.94[a,***]	-0.90 ± 0.94[a,***]	-1.06 ± 1.34[a,**]	*ns*	*ns*	*ns*
Fat mass (%)							
Baseline	48.80 ± 3.41	46.91 ± 2.35	48.40 ± 3.98	48.03 ± 2.92			
Change	-2.31 ± 1.15[a,***]	-1.57 ± 1.35[a,***]	-1.80 ± 1.49[a,***]	-2.13 ± 2.45[a,**]	*ns*	*ns*	*ns*
Visceral fat (kg)							
Baseline	1.30 ± 0.44	1.37 ± 0.44	1.27 ± 0.51	1.18 ± 0.47			
Change	-0.20 ± 0.19[a,***]	-0.11 ± 0.14[a,**]	-0.11 ± 0.18[b,*]	-0.12 ± 0.23[a,†]	*ns*	*ns*	*ns*
Adjusted change[d]	-0.20(0.04)[a,***]	-0.11(0.02)[a,***]	-0.12(0.03)[b,**]	-0.12(0.03)[a,**]	*ns*	*ns*	*ns*
Muscle mass (%)							
Baseline	49.76± 3.20	51.52 ± 2.18	50.16 ± 3.78	50.53 ± 2.76			
Change	2.21 ± 1.08[a,***]	1.49 ± 1.26[a,***]	1.70 ± 1.43[a,***]	2.01 ± 2.34[a,**]	*ns*	*ns*	*ns*
BMC (g)							
Baseline	2152.65 ± 308.05	2366.33 ± 332.76	2156.10 ± 231.21	2240.31 ± 258.36			
Change	-27.60 ± 17.36[**,]	-17.53 ± 20.22[b,*]	1.40 ± 30.33	-1.38 ± 32.81	*ns*	0.001	*ns*
Adjusted change[d]	-25.95(4.19)***	-17.50(5.08)[b,**]	-2.08(6.63)	-1.26(7.10)	*ns*	*P*<0.001	*ns*
Waist circumference (cm)							
Baseline	93.11 ± 4.57	95.00 ± 7.63	92.67 ± 5.47	93.90 ± 7.16			
Change	-3.45 ± 2.62[a,***]	-3.15 ± 2.94[a,**]	-3.01 ± 1.80[a,***]	-4.04 ± 3.73[a,***]	*ns*	*ns*	*ns*
Adjusted change[d]	-3.35(0.26)[a,***]	-3.18(0.65)[a,***]	-3.08(0.38)[b,***]	-4.03(0.62)[a,***]	*ns*	*ns*	*ns*
Hip circumference (cm)							
Baseline	110.68 ± 7.14	112.50 ± 5.78	110.65 ± 5.74	113.35 ± 6.82			
Change	-2.40 ± 2.97[a,**]	-3.00 ± 3.12[a,**]	-3.17 ± 4.99[a,*]	-3.06 ± 3.52[a,**]	*ns*	*ns*	*ns*
Adjusted change[d]	-2.42(0.37)[a,***]	-2.87(0.57)[a,***]	-3.33(1.17)[a,*]	-2.89(0.91)[a,**]	*ns*	*ns*	*ns*
Waist/hip ratio							
Baseline	0.84 ± 0.04	0.85 ± 0.06	0.84 ± 0.06	0.83 ± 0.08			
Change	-0.01 ± 0.01[a,***]	-0.01 ± 0.01[a,*]	-0.00 ± 0.04	-0.02 ± 0.03	*ns*	*ns*	*ns*
Adjusted change[d]	-0.01(0.00)[a,**]	-0.01(0.00)	-0.00(0.01)	-0.02(0.01)[a,*]	*ns*	*ns*	*ns*

Note: P: Placebo group, n-3: DHA-rich n-3 PUFA supplemented group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplemented + resistance training group; BMI: Body mass index; BMC: Bone mineral content. Data are mean ± SD.

[a]Paired Student's *t*-test, [b]Wilcoxon's signed-rank test. [c]Differences between groups for changes were evaluated by two-way ANOVA. [d]Means(SEM) adjusted by changes in body weight.

***P<0.001, **P<0.01, *P<0.05, [†]P trend (P=0.056-0.061) *vs.* baseline; *ns*, nonsignificant (P>0.05).

3.2. Effects on whole body composition and anthropometric measurements

After the 16-week intervention, all groups showed a moderate but statistically significant reductions in body weight, BMI, and the percentage of fat mass (**Table 1**). Visceral fat mass was also significantly reduced after the intervention in all groups except for n-3+RT group, but a reduction was observed when adjusted for total weight loss. Interestingly, muscle mass percentage increased in the four groups after the intervention. However, the analysis of the changes between groups by two-way ANOVA revealed no significant statistical differences between the four experimental groups for any of the previously described body composition parameters. Noteworthy, bone mineral content (BMC) significantly decreased in those groups that were not allocated to the RT program **(Table 1**). In fact, the analysis of the changes between groups revealed that the RT program was able to significantly prevent this reduction in BMC observed in the untrained groups (**Table 1**).

Regarding anthropometric measurements, all groups showed a decrease in waist, and hip circumferences, but the waist/hip ratio was significantly reduced only in the P and n-3 groups. Statistical differences were maintained in P group and appeared in n-3+RT when adjusting for weight loss, while they disappeared in n-3 group (**Table 1**). When looking for differences in changes between groups due to RT and/or n-3-PUFA supplementation, no significant differences were found (**Table 1**).

3.3. Effects on segmented body composition and anthropometric measurements

Fat and muscle composition as well as anthropometric measurements of arms and legs were studied. A significant reduction in arms weight was observed only in untrained groups after the trial. Arms muscle mass tended to decrease in the non-trained groups. Thus, the two-way ANOVA analysis revealed that the RT program significantly prevented the muscle mass and arms weight loss compared to the untrained groups (**Table 2**). Arms fat mass was reduced in the four intervention groups, although it did not reach statistical significance in the n-3+RT group. The analysis of changes between groups showed that the reduction in arms fat mass was significantly lower in the groups receiving n-3 PUFA supplementation (**Table 2**). Nevertheless, arms circumference and tricipital skinfold were reduced in all groups after the intervention, without significant differences for RT or n-3-PUFA supplementation when changes between groups were analyzed (**Supplementary Table 3**).

Regarding lower body composition, legs weight was reduced in all intervention groups (**Table 2**). Although reductions tended to be higher in RT *vs.* untrained groups, two-way ANOVA did not reach statistical significance (P=0.098). Legs muscle mass did not change in any of the intervention groups, neither when baseline-endpoint nor when changes between groups were analyzed. Nevertheless, legs fat mass did decrease in the four groups of study, with a significantly higher decrease in the RT *vs.* the non-trained groups. Anthropometric measurements reflected similar results, as calf circumference was reduced in all study groups, with significantly higher losses only in RT groups when changes between groups were compared (**Table 2**). Thigh circumference was also moderately reduced in the four experimental groups but did not change significantly by either RT or n-3-PUFA supplementation when analyzed by two-way ANOVA. Similar to calf circumference, calf and thigh skinfolds reductions were significantly higher in the RT groups compared to the untrained groups (**Table 2**).

Table 2. Effects of 16 weeks of DHA-rich n-3 PUFA (n-3) supplementation and/or resistance training (RT) on segmented body composition and anthropometric measures in overweight/obese postmenopausal women.

	P	**n-3**	**P+RT**	**n-3+RT**	**Two-way ANOVA[c]**		
N	20	15	20	16	n-3	RT	n-3xRT
Arms weight (kg)							
Baseline	8.65 ± 0.89	8.60 ± 1.17	8.73 ± 1.21	8.83 ± 1.04			
Change	-0.37 ± 0.40[a,***]	-0.33 ± 0.42[a,**]	-0.13 ± 0.40	0.01 ± 0.60	*ns*	0.010	*ns*
Adjusted change[d]	-0.38(0.07)[a,***]	-0.32(0.11)[a,*]	-0.15(0.09)	0.02(0.11)	*ns*	0.003	*ns*
Arms fat mass (g)							
Baseline	4.36 ± 0.60	4.01 ± 0.68	4.32 ± 0.68	4.24 ± 0.71			
Change	-0.33 ± 0.27[a,***]	-0.24 ± 0.24[b,**]	-0.22 ± 0.28[a,**]	-0.11 ± 0.41	*ns*	*ns*	*ns*
Adjusted change[d]	-0.34(0.04)[a,***]	-0.23(0.06)[a,**]	-0.24(0.05)[a,***]	-0.10(0.07)	0.041	*ns*	*ns*
Arms muscle mass (g)							
Baseline	4.02 ± 0.56	4.30 ± 0.60	4.13 ± 0.66	4.29 ± 0.47			
Change	-0.04 ± 0.18	-0.09 ± 0.22	0.09 ± 0.19[a,†]	0.13 ± 0.30	*ns*	0.002	*ns*
Adjusted change[d]	-0.05(0.04)	-0.09(0.06)	0.09(0.04)[a,†]	0.13(0.07)[a,†]	*ns*	0.002	*ns*
Legs weight (kg)							
Baseline	25.10 ± 3.59	27.52 ± 3.28	26.55 ± 3.91	27.11 ± 3.95			
Change	-0.60 ± 1.79[b,†]	-0.91 ± 0.97[a,**]	-0.95 ± 1.12[a,**]	-0.98 ± 1.45[a,*]	*ns*	*ns*	*ns*
Adjusted change[d]	-0.51(0.22)[b,**]	-0.86(0.10)[a,***]	-1.08(0.22)[a,***]	-0.91(0.10)[a,***]	*ns*	*ns*	*ns*
Legs fat mass (g)							
Baseline	11.29 ± 2.58	12.16 ± 2.38	12.36 ± 2.88	12.18 ± 2.47			
Change	-0.62 ± 0.90[b,**]	-0.73 ± 0.66[a,***]	-0.96 ± 0.73[a,***]	-0.97 ± 1.02[a,**]	*ns*	*ns*	*ns*
Adjusted change[d]	-0.60(0.12)[b,**]	-0.70(0.08)[a,***]	-1.02(0.14)[a,***]	-0.92(0.12)[a,***]	*ns*	0.005	*ns*
Legs muscle mass (g)							
Baseline	13.03 ± 1.57	14.51 ± 1.66	13.40 ± 1.64	14.11 ± 1.88			
Change	0.02 ± 0.98	-0.16 ± 0.50	0.02 ± 0.57	0.02 ± 0.63	*ns*	*ns*	*ns*
Adjusted change[d]	0.01(0.14)	-0.15(0.10)	-0.05(0.13)	0.03(0.99)	*ns*	*ns*	*ns*
Thigh circumference (cm)							
Baseline	56.42 ± 4.54	56.75 ± 3.48	59.71 ± 5.21	60.78 ± 6.47			
Change	-1.49 ± 1.75[b,**]	-0.84 ± 1.18[a,*]	-2.38 ± 3.00[a,**]	-1.50 ± 2.52[a,*]	*ns*	*ns*	*ns*
Adjusted change[d]	-1.07(0.24)[b,**]	-0.91(0.40)[a,*]	-2.71(0.57)[a,***]	-1.60(0.56)[a,*]	*ns*	*ns*	*ns*
Calf circumference (cm)							
Baseline	38.05 ± 2.12	39.59 ± 2.58	40.15 ± 2.41	39.30 ± 2.55			
Change	-0.32 ± 0.54[a,*]	-0.39 ± 0.67[a,*]	-0.81 ± 0.78[b,***]	-0.68 ± 0.69[a,**]	*ns*	0.017	*ns*
Adjusted change[d]	-0.26(0.09)[a,*]	-0.38(0.15)[b,*]	-0.85(0.19)[a,***]	-0.68(0.13)[a,***]	*ns*	0.005	*ns*
Thigh skinfold (mm)							
Baseline	39.92 ± 4.76	41.25 ± 5.49	42.67 ± 2.99	41.06 ± 5.37			
Change	0.43 ± 3.36	-1.23 ± 2.28[b,*]	-3.43 ± 3.15[a,***]	-5.19 ± 7.61[b,**]	*ns*	*P*<0.001	*ns*
Adjusted change[d]	-0.28(0.61)	-0.70(0.54)[b,*]	-3.31(0.66)[a,***]	-4.64(1.92)[b,*]	*ns*	*P*<0.001	*ns*
Calf skinfold (mm)							
Baseline	31.07 ± 5.28	32.02 ± 5.60	33.26 ± 4.88	34.76 ± 5.45			
Change	-1.26 ± 3.65	-2.51 ± 3.05[a,**]	-5.17 ± 4.63[a,***]	-7.73 ± 7.40[b,**]	*ns*	*P*<0.001	*ns*
Adjusted change[d]	-1.46(0.70)[a,†]	-2.25(0.80)[a,*]	-5.05(1.92)[a,***]	-7.44(1.82)[b,**]	*ns*	*P*<0.001	*ns*

Note. P: Placebo group, n-3: DHA-rich n-3 PUFA supplemented group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplemented + resistance training group. Data are mean ± SD.

[a]Paired Student's *t*-test, [b]Wilcoxon's signed-rank test. [c]Differences between groups for changes were evaluated by two-way ANOVA. [d]Means(SEM) adjusted by changes in body weight.

***P<0.001, **P<0.01, *P<0.05, [†]P trend (P=0.052-0.061) *vs.* baseline; *ns*, nonsignificant (P>0.05).

3.4. Effects on muscle strength and quality

Muscle strength values (kg, 1-RM tests) were normalized to the subjects' body weight at baseline and at the end of the trial. Likewise, muscle quality was calculated as previously described[55] for both upper and lower limbs, by dividing muscle strength (kg lifted in the chest and leg press 1-RM tests)/muscle mass (kg measured in arms and legs DXA segmented analyses). As expected, the RT groups significantly increased their muscle strength and quality (P<0.001) compared to the untrained groups, both in upper and lower limbs (**Figure 2**). Noteworthy, the DHA-rich supplement did not influence muscle strength but revealed a tendency to promote this effect in lower limbs (P=0.067) that could rely on the local improvement in muscle quality (P<0.01). However, these effects observed in lower limbs by n-3 PUFA supplementation were not mimicked by the results observed in upper limbs. No synergistic effects were observed for the DHA-rich supplementation on strength and muscle quality gains derived from the RT program.

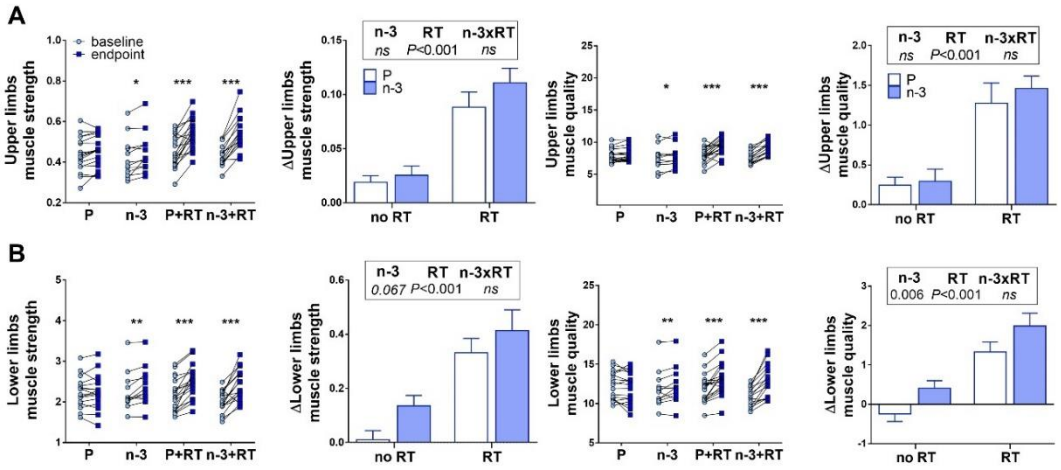


Figure 2. Effects of 16 weeks of DHA-rich n-3 PUFA (n-3) supplementation and/or resistance training (RT) on upper (**A**) and lower (**B**) limbs muscle strength, calculated as 1-RM (kg) / body weight (kg); and muscle quality, calculated as 1-RM (kg) / local muscle mass (kg). Baseline-endpoint differences were studied by paired Student's *t*-test or Wilcoxon's signed-rank test after testing for normality. Differences in changes between groups were compared by two-way ANOVA (*ns*, nonsignificant, P>0.05). Data are mean ± SEM. ***P<0.001; **P<0.01; *P<0.05 *vs.* baseline. P: P: Placebo group, n-3: DHA-rich n-3 PUFA supplemented group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplemented + resistance training group.

3.5. Effects on blood pressure and lipid metabolism biomarkers

With respect to blood pressure measurements, systolic and diastolic blood pressure did not show significant changes in any group except for a significant reduction in diastolic blood pressure in the n-3+RT group (**Table 3**). However, two-way ANOVA revealed a significant effect for the DHA-rich supplement on lowering diastolic blood pressure in n-3 compared to P-supplemented groups.

Table 3. Effects of 16 weeks of DHA-rich n-3 PUFA (n-3) supplementation and/or resistance training (RT) on blood pressure and glucose and lipid metabolism biomarkers in overweight/obese postmenopausal women.

	P	**n-3**	**P+RT**	**n-3+RT**	**Two-way ANOVA[c]**		
N	20	15	20	16	n-3	RT	n-3xRT
SBP (mm/Hg)							
Baseline	121.83 ± 19.68	119.18 ± 7.98	122.73 ± 14.96	123.67 ± 9.01	*ns*	*ns*	*ns*
Change	-2.40 ± 10.84	0.20 ± 10.96	-1.22 ± 14.45	-6.00 ± 11.79	*ns*	*ns*	*ns*
DBP (mm/Hg)							
Baseline	80.04 ± 12.38	79.71 ± 6.05	79.08 ± 7.88	81.62 ± 6.67	*ns*	*ns*	*ns*
Change	-1.40 ± 7.13	-2.09 ± 6.97	1.83 ± 7.05	-4.94 ± 7.70[a,*]	0.035	*ns*	*ns*
Triglycerides (mg/dL)							
Baseline	92.64 ± 29.47	118.2 ± 55.31	110.90 ± 51.66	101.34 ± 33.26			
Change	1.94 ± 26.29	-28.87 ± 52.97[b,*]	-17.10 ± 23.78[b,*]	-18.88 ± 28.73[b,*]	0.047	*ns*	*ns*
Adjusted change[d]	2.99(6.14)	-22.09(7.34)[a,**]	-13.98(4.02)a[,**]	-20.75(5.06)[a,**]	0.038	*ns*	*ns*
Total chol (mg/dL)							
Baseline	237.40 ± 30.79	239.73 ± 46.41	254.50 ± 27.83	250.31 ± 45.89			
Change	-8.10 ± 25.20	-9.00 ± 44.86	-21.45 ± 24.34[b,**]	-14.41 ± 43.32	*ns*	*ns*	*ns*
Adjusted change[d]	-12.66(4.87)[a,*]	-12.66(8.06)	-17.42(3.83)[a,***]	-10.41(8.62)	*ns*	*ns*	*ns*
LDL-chol (mg/dL)							
Baseline	153.31 ± 32.65	154.20 ± 36.89	168.40 ± 24.49	164.90 ± 44.04			
Change	-6.98 ± 19.29	-4.22 ± 35.87	-14.20 ± 23.09[b,**]	-8.14 ± 38.54	*ns*	*ns*	*ns*
Adjusted change[d]	-10.46(3.65)[a,*]	-7.79(7.47)	-10.69(3.95)[a,*]	-5.29(7.60)	*ns*	*ns*	*ns*
HDL-chol (mg/dL)							
Baseline	65.74 ± 16.77	61.89 ± 16.38	63.92 ± 14.61	65.15 ± 11.03			
Change	-0.55 ± 9.45	-0.99 ± 13.97	-3.83 ± 8.32[a,†]	-2.50 ± 9.46	*ns*	*ns*	*ns*
Adjusted change[d]	-0.08(1.70)	-0.07(2.68)	-4.35(1.83)[a,*]	-1.63(2.08)	*ns*	*ns*	*ns*
Glucose (mg/dL)							
Baseline	98.57 ± 13.03	103.90 ± 15.72	109.14 ± 18.90	108.35 ± 11.32			
Change	-1.83 ± 9.22	-0.86 ± 11.87	-3.59 ± 20.78	-4.73 ± 9.36[a,†]	*ns*	*ns*	*ns*
Adjusted change[d]	-3.49(2.85)	-2.73(2.68)	-1.26(3.17)	-3.85(1.61)[a,*]	*ns*	*ns*	*ns*
Insulin (mU/L)							
Baseline	10.02 ± 4.41	9.90 ± 5.21	9.49 ± 5.00	10.54 ± 4.05			
Change	-2.63 ± 3.91[b,*]	-0.84 ± 2.75	-1.35 ± 4.03	-1.96 ± 2.76[b,*]	*ns*	*ns*	*ns*
Adjusted change[d]	-2.14(0.64)[a,**]	-1.20(0.77)	-1.82(0.64)[a,**]	-1.73(0.47)[a,*]	*ns*	*ns*	*ns*
HOMA-IR index							
Baseline	2.51 ± 1.43	2.48 ± 1.19	2.67 ± 1.86	2.82 ± 1.14			
Change	-0.71 ± 1.05[b,*]	-0.23 ± 0.87	-0.54 ± 1.52	-0.60 ± 0.74[b,**]	*ns*	*ns*	*ns*
Adjusted change[d]	-0.59(0.15)[a,***]	-0.46(0.22)[a,†]	-0.56(0.18)[a,**]	-0.52(0.13)[a,**]	*ns*	*ns*	*ns*
TyG index							
Baseline	8.45 ± 0.45	8.64 ± 0.41	8.64 ± 0.47	8.59 ± 0.39			
Change	-0.05 ± 0.31	-0.15 ± 0.37	-0.17 ± 0.27[a,*]	-0.23 ± 0.33[a,*]	*ns*	*ns*	*ns*
Adjusted change[d]	-0.06(0.07)	-0.15(0.09)	-0.15(0.06)[a,*]	-0.22(0.06)[a,**]	*ns*	*ns*	*ns*

Note. P: Placebo group, n-3: DHA-rich n-3 PUFA supplemented group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplemented + resistance training group; SBP: systolic blood pressure; DBP: diastolic blood pressure; Total chol: Total cholesterol; HDL-chol: HDL-cholesterol; LDL-chol: LDL-cholesterol; TyG: Triglycerides to Glucose index. Data are mean ± SD.

[a]Paired Student's *t*-test, [b]Wilcoxon's signed-rank test. [c]Differences between groups for changes were evaluated by two-way ANOVA. [d]Means(SEM) adjusted by changes in fat mass and values at baseline.

***P<0.001, **P<0.01, *P<0.05, [†]P trend (P=0.053-0.062) *vs.* baseline; *ns*, nonsignificant (P>0.05).

Triglycerides were significantly reduced after the intervention in all groups, except the P group. The analysis of the differences of changes between groups showed that this reduction in triglycerides was significantly higher in the n-3 supplemented groups as compared with those receiving placebo (**Table 3**). Total cholesterol, LDL-cholesterol and HDL-cholesterol were reduced only in P+RT group after the intervention when values were adjusted for fat mass loss and the corresponding baseline value. Nonetheless, when comparing the differences between groups no statistical differences were found for the changes in cholesterol parameters (**Table 3**).

3.6.Effects on serum glucose metabolism biomarkers

Fasting glucose tended to decrease in all intervention groups, but without statistical significance in any of them, neither when baseline-endpoint nor when changes between groups were compared (**Table 3**).

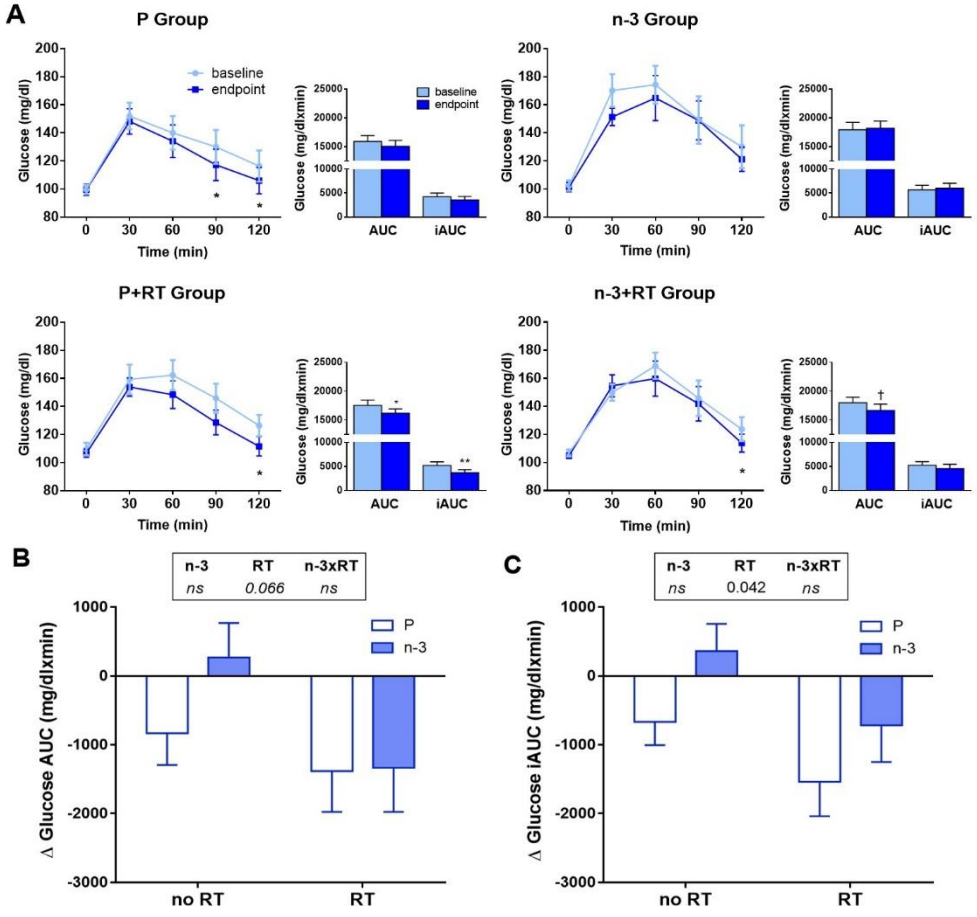


Figure 3. Effects of 16 weeks of DHA-rich n-3 PUFA (n-3) supplementation and/or resistance training (RT) on (**A**) oral glucose tolerance (OGTT) excursion curves, AUC and iAUC at baseline and endpoint. Comparisons between baseline and endpoint values were assessed using paired Student's *t*-test or Wilcoxon's signed-rank test. B-C Panels show changes (endpoint-baseline) between the experimental groups for AUC (**B**) and iAUC (**C**) respectively. Comparison of changes between groups was evaluated by two-way ANOVA (*ns*, nonsignificant, $P>0.05$). Data are mean ± SEM. *$P<0.05$, **$P<0.01$, $^{\dagger}P=0.060$ *vs.* baseline. P: Placebo group, n-3: DHA-rich n-3 PUFA supplemented group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplemented + resistance training group.

Fasting insulin and HOMA-IR index were significantly reduced in P-supplemented and n-3+RT groups after the intervention. However, no significant differences induced by RT or n-3-PUFA supplementation were observed when the changes between groups were analyzed (**Table 3**). The TyG index, a reliable marker for insulin resistance, was significantly reduced after the intervention in both groups performing the RT program (P+RT and n-3+RT); yet, when the differences in changes between the intervention groups were evaluated, no significant effects were reached (**Table 3**).

The effect of a 16-week RT program alone or in combination with DHA supplementation on glucose tolerance was also studied through an OGTT. For that, a high-glucose beverage (75 g) was administered to the volunteers in order to evaluate the changes in serum glucose. The glucose excursions after OGTT are represented in **Figure 3A**, which shows the levels before and after the intervention in the four intervention groups. After the intervention, all groups, except the n-3 group, exhibited a better response to the OGTT than at baseline. However, when the area under the curves (AUC) were calculated, only a significant reduction when comparing before and after was found in the P+RT group, and a tendency to decrease was observed in n-3+RT group. To adjust for basal glucose, iAUC was also calculated, with significant reductions in the P+RT group. When differential changes between groups were analyzed by two-way ANOVA, the AUC showed a tendency to decrease in RT groups (P=0.066), and a significant decrease in iAUC was found in RT groups *vs.* not trained groups (**Figure 3B-C**). Nevertheless, the DHA supplementation had no remarkable effect on any of the glucose tolerance parameters analyzed.

4. Discussion

Here, we describe the effects of a 16-week intervention with DHA-rich supplement combined or not with a RT program on body composition, serum glucose and lipid metabolism biomarkers, blood pressure and muscle strength and quality in overweight and obese postmenopausal women. While few previous studies in postmenopausal women have combined n-3 PUFA supplementation and RT, these trials were focused on muscle metabolism, strength and function; and some lacked either a placebo group, or an untrained group[23–26].

In the present study, the four experimental groups showed moderate but significant reductions in body weight, BMI, and fat percentage, along with visceral adipose tissue, and waist and hip circumferences after the intervention. These results suggest the efficacy of dietary advice for a healthy diet on remodeling body composition without a hypocaloric dietary approach. It should be considered that several studies have suggested that olive oil supplementation, the placebo used in our trial, can also promote moderate weight loss[60]. Therefore, although the amount of olive oil supplemented (3.0 g/day) was equivalent to approximately 10% daily recommendations, it could have been relevant, since it increases the amount of dietary olive oil usually ingested by the participants of the study, and thus might have contributed to the unexpected weight loss observed in the placebo groups. On the other hand, our data in the RT groups may apparently contrast with previous studies observing effects for RT on inducing fat mass loss in adults as compared to non-trained subjects[15,19]. However, RT effects in postmenopausal women without dietary treatment only had such effects in body composition when longer intervention periods[20,61], higher training volumes[9,23,26], groups mixing women and men[14,19,62,63], or only overweight or few proportions of obese women were included[18,61,64]. Even in those circumstances, no effects in body composition have been reported in postmenopausal women[11–13].

The lack of effects for RT on whole-body muscle mass, namely fat free mass or lean mass, has been reported in postmenopausal women[11–13,21]. Similar to those showing effects on adiposity, those reporting effects on increasing muscle mass used rather long exercise protocols, applied higher training volumes, mixed men and women, or were performed only in overweight subjects[9,19,25,65,66]. Here, we provide evidence of a 16-week protocol that achieved muscle strength and quality gains, alongside high adherence rates (above 95%), with a moderate RT program (10 out of 16 weeks with 70-80% RM loads) in previously untrained, overweight, and obese post-menopausal women. Others have reported increased muscle strength, quality, cross sectional area or muscle protein synthesis that were not translated to higher muscle mass in older populations nor in postmenopausal women[9,12,13,67]. Noteworthy, Churchward-Venne *et al.*[68] compared two different training programs in older overweight women and concluded that, although there are no non-responders to exercise, the time-effect response is highly individualized. Similarly, Ahtiainen *et al.*[19] collected studies of their group developing the same RT program in different populations and observed a large interindividual variations in the training response that could not be explained by sex, age, body composition or nutritional status. Therefore, the physiological adaptations secondary to RT might rely on individual features whose causative role are beyond the purpose of this study.

Analyses of upper and lower body composition by DXA segmented analyses revealed principal effects for the RT program when studying changes among groups. In upper limbs, our RT program exhibited effects on arms muscle hypertrophy and weight maintenance compared to the untrained groups, who showed a moderate weight and muscle loss. To our knowledge, it is the first time these effects of RT compared to untrained postmenopausal women are reported in the literature with shorter training programs, as improvements in segmented muscle mass have been reported previously only for overweight older women and longer training periods and/or higher frequencies[20,55]. Regarding lower body composition, RT did not show an effect in legs weight and muscle mass, but it did increase fat mass loss as compared to untrained groups. Such effects could be explained by women's natural distribution of adiposity in gluteofemoral areas, and thus could be the main effect of our RT program on fat mass losses. In fact, those studies reporting increases in lower limbs lean mass after RT were conducted in leaner women[21], and no differences have been reported on lower limbs fat mass[21,23,55]. Anthropometric measurements results agreed with those found in DXA for lower limbs body composition, as RT had a significant effect in decreasing leg skinfolds and calf circumference as compared to untrained groups. On the contrary, thigh circumference did not change with RT, indicating a possible hypertrophy in thighs and higher fat mass losses at calves. Upper limbs anthropometric measurements did match those of DXA regarding arms hypertrophy with RT, as arms circumference did not change. Thus, these results support that segmented body composition changes measured by DXA were comparable to those obtained with the anthropometric measurements including the observed upper limbs hypertrophy and lower limbs fat mass loss after RT. Moreover, these results establish anthropometry as a reliable tool for evaluating changes in upper and lower body composition in overweight/obese postmenopausal women under exercise programs.

Findings of RT effects improving upper and lower body muscle mass, quality and strength were consistent with resistance training improving also whole-body BMC. A clear effect of RT on BMC maintenance was observed, despite small changes and high variability. However, there were no effects on bone mineral density (BMD) (data not shown). Data of exercise programs effects on bone metabolism in postmenopausal women is large, and thus several meta-analyses have been conducted, concluding that combined RT and high impact exercises are the best to maintain BMD after longer interventional periods[10], while local effects

of RT on BMD have also been observed[69]. Although BMC results can be found in trials, such results were not included in the statistical analyses of such meta-analyses. In fact, BMC has less clinical relevance compared to BMD, and thus studies of BMC changes in postmenopausal women after RT are lacking. Despite this, both BMD and BMC are predictors of fracture risk[70]. BMC improvements without concomitant effects on BMD after RT in postmenopausal women with low bone mass have been reported before[71]. Thus, our finding supports a beneficial effect of RT on bone mass independent of n-3-PUFA supplementation and regardless of having or not low BMD and BMC in a population at risk of bone mass loss.

As expected, no effects were observed for n-3 PUFA supplementation alone or combined with RT on body composition changes[37]. Other groups have observed similar results to ours using DHA and EPA at similar doses (1.62 g/day, 1.9:1 DHA:EPA)[46,47]. Such studies did not show differences between the placebo (6 capsules x 1g sunola oil per day) and fish oil supplemented group in any of the body weight and composition parameters after 12 weeks of supplementation[46], neither when combining it with a very low energy diet[47]. Surprisingly, a significant effect of n-3-PUFA supplementation was found for smaller fat mass losses in arms than placebo supplementation. Conversely, these effects were not translated to smaller subcutaneous fat mass losses, as both tricipital skinfold and arm circumference decreased in the four groups of study without differences in changes. In fact, it has been described that the main synergistic effects of n-3 PUFA to RT on body composition are the promotion of muscular protein synthesis together with beneficial effects on the neuromuscular system[24,72], with no further effects on body composition. With this regard, our results showed effects for RT on increasing muscular strength and quality significantly in lower and upper limbs, while n-3 PUFA revealed an effect on muscle quality in lower limbs. Such effects are supported by those observed by Rodacki *et al.*[26] and Strandberg *et al.*[25], who showed increased activation and neuromuscular response to RT in groups supplemented with n-3-PUFA (2g, EPA 29.5 ± 0.7% and DHA 23.6 ± 0.2%, for 90 or 150 days) or with a n-3 PUFA enriched diet (ratio n-6/n-3 < 2, 24 weeks) respectively, coupled to a RT program. It must be noted that their RT programs used high training intensities for longer training periods (80% of 1-RM for 10 and 20 weeks), and that subjects were leaner than those included in our study, according to BMI (mean BMI ~24.7-27.7 kg/m^2).

The DHA-rich n-3 PUFA supplementation exerted two remarkable effects in main cardiovascular risk factors, blood pressure and circulating lipids. Firstly, diastolic blood pressure was reduced by the DHA-rich supplementation when compared to the placebo supplementation. This result is in line with others who have observed inverse associations between erythrocyte DHA content and hypertension in postmenopausal women[33,73]. Although a recent trial in middle aged women showed no results after n-3 PUFA supplementation with similar DHA dose to those used in our study, a smaller sample size (n=6, 1600 mg DHA + 400 mg EPA) was studied[74]. On the other hand and similar to what has been demonstrated in older subjects and in postmenopausal women[33,75], the DHA-rich n-3 PUFA supplementation also induced a reduction in circulating triglycerides, which is in agreement with the recognized n-3 PUFA hypotriglyceridemic claimed effects. Concerning total cholesterol, LDL-cholesterol and HDL-cholesterol no effects were observed for the DHA-rich supplementation on improving their levels when compared to the Placebo groups, nor when comparing within groups. Accordingly, a recent meta-analysis concluded that n-3 PUFA exert effects only in lowering triglycerides[75]. These effects of DHA supplementation on cardiovascular risk factors are especially relevant in the postmenopausal population, in which cardiovascular-mortality exhibits a sudden rise[76].

Regarding the RT program, no effects were observed on any of the cardiovascular risk parameters when comparing trained groups to untrained groups, although circulating levels of Total chol, LDL-cholesterol and HDL-cholesterol were decreased in the P+RT group, and both trained groups revealed a decrease in triglycerides. Despite the recent meta-analyses describing beneficial effects of RT on circulating lipid levels in adults[15,77], multiple trials investigating RT effects on blood lipid levels observed no effects in postmenopausal women and older adults[19,21,63,78]. Thus, several studies have assessed this question by examining the individual response to RT in older adults. Although the occurrence of non-responders to RT was discarded recently[68,79], the individual response to RT was quantified in the aforementioned studies and described to be highly heterogeneous, leading to a great variability in the measured outcomes especially in blood lipids[19,68], as observed in our n-3+RT group. On the other hand, the presence of subjects with delayed time-response effect might have led to little changes in body composition[62,68], that in turn mediate circulating lipid levels[64,80]. Nevertheless, it seems that the most consistent outcome of RT in blood lipid levels in older adults and postmenopausal women is the increase in HDL-cholesterol, even when the rest of lipids do not change[19,62,81]. By contrast, our HDL-cholesterol levels were decreased in both trained groups and significantly in P+RT group, possibly due to the higher levels at baseline observed in our participants compared to those participating in the aforementioned trials. Moreover, such levels could be a consequence of increased levels of the atherogenic HDL-cholesterol fraction.

A remarkable effect exerted by RT was the improvement in glucose tolerance. Thus, RT groups showed an effect in OGTT-iAUC compared to untrained groups, regardless of n-3-PUFA supplementation. These effects of RT on improving glucose tolerance in postmenopausal women can rely on the local increases in muscle mass and the muscle strength and quality gains. In fact, sarcopenic older subjects under RT exhibited lower glucose AUC after the intervention[82], demonstrating muscle's essential role in glucose metabolism. However, the improved glucose tolerance was not accompanied by similar outcomes in fasting glucose, insulin, and HOMA-IR index, although small but significant improvements were observed in TyG index for insulin resistance in both trained groups. Insulin sensitivity measurements are lacking in this study, but it might be the causative factor for the improved glucose metabolism and TyG index, regardless of fasting insulin and HOMA-IR levels, and secondary to the increased glucose tolerance. In fact, RT has been shown to have lower effects on insulin resistance as compared to aerobic or combined (resistance + aerobic) training[14] which are, in turn, demonstrated to elicit higher fat mass losses[83]. Moreover, the higher variability in responses to the intervention observed in the n3-PUFA+RT group may have blunted both RT and n-3 PUFA principal effects when changes between groups were studied. Nevertheless, it has to be noted that lack of synergistic effects for RT and fish oil supplementation on fasting glucose and insulin was also observed in postmenopausal women in the study of Da Boit *et al.*[24].

There are limitations in the current study that need to be considered. Some previous clinical trials with n-3 PUFA derived from fish oil have reported difficulties to successful blinding because of the fishy taste and odor of these fatty acids[84]. We must report a difficulty with the double blinding of our study, due to the fishy taste reported by some of the participants allocated in the DHA-rich supplement group and a small difference in the thickness/color of the olive (placebo) and the fish oil concentrate, as they were provided in transparent liquid fill capsules. Although some of the investigators providing the capsules to the volunteers suspect about the type of supplements, most of the participants were blinded concerning the type of supplement they were receiving. Moreover, the researchers in charge to carry out the biochemical analysis of the blood samples

and DXA analysis were totally blinded and therefore we consider that this minor incident with the blinding has not affected the results reported in this study.

Another limitation of the study is that we had few participants between 65-70 years, and the mean ages of subjects were closer to the middle-age, and thus interrelationships between obesity and menopause may have had a more relevant role than expected in the response to both RT and n-3-PUFA supplementation. Moreover, evidence in the current literature for both RT and n-3-PUFA interventions in postmenopausal women is largely heterogenous regarding methodologies conducted and characteristics of the study populations, making it difficult to compare effects between studies. These facts and the applicability of the findings obtained in the present study highlight the relevance of performing future trials involving a higher number of postmenopausal older women.

5. Conclusions

In summary, we conclude that progressive intensity RT has beneficial effects on upper limbs muscular hypertrophy and lower limbs fat mass loss, on muscle strength and muscle quality, along with whole body BMC maintenance and improved glucose tolerance in postmenopausal women. The DHA-rich oil supplement had the previously documented effect on lowering fasting triglycerides levels and lowered diastolic blood pressure. However, no effects were found on insulin resistance or other biomarkers of lipid metabolism, and no relevant synergistic effects for n-3-PUFA and RT were observed.

References

1. Gold EB. The timing of the age at which natural menopause occurs. *Obstet Gynecol Clin North Am*. 2011;38:425-440. doi:10.1016/j.ogc.2011.05.002
2. Zhang X, Liu L, Song F, Song Y, Dai H. Ages at menarche and menopause, and mortality among postmenopausal women. *Maturitas*. 2019;130:50-56. doi:10.1016/j.maturitas.2019.10.009
3. Polotsky HN, Polotsky AJ. Metabolic implications of menopause. *Semin Reprod Med*. 2010;28:426-434. doi:10.1055/s-0030-1262902.
4. Mauvais-Jarvis F. Epidemiology of gender differences in diabetes and obesity. *Adv Exp Med Biol*. 2017;1043:3-8. doi:10.1007/978-3-319-70178-3_1
5. Anand SS, Islam S, Rosengren A, et al. Risk factors for myocardial infarction in women and men: insights from the INTERHEART study. *Eur Heart J*. 2008;29:932-940. doi:10.1093/eurheartj/ehn018
6. Al-Safi ZA, Polotsky AJ. Obesity and menopause. *Best Pract Res Clin Obstet Gynaecol*. 2015;29:548-553. doi:10.1016/j.bpobgyn.2014.12.002
7. Elhakeem A, Cooper R, Whincup P, Brage S, Kuh D, Hardy R. Physical activity, sedentary time, and cardiovascular disease biomarkers at age 60 to 64 years. *J Am Heart Assoc*. 2018;7:e007459. doi:10.1161/JAHA.117.007459
8. Peterson MD, Sen A, Gordon PM. Influence of resistance exercise on lean body mass in aging adults: a meta-analysis. *Med Sci Sports Exerc*. 2011;43:249-258. doi:10.1249/MSS.0b013e3181eb6265.
9. Caserotti P, Aagaard P, Buttrup Larsen J, Puggaard L. Explosive heavy-resistance training in old and very old adults: Changes in rapid muscle force, strength and power. *Scand J Med Sci Sport*. 2008;18:773-782. doi:10.1111/j.1600-0838.2007.00732.x
10. Zhao R, Zhao M, Xu Z. The effects of differing resistance training modes on the preservation of bone mineral density in postmenopausal women: a meta-analysis. *Osteoporos Int*. 2015;26:1605-1618. doi:10.1007/s00198-015-3034-0
11. Häkkinen K, Pakarinen A, Kraemer WJ, Newton RU, Alen M. Basal concentrations and acute responses of serum hormones and strength development during heavy resistance training in middle-

aged and elderly men and women. *Journals Gerontol Ser A Biol Sci Med Sci*. 2000;55:B95-105. doi:10.1093/gerona/55.2.b95

12. Hanson ED, Srivatsan SR, Agrawal S, et al. Effects of strength training on physical function: influence of power, strength, and body composition. *J Strength Cond Res*. 2010;23:2627-2637. doi:10.1519/JSC.0b013e3181b2297b.
13. Ryan AS, Hurlbut DE, Lott ME, et al. Insulin action after resistive training in insulin resistant older men and women. *J Am Geriatr Soc*. 2001;49:247-253. doi:10.1046/j.1532-5415.2001.4930247.x.
14. Davidson LE, Hudson R, Kilpatrick K, et al. Effects of exercise modality on insulin resistance and functional limitation in older adults: a randomized controlled trial. *Arch Intern Med*. 2009;169:122-131. doi:10.1001/archinternmed.2008.558.7/s12170-009-0037-4
15. Cornelissen VA, Fagard RH, Coeckelberghs E, Vanhees L. Impact of resistance training on blood pressure and other cardiovascular risk factors: a meta-analysis of randomized, controlled trials. *Hypertension*. 2011;58:950-958. doi:10.1161/HYPERTENSIONAHA.111.177071
16. da Cunha Nascimento D, da Silva CR, Valduga R, et al. Blood pressure response to resistance training in hypertensive and normotensive older women. *Clin Interv Aging*. 2018;13:541-553. doi:10.2147/CIA.S157479
17. Wooten JS, Phillips MD, Mitchell JB, et al. Resistance exercise and lipoproteins in postmenopausal women. *Int J s*. 2011;32:7-13. doi:10.1055/s-0030-1268008.
18. Ribeiro AS, Tomeleri CM, Souza MF, et al. Effect of resistance training on C-reactive protein, blood glucose and lipid profile in older women with differing levels of RT experience. *Age (Omaha)*. 2015;37:109. doi:10.1007/s11357-015-9849-y
19. Ahtiainen JP, Sallinen J, Häkkinen K, Sillanpää E. Inter-individual variation in response to resistance training in cardiometabolic health indicators. *Scand J Med Sci Sport*. 2020;30:1040-1053. doi:10.1111/sms.13650
20. Botero JP, Shiguemoto GE, Prestes J, et al. Effects of long-term periodized resistance training on body composition, leptin, resistin and muscle strength in elderly post-menopausal women. *J Sports Med Phys Fitness*. 2013;53:1460.
21. Sillanpää E, Laaksonen DE, Häkkinen A, et al. Body composition, fitness, and metabolic health during strength and endurance training and their combination in middle-aged and older women. *Eur J Appl Physiol*. 2009;106:285-296. doi:10.1007/s00421-009-1013-x
22. Lalia AZ, Dasari S, Robinson MM, et al. Influence of omega-3 fatty acids on skeletal muscle protein metabolism and mitochondrial bioenergetics in older adults. *Aging (Albany NY)*. 2017;9:1096-1129. doi:10.18632/aging.101210
23. Strandberg E, Ponsot E, Piehl-Aulin K, Falk G, Kadi F. Resistance training alone or combined with n-3 PUFA-rich diet in older women: effects on muscle fiber hypertrophy. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2019;74:489-493. doi:10.1093/gerona/gly130
24. Da Boit M, Sibson R, Sivasubramaniam S, et al. Sex differences in the effect of fish-oil supplementation on the adaptive response to resistance exercise training in older people: a randomized controlled trial. *Am J Clin Nutr*. 2017;105:151-158. doi:10.3945/ajcn.116.140780
25. Strandberg E, Edholm P, Ponsot E, et al. Influence of combined resistance training and healthy diet on muscle mass in healthy elderly women: a randomized controlled trial. *J Appl Physiol*. 2015;119:918-925. doi:10.1152/japplphysiol.00066.2015
26. Rodacki CLN, Rodacki ALF, Pereira G, et al. Fish-oil supplementation enhances the effects of strength training in elderly women. *Am J Clin Nutr*. 2012;95:428-436. doi:10.3945/ajcn.111.021915
27. Lorente-Cebrián S, Costa AG V, Navas-Carretero S, Zabala M, Alfredo Martínez J, Moreno-Aliaga MJ. Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: a review of the evidence. *J Physiol Biochem*. 2013;69:633-651. doi:10.1007/s13105-013-0265-4
28. Zhuang P, Lu Y, Shou Q, et al. Differential anti-adipogenic effects of eicosapentaenoic and docosahexaenoic acids in obesity. *Mol Nutr Food Res*. 2019;63:e1801135. doi:10.1002/mnfr.201801135
29. Erkkilä AT, Matthan NR, Herrington DM, Lichtenstein AH. Higher plasma docosahexaenoic acid is associated with reduced progression of coronary atherosclerosis in women with CAD. *J Lipid Res*. 2006;47:2814-2819. doi:10.1194/jlr.P600005-JLR200

30. Rhee Y, Paik MJ, Kim KR, et al. Plasma free fatty acid level patterns according to cardiovascular risk status in postmenopausal women. *Clin Chim Acta*. 2008;392:11-16. doi:10.1016/j.cca.2008.02.012

31. Woodman RJ, Mori TA, Burke V, et al. Effects of purified eicosapentaenoic acid and docosahexaenoic acid on platelet, fibrinolytic and vascular function in hypertensive type 2 diabetic patients. *Atherosclerosis*. 2003;166:85-93. doi:10.1016/S0021-9150(02)00307-6

32. Sparkes C, Gibson R, Sinclair A, Else PL, Meyer BJ. Effect of low dose docosahexaenoic acid-rich fish oil on plasma lipids and lipoproteins in pre-menopausal women: a dose-response randomized placebo-controlled trial. *Nutrients*. 2018;10:1460. doi:10.3390/nu10101460

33. Griffin MD, Sanders TAB, Davies IG, et al. Effects of altering the ratio of dietary n-6 to n-3 fatty acids on insulin sensitivity, lipoprotein size, and postprandial lipemia in men and postmenopausal women aged 45-70 y: The OPTILIP study. *Am J Clin Nutr*. 2006;84:1290-1298. doi:10.1093/ajcn/84.6.1290

34. AbuMweis S, Jew S, Tayyem R, Agraib L. Eicosapentaenoic acid and docosahexaenoic acid containing supplements modulate risk factors for cardiovascular disease: a meta-analysis of randomised placebo-control human clinical trials. *J Hum Nutr Diet*. 2018;31:67-84. doi:10.1111/jhn.12493

35. Gao H, Geng T, Huang T, Zhao Q. Fish oil supplementation and insulin sensitivity: a systematic review and meta-analysis. *Lipids Health Dis*. 2017;16:131. doi:10.1186/s12944-017-0528-0

36. Akinkuolie AO, Ngwa JS, Meigs JB, Djoussé L. Omega-3 polyunsaturated fatty acid and insulin sensitivity: a meta-analysis of randomized controlled trials. *Clin Nutr*. 2011;30:702-707. doi:10.1016/j.clnu.2011.08.013.

37. Bender N, Portmann M, Heg Z, Hofmann K, Zwahlen M, Egger M. Fish or n3-PUFA intake and body composition: a systematic review and meta-analysis. *Obes Rev*. 2014;15:657-665. doi:10.1111/obr.12189

38. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *J Am Coll Dent*. 2014;81:14-18. doi:10.1093/acprof:oso/9780199241323.003.0025

39. World Health Organization. *Men, Ageing and Health: Achieving Health across the Life Span*.; 2001.

40. World Health Organization. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. 2000;894:i-xii, 1-253.

41. Huerta AE, Prieto-Hontoria PL, Fernández-Galilea M, et al. Circulating irisin and glucose metabolism in overweight/obese women: effects of α-lipoic acid and eicosapentaenoic acid. *J Physiol Biochem*. 2015;71:547-558. doi:10.1007/s13105-015-0400-5

42. Aranceta Bartrina J, Arija Val V V., Maíz Aldalur E, et al. Dietary Guidelines for the Spanish population (SENC, diciembre 2016); the new graphic icon of healthy food. *Nutr Hosp*. 2016;33:e95581. doi:10.20960/nh.827

43. Schröder H, Fitó M, Estruch R, et al. A short screener is valid for assessing mediterranean diet adherence among older spanish men and women. *J Nutr*. 2011;141:1140-1145. doi:10.3945/jn.110.135566

44. Martínez-González MA, López-Fontana C, Varo JJ, Sánchez-Villegas A, Martinez JA. Validation of the Spanish version of the physical activity questionnaire used in the Nurses' Health Study and the Health Professionals' Follow-up Study. *Public Health Nutr*. 2005;8:920-927. doi:10.1079/phn2005745

45. Munro IA, Garg ML. Prior supplementation with long chain omega-3 polyunsaturated fatty acids promotes weight loss in obese adults: a double-blinded randomised controlled trial. *Food Funct*. 2013;4:650-658. doi:10.1039/c3fo60038f

46. Munro IA, Garg ML. Dietary supplementation with long chain omega-3 polyunsaturated fatty acids and weight loss in obese adults. *Obes Res Clin Pract*. 2013;7:e173-e181. doi:10.1016/j.orcp.2011.11.001

47. Munro IA, Garg ML. Dietary supplementation with n-3 PUFA does not promote weight loss when combined with a very-low-energy diet. *Br J Nutr*. 2012;108:1466-1474. doi:10.1017/S0007114511006817

48. U.S. Food and Drug Administration. Qualified Health Claims: Letters of Enforcement Discretion | FDA. Omega-3 fatty acids and reduced risk of coronary heart disease (Martek petition). Published 2004. https://www.fda.gov/food/food-labeling-nutrition/qualified-health-claims-letters-enforcement-discretion

49. Mataix-Verdú J, Mañas Almendros M, Llopis González J, Martínez de Victoria E. *Tablas de Composición de Alimentos*. Universidad de Granada; 1993.

50. Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). *EFSA J*. 2012;10:2815. doi:10.2903/j.efsa.2012.2815

51. Ibañez J, Izquierdo M, Argüelles I, et al. Twice-weekly progressive resistance training decreases abdominal fat and improves insulin sensitivity in older men with type 2 diabetes. *Diabetes Care*. 2005;28:662-667. doi:10.2337/diacare.28.3.662

52. Ibáñez J, Gorostiaga EM, Alonso AM, et al. Lower muscle strength gains in older men with type 2 diabetes after resistance training. *J Diabetes Complications*. 2008;22:112-118. doi:10.1016/j.jdiacomp.2007.06.008

53. Cuff DJ, Meneilly GS, Martin A, Ignaszewski A, Tildesley HD, Frohlich JJ. Effective exercise modality to reduce insulin resistance in women with type 2 diabetes. *Diabetes Care*. 2003;26:2977-2982. doi:10.2337/diacare.26.11.2977

54. American College of Sports Medicine. Progression models in resistance training for healthy adults. *Med Sci Sports Exerc*. 2009;41:687-708. doi:10.1249/MSS.0b013e3181915670

55. Pina FLC, Nunes JP, Nascimento MA, Ribeiro AS, Mayhew JL, Cyrino ES. Similar Effects of 24 Weeks of Resistance Training Performed with Different Frequencies on Muscle Strength, Muscle Mass, and Muscle Quality in Older Women. *Int J Exerc Sci*. 2019;12:623-635. http://www.ncbi.nlm.nih.gov/pubmed/31156757%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC6533095

56. Huerta AE, Navas-Carretero S, Prieto-Hontoria PL, Martínez JA, Moreno-Aliaga MJ. Effects of α-lipoic acid and eicosapentaenoic acid in overweight and obese women during weight loss. *Obesity*. 2015;23:313-321. doi:10.1002/oby.20966

57. Stewart A, Marfell-Jones M, Olds T, de Ridder H. *International Standards for Anthropometric Assessment*. New Zealand: ISAK: LowerHutt; 2011.

58. Hill AM, Buckley JD, Murphy KJ, Howe PRC. Combining fish-oil supplements with regular aerobic exercise improves body composition and cardiovascular disease risk factors. *Am J Clin Nutr*. 2007;85:1267-1274. doi:10.1093/ajcn/85.5.1267

59. DeFina LF, Marcoux LG, Devers SM, Cleaver JP, Willis BL. Effects of omega-3 supplementation in combination with diet and exercise on weight loss and body composition. *Am J Clin Nutr*. 2011;93:455-462. doi:10.3945/ajcn.110.002741

60. Zamora Zamora F, Miguel Martínez Galiano J, Juan Gaforio Martínez J, Delgado Rodríguez M. Olive oil and body weight. Systematic review and meta-analysis of randomized controlled trials. *Rev Esp Salud Pública*. 2018;92:e201811083. Accessed November 23, 2020. www.msc.es/resp

61. Hunter GR, Bryan DR, Wetzstein CJ, Zuckerman PA, Bamman MM. Resistance training and intra-abdominal adipose tissue in older men and women. *Med Sci Sports Exerc*. 2002;34:1023-1028. doi:10.1097/00005768-200206000-00019

62. Ihalainen JK, Inglis A, Mäkinen T, et al. Strength training improves metabolic health markers in older individual regardless of training frequency. *Front Physiol*. 2019;10:32. doi:10.3389/fphys.2019.00032

63. Phillips BE, Williams JP, Greenhaff PL, Smith K, Atherton PJ. Physiological adaptations to resistance exercise as a function of age. *JCI insight*. 2017;2:1-16. doi:10.1172/jci.insight.95581

64. Tomeleri CM, Ribeiro AS, Souza MF, et al. Resistance training improves inflammatory level, lipid and glycemic profiles in obese older women: a randomized controlled trial. *Exp Gerontol*. 2016;84:80-87. doi:10.1016/j.exger.2016.09.005

65. Ribeiro AS, Schoenfeld BJ, Souza MF, et al. Traditional and pyramidal resistance training systems improve muscle quality and metabolic biomarkers in older women: A randomized crossover study. *Exp Gerontol*. 2016;79:8-15. doi:10.1016/j.exger.2016.03.007

66. Sardeli AV, Tomeleri CM, Cyrino ES, Fernhall B, Cavaglieri CR, Chacon-Mikahil MPT. Effect of resistance training on inflammatory markers of older adults: a meta-analysis. *Exp Gerontol*. 2018;111:188-196. doi:10.1016/j.exger.2018.07.021

67. Phillips MD, Patrizi RM, Cheek DJ, Wooten JS, Barbee JJ, Mitchell JB. Resistance training reduces subclinical inflammation in obese, postmenopausal women. *Med Sci Sports Exerc*. 2012;44:2099-2110. doi:10.1249/MSS.0b013e3182644984

68. Churchward-Venne TA, Tieland M, Verdijk LB, et al. There are no nonresponders to resistance-type exercise training in older men and women. *J Am Med Dir Assoc*. 2015;16:400-411. doi:10.1016/j.jamda.2015.01.071

69. Howe T, Shea B, LJ D, et al. Exercise for preventing and treating osteoporosis in postmenopausal women (Review). *Cochrane Database Syst Rev*. 2011;7:CD000333. doi:10.1002/14651858.CD000333.pub2.

70. Curtis EM, Harvey NC, D'Angelo S, et al. Bone mineral content and areal density, but not bone area, predict an incident fracture risk: a comparative study in a UK prospective cohort. *Arch Osteoporos*. 2016;11:39. doi:10.1007/s11657-016-0293-0

71. Mosti MP, Kaehler N, Stunes AK, Hoff J, Syversen U. Maximal strength training in postmenopausal women with osteoporosis or osteopenia. *J Strength Cond Res*. 2013;27:2879-2886. doi:10.1519/JSC.0b013e318280d4e2

72. Smith GI, Atherton P, Reeds DN, et al. Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperinsulinaemia-hyperaminoacidaemia in healthy young and middle-aged men and women. *Clin Sci*. 2011;121:267-278. doi:10.1042/CS20100597

73. Zhang G, Sun Q, Hu FB, et al. Erythrocyte n-3 fatty acids and metabolic syndrome in middle-aged and older Chinese. *J Clin Endocrinol Metab*. 2012;97:E973-7. doi:10.1210/jc.2011-2997

74. Howe PRC, Evans HM, Kuszewski JC, Wong RHX. Effects of long chain omega-3 polyunsaturated fatty acids on brain function in mildly hypertensive older adults. *Nutrients*. 2018;10:1413. doi:10.3390/nu10101413

75. Zhang YY, Liu W, Zhao TY, Tian HM. Efficacy of omega-3 polyunsaturated fatty acids supplementation in managing overweight and obesity: a meta-analysis of randomized clinical trials. *J Nutr Heal Aging*. 2017;21:187-192. doi:10.1007/s12603-016-0755-5

76. Bassan R. Cardiovascular changes and cardiac morbidity of menopause. Effects of hormone replacement therapy. *Arq Bras Cardiol*. 1999;72:92-98. doi:10.1590/S0066-782X1999000100008

77. Costa RR, Buttelli ACK, Vieira AF, et al. Effect of strength training on lipid and inflammatory outcomes: Systematic review with meta-analysis and meta-regression. *J Phys Act Heal*. 2019;16:477-491. doi:10.1123/jpah.2018-0317

78. Marques E, Carvalho J, Soares JMC, Marques F, Mota J. Effects of resistance and multicomponent exercise on lipid profiles of older women. *Maturitas*. 2009;63:84-88. doi:10.1016/j.maturitas.2009.03.003

79. Barbalho M de SM, Gentil P, Izquierdo M, Fisher J, Steele J, Raiol R de A. There are no no-responders to low or high resistance training volumes among older women. *Exp Gerontol*. 2017;99:18-26. doi:10.1016/j.exger.2017.09.003

80. Cunha PM, Tomeleri CM, Nascimento MA, et al. Comparision of low and high volume of resistance training on body fat and blood biomarkers in untrained older women: a randomized clinical trial. *J Strength Cond Res*. 2019;35:1-8. doi:10.1519/jsc.0000000000003245

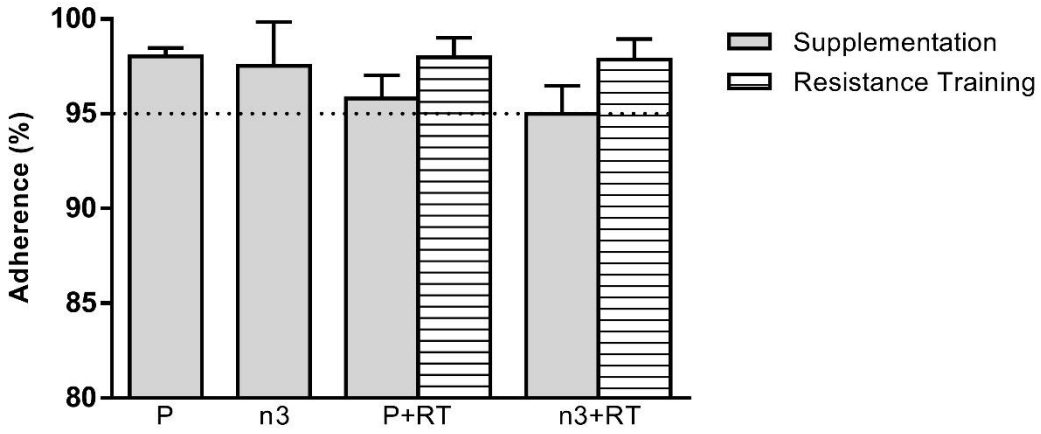
81. Dos Santos ES, Asano RY, Filho IG, et al. Acute and chronic cardiovascular response to 16 weeks of combined eccentric or traditional resistance and aerobic training in elderly hypertensive women: a randomized controlled trial. *J Strength Cond Res*. 2014;28:3073-3084. doi:10.1519/JSC.0000000000000537

82. Spolador de Alencar Silva B, Santos Lira F, Eduardo Rossi F, et al. Elastic resistance training improved glycemic homeostasis, strength, and functionality in sarcopenic older adults: a pilot study. *J Exerc Rehabil*. 2018;14:1085-1091. doi:10.12965/jer.1836412.206

83. Slentz CA, Bateman LA, Willis LH, et al. Effects of aerobic vs. resistance training on visceral and liver fat stores, liver enzymes, and insulin resistance by HOMA in overweight adults from STRRIDE AT/RT. *Am J Physiol - Endocrinol Metab*. 2011;301:1033-1039. doi:10.1152/ajpendo.00291.2011

84. Liu JC, Ang RP, Fung DS. Something fishy: The issue of omega-3 blinding in psychiatric clinical trials. *Aust N Z J Psychiatry*. 2013;47:201-205. doi:10.1177/0004867412451343

Supporting information



Supplementary Figure 1. Adherence to supplements intake and resistance training program in the four intervention groups. P: Placebo group, n-3: DHA-rich n-3 PUFA supplemented group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplemented + resistance training group. Data are mean ± SEM.

Supplementary Table 1. Baseline characteristics of the study population.

	P	n-3	P+RT	n-3+RT	
Number of subjects	20	15	20	16	*P*[a]
Age (years)	58.75 ± 3.39	58.00 ± 2.78	58.95 ± 3.46	58.13 ± 3.14	*ns*
Weight (kg)	76.75 ± 6.04	80.34 ± 8.51	77.76 ± 7.92	80.57 ± 6.60	*ns*
Height (m)	1.59 ± 0.06	1.62 ± 0.06	1.59 ± 0.06	1.61 ± 0.06	*ns*
BMI (kg/m^2)	30.25 ± 2.30	30.39 ± 1.94	30.79 ± 2.34	31.07 ± 1.82	*ns*
Fat mass (%)	48.80 ± 3.41	46.91 ± 2.35	48.40 ± 3.98	48.03 ± 2.92	*ns*
Muscle mass (%)	49.76 ± 3.20	51.52 ± 2.18	50.16 ± 3.78	50.53 ± 2.76	*ns*
Visceral fat (kg)	1.30 ± 0.44	1.37 ± 0.44	1.27 ± 0.51	1.18 ± 0.47	*ns*
BMC (g)	2152.65 ± 308.05	2366.33 ± 332.76	2156.10 ± 231.21	2240.31 ± 258.36	*ns*
Waist circumference (cm)	93.11 ± 4.57	95.00 ± 7.63	92.67 ± 5.47	93.90 ± 7.16	*ns*
Hip circumference (cm)	110.68 ± 7.14	112.50 ± 5.78	110.65 ± 5.74	113.35 ± 6.82	*ns*
Waist/Hip Ratio	0.84 ± 0.04	0.85 ± 0.06	0.84 ± 0.06	0.83 ± 0.08	*ns*
Arms weight (kg)	8.65 ± 0.89	8.60 ± 1.17	8.73 ± 1.21	8.83 ± 1.04	*ns*
Arms fat mass (kg)	4.36 ± 0.60	4.01 ± 0.68	4.32 ± 0.68	4.24 ± 0.71	*ns*
Arms muscle (kg)	4.02 ± 0.56	4.30 ± 0.60	4.13 ± 0.66	4.29 ± 0.47	*ns*
Legs weight (kg)	25.10 ± 3.59	27.52 ± 3.28	26.55 ± 3.91	27.11 ± 3.95	*ns*
Legs fat mass (kg)	11.29 ± 2.58	12.16 ± 2.38	12.36 ± 2.88	12.18 ± 2.47	*ns*
Legs muscle (kg)	13.03 ± 1.57	14.51 ± 1.66	13.40 ± 1.64	14.11 ± 1.88	*ns*
Arm circumference (cm)	34.87 ± 1.49	34.02 ± 2.30	34.59 ± 2.31	35.56 ± 2.39	*ns*
Tricipital skinfold (mm)	31.85 ± 5.15	31.04 ± 3.91	32.95 ± 3.88	33.28 ± 5.42	*ns*
Thigh circumference (cm)	56.42 ± 4.54	56.75 ± 3.48	59.71 ± 5.21	60.42 ± 6.42	*ns*
Calf circumference (cm)	38.05 ± 2.12	39.59 ± 2.58	40.15 ± 2.41	39.30 ± 2.55	*ns*
Thigh skinfold (mm)	39.92 ± 4.76	41.25 ± 5.49	42.67 ± 2.99	41.06 ± 5.37	*ns*
Calf skinfold (mm)	31.07 ± 5.28	32.02 ± 5.60	33.26 ± 4.88	34.76 ± 5.45	*ns*
Lower limbs muscle strength	2.24 ± 0.38	2.26 ± 0.46	2.19 ± 0.40	2.01 ± 0.28	*ns*
Upper limbs muscle strength	0.44 ± 0.08	0.44 ± 0.10	0.45 ± 0.08	0.44 ± 0.05	*ns*
Lower limbs muscle quality	11.64 ± 3.38	11.86 ± 2.24	11.98 ± 1.81	10.89 ± 1.26	*ns*
Upper limbs muscle quality	7.40 ± 2.07	7.94 ± 1.59	8.05 ± 1.14	7.62 ± 0.99	*ns*
SBP (mmHg)	121.83 ± 19.68	119.18 ± 7.98	122.73 ± 14.96	123.67 ± 9.01	*ns*
DBP (mmHg)	80.04 ± 12.38	79.71 ± 6.05	79.08 ± 7.88	81.62 ± 6.67	*ns*
Triglycerides (mg/dL)	92.64 ± 29.47	118.20 ± 55.31	110.90 ± 51.66	101.34 ± 33.26	*ns*
Total chol (mg/dL)	237.40 ± 30.79	239.73 ± 46.41	254.50 ± 27.83	250.31 ± 45.89	*ns*
LDL-chol (mg/dL)	153.31 ± 32.65	154.20 ± 36.89	168.40 ± 24.49	164.90 ± 44.04	*ns*
HDL-chol (mg/dL)	65.74 ± 16.77	61.89 ± 16.38	63.92 ± 14.61	65.15 ± 11.03	*ns*
Glucose (mg/dL)	98.57 ± 13.03[1,2]	103.90 ± 15.72	109.14 ± 18.90[1]	108.35 ± 11.32[2]	*0.034*
AUC (mg min/dL)	15901.53 ± 4295.75	17942.80 ± 4904.76	17468 ± 3923.79	18006.69 ± 3890.92	*ns*
iAUC (mg min/dL)	4227.82 ± 3147.54	5652.67 ± 3787.17	5128.40 ± 3195.59	5172.44 ± 2983.79	*ns*
Insulin	10.02 ± 4.41	9.90 ± 5.21	9.49 ± 5.00	10.54 ± 4.05	*ns*
HOMA-IR index	2.51 ± 1.43	2.48 ± 1.19	2.67 ± 1.86	2.82 ± 1.14	*ns*
TyG index	8.45 ± 0.45	8.64 ± 0.41	8.64 ± 0.47	8.59 ± 0.39	*ns*
PA (METs-h/week)[b]	32.78 ± 17.26	22.65 ± 16.99	22.69 ± 25.08	19.99 ± 13.47	*ns*
MedDiet adherence (p14)	7.95 ± 1.91	8.13 ± 1.77	8.11 ± 2.06	7.81 ± 1.38	*ns*

Note: P: Placebo group, n-3: DHA-rich n-3 PUFA supplemented group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplemented + resistance training group; BMC: Bone mineral content; SBP: systolic blood pressure; DBP: diastolic blood pressure; Total chol: Total cholesterol; LDL-chol: LDL-cholesterol; HDL-chol: HDL-cholesterol; TyG: Triglycerides to Glucose index; PA: Physical Activity; MET: Metabolic equivalent of the task; MedDiet adherence: Mediterranean Diet adherence. Data are mean ± SD.

[a]Data were analyzed by one-way ANOVA or Kruskal-Wallis after testing for normality and homoscedasticity of the samples. Tukey or Dunn's multiple testing correction were used accordingly.

[b]Data obtained from the validated physical activity questionnaire[44].

[1, 2]Means that share a common superscript number were significantly different; *ns*, nonsignificant (*P*>0.05).

Supplementary Table 2. Physical activity data and adherence to the Mediterranean Diet in the four intervention groups.

	P	n-3	P+RT	n-3+RT	Two-way ANOVA[b]		
Number of subjects	20	15	20	16	n-3	RT	n-3xRT
PA (METs-h/week)[c]							
Baseline	32.78 ± 17.26	22.65 ± 16.99	22.69 ± 25.08	19.99 ± 13.47			
Change	3.15 ± 26.25	0.50 ± 14.56	4.90 ± 18.59	3.98 ± 10.88	*ns*	*ns*	*ns*
SPA (%)[d]	62.98 ± 11.16	61.91 ± 11.76	57.96 ± 11.72	58.21 ± 11.11	*ns*	*ns*	*ns*
LPA (%)[d]	29.48 ± 6.97	32.67 ± 8.50	31.50 ± 5.79	33.69 ± 6.96	*ns*	*ns*	*ns*
MPA (%)[d]	7.18 ± 7.04	5.37 ± 6.98	10.32 ± 10.54	8.07 ± 7.70	*ns*	*ns*	*ns*
VPA (%)[d]	0.37 ± 0.73	0.06 ± 0.13	0.23 ± 0.81	0.03 ± 0.08	*ns*	*ns*	*ns*
MedDiet adherence							
Baseline	7.95 ± 1.91	8.13 ± 1.77	8.11 ± 2.06	7.81 ± 1.38			
Change	1.55 ± 2.42[a,**]	0.80 ± 2.11	0.50 ± 2.18	1.06 ± 2.18[a,†]	*ns*	*ns*	*ns*

Note: P: Placebo group, n-3: DHA-rich n-3 PUFA supplemented group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplemented + resistance training group; PA: Physical Activity; MET: Metabolic equivalent of the task; SPA: sedentary physical activity; LPA: light physical activity; MPA: moderate physical activity; VPA: vigorous physical activity; MedDiet adherence: Mediterranean Diet adherence. Data are mean ± SD. [a]Paired Student's *t* test. [b]Differences in changes (16 weeks – baseline) between groups were evaluated by two-way ANOVA. [c]Data obtained from a validated physical activity questionnaire[44]. [d] Data obtained from 1-week recorded accelerometry.

**P<0.01, †P trend (P=0.07) *vs*. baseline; *ns*, nonsignificant (P>0.05).

Supplementary Table 3. Effects of 16 weeks of DHA-rich n-3 PUFA (n-3) supplementation and/or resistance training (RT) on arms anthropometric measurements in overweight/obese postmenopausal women.

	P	n-3	P+RT	n-3+RT	Two-way ANOVA[b]		
N	20	15	20	16	n-3	RT	n-3xRT
Arm circumference (cm)							
Baseline	34.87 ± 1.49	34.02 ± 2.30	34.59 ± 2.31	35.56 ± 2.39			
Change	-1.18 ± 1.32[a,***]	-1.09 ± 0.82[a,***]	-0.98 ± 0.96[a,***]	-1.35 ± 1.16[a,***]	*ns*	*ns*	*ns*
Adjusted change[c]	-1.22(0.21)[a,***]	-1.05(0.14)[a,***]	-1.01(0.20)[a,***]	-1.30(0.20)[a,***]	*ns*	*ns*	*ns*
Tricipital skinfold (mm)							
Baseline	31.85 ± 5.15	31.04 ± 3.91	32.95 ± 3.88	33.28 ± 5.42			
Change	-2.99 ± 4.31[a,**]	-2.94 ± 2.88[a,**]	-4.18 ± 3.69[a,***]	-4.05 ± 3.49[a,**]	*ns*	*ns*	*ns*
Adjusted change[c]	-3.35(1.05)[a,**]	-2.80(0.70)[a,**]	-4.05(0.85)[a,***]	-3.89(0.73)[a,***]	*ns*	*ns*	*ns*

Note: P: Placebo group, n-3: DHA-rich n-3 PUFA supplemented group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplemented + resistance training group.

[a]Paired Student's *t* test. [b]Differences in changes (16 weeks – baseline) between groups were evaluated by two-way ANOVA. [c]Means(SEM) adjusted by changes in body weight.

***P<0.001, **P<0.01 *vs*. baseline; *ns*, nonsignificant (P>0.05)

CHAPTER 5

DHA supplementation and resistance training differentially modulate the inflammatory status in serum and adipose tissue of overweight and obese postmenopausal women

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Abstract

During menopause, women experience a sudden increase in the pace of aging and in adiposity. Among adipose depots, subcutaneous adipose tissue (SAT) is the largest fat depot and contributes to the chronic, low-grade inflammation that occurs in obesity and aging. This randomized, placebo-controlled trial aimed to investigate the effects of 16 weeks of a docosahexaenoic acid (DHA)-rich fish oil concentrate supplementation and/or a resistance training (RT) program on systemic and adipose tissue inflammation in healthy, overweight/obese postmenopausal women. At baseline and end of the trial, blood and periumbilical subcutaneous adipose tissue (SAT) samples were obtained to study markers of systemic and adipose tissue inflammation. DHA supplementation lowered the inflammatory platelet-to-lymphocyte ratio. Moreover, DHA supplementation also reduced the circulating C-reactive protein (CRP) and SAT gene expression of *LEPTIN*, *CHEMERIN*, *IL6* and *CD11c* compared to placebo untrained subjects. On the other hand, RT lowered serum CRP levels and SAT mRNA levels of *LEPTIN*, *CHEMERIN* and *IL6* in subjects supplemented with placebo but not with DHA. However, RT increased SAT gene expression of *ADIPONECTIN*. The combination of the DHA-rich supplement and the RT program did not have synergistic beneficial effects on inflammation-related parameters. These data suggest the recommendation of RT and n-3 PUFA consumption to ameliorate inflammation and improve metabolic health in overweight/obese postmenopausal women.

Keywords: obesity; postmenopause; DHA; resistance training; inflammation; adipose tissue.

1. Introduction

The global burden of obesity and related non-communicable diseases has reached pandemic proportions, with more than 2 billion people expected to be obese by 2030[1]. In parallel, one in six people in the world (16%) will be over the age of 65 by 2050[2]. Within the middle-aged obese population, menopausal women experience a sudden acceleration in the pace of aging[3] and in fat mass redistribution towards abdominal depots[4], thus becoming a population at high risk of developing metabolic diseases.

Aging is characterized by processes of *inflammaging*, with an increase in circulating proinflammatory molecules, and *immunosenescence*, characterized by increased circulating white blood cells that show impaired phagocytic activity[5]. After menopause, lack of estrogens regulatory actions on immune system and a sudden expansion in abdominal white adipose tissue (WAT) depots, favor the advance of inflammation in aging[6,7]. Indeed, abdominal WAT depots are strongly related to proinflammatory phenotypes both in aged and obese subjects[5,8]. In obesity, inflamed WAT shows a spoiled secretion pattern of pro and antiinflammatory cytokines and adipokines, characterized by lower production of the antiinflammatory adiponectin, and increased levels of the proinflammatory leptin, chemerin, and IL-6. This altered secretion pattern occurs in parallel with higher recruitment and infiltration of M1 proinflammatory macrophages [9]. Also, WAT ability to acquire a leaner and more thermogenic phenotype (browning/beiging of WAT) is inversely associated with aging and obesity[10], and mediated by the unresolved inflammation that underlies both processes[11].

Large population studies have found increased white blood cell count and C-reactive protein (CRP) with age and BMI specially in women[12], while increased lymphocyte and neutrophil counts have been associated with increased waist circumference[13]. In this regard, the derived platelet-to-lymphocyte ratio (PLR), neutrophil-to-lymphocyte ratio (NLR) and systemic-immune inflammatory index (SII index) are biomarkers of systemic inflammation and cardiometabolic health[14] which could be of special interest in the postmenopause, since they are increased/affected by obesity[15,1617], and associated with CRP in obese healthy adults[18]. With regard to adipokines, menopause contributes to the negative association described between adiponectin and the development of metabolic syndrome[19,20]. Recently, altered levels of leptin, adiponectin, and chemerin have been described as predictors of length of disease in older subjects with type 2 Diabetes Mellitus, with chemerin predicting adipocyte dysfunction independently from age or BMI[21].

With this regard, establishing effective dietary and lifestyle interventions to prevent or attenuate systemic inflammation and WAT dysfunction in overweight/obese women during menopause could be of special interest. Regular physical exercise is a key factor for a healthy aging because of its beneficial effects on improving cardiovascular risk, insulin sensitivity, and inflammation, among others. Indeed, reduced circulating levels of CRP, leptin or interleukin 6 (IL-6), and higher levels of adiponectin were observed in physically active aging population compared to their sedentary counterparts[22]. These associations were stronger for women, largely mediated by sex differences in fat mass[22]. Likewise, fitness levels were inversely correlated with white blood cell counts[23]. Among physical exercise approaches, resistance training (RT) is especially convenient for the aging population, as it elicits an increase in muscle strength, function and mass that directly correlate with decreased risk of frailty and mortality[24,25]. Despite RT has been shown to beneficially modulate immune function in postmenopausal and aging populations[26–28], effects on biomarkers of systemic inflammation (CRP, IL-6 or tumor necrosis factor-α (TNF-α)) are inconclusive[29], and no effects

have been described for the PLR, NLR and SII indexes. While various studies have analyzed the effects of RT on circulating adipokines[30,31], trials investigating the effects of RT in WAT gene expression in postmenopausal overweight/obese women are scarce or found no effects[32].

Several trials have evidenced beneficial effects of n-3 polyunsaturated fatty acids (n-3 PUFA) on the inflammatory status of the obese and aging population at both systemic and WAT level[33,34,35], through the regulation of the production of adipokines such as adiponectin[36] and leptin[37]. Importantly, circulating adiponectin and WAT mRNA levels correlated to plasma n-3 PUFA in obese subjects[38]. Moreover, improvements in the inflammatory secretion pattern have been described to occur alongside with browning of WAT in animal models of obesity supplemented with n-3 PUFA[39]. However, controversial results have been found between trials. Beneficial effects of n-3 PUFA also include the modulation of platelet activity[40]. No effects have been described for n-3 PUFA supplementation on NLR, PLR or SII index, but plasma levels of n-3 PUFA have been recently described to be an independent factor modulating fluctuations on white blood cells count and PLR[41]. Several studies have suggested differential effects for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on inflammatory markers and metabolic health[42]. Indeed, lower circulating levels of DHA, and not of EPA, have been related to higher risk of developing metabolic syndrome in observational studies[43], being DHA inversely associated with BMI only in women[44]. Moreover, systematic reviews have suggested more beneficial effects for DHA than EPA in markers of metabolic inflammation[40]. Although previous trials in postmenopausal women have combined RT programs and n-3 PUFA, DHA was not the primary component of the supplementation[45].

On the other hand, while the ability of exercise and n-3 PUFA supplementation to induce WAT browning and metabolic improvement in rodents is clear[46,47], the studies in humans are few and with inconclusive results[48].

Thus, the aim of the present study was to investigate the effects of a RT program alone or in combination with a DHA-rich n-3 PUFA supplementation on systemic biomarkers of inflammation as well as on subcutaneous WAT gene expression of inflammatory and beiging markers, in a group of overweight and obese postmenopausal women.

2. Materials and methods

2.1 Study design and participants

In this randomized double-blind placebo-controlled trial (OBELEX trial), participants were allocated into four parallel intervention groups for 16 weeks: 1) Placebo group (P) received placebo capsules containing olive oil, 2) Omega-3 group (n-3) received DHA-rich n-3-PUFA capsules providing 1.650 mg/day of DHA and 150 mg/day of EPA, 3) Placebo + Resistance Training group (P+RT) received placebo capsules and followed a progressive RT program of 2 sessions/week, and 4) Omega-3 + Resistance Training group (n-3+RT) received DHA-rich n-3-PUFA capsules and followed a progressive RT program of 2 sessions/week.

Women aged 55-70 years old with a BMI between 27.5-35 kg/m^2 were recruited. Inclusion criteria included at least 1 year of amenorrhea, a stable weight (± 3 kg) in the 3 months prior the start of the trial, and an overall physical and physiological healthy condition in accordance with the aim of the study. Exclusion criteria included regular prescription medication (hormonal therapy, oral antidiabetic drugs, hypolipidemic drugs, proton pump inhibitors); recently (3 months) prescribed or changed medication; to suffer from any chronic severe metabolic, hepatic, renal, cardiovascular, neuromuscular, arthritic, pulmonary or other debilitating

diseases; or to follow any special diets prior 3 months the start of trial; and a history of eating disorders, surgically treated obesity, or alcohol/drug abuse. Candidates were screened by a physician of the Metabolic Unit (MU) of the University of Navarra and gave their written consent prior to enrolment in the study. Thereafter, volunteers were randomly allocated to one of the four groups using MATLAB® (The Mathworks™, Natick, USA) according to WHO criteria for age corresponding to adult/older adult (55-59/60-70 years old) and for BMI corresponding to overweight type II/obesity type I (27.5-29.9/30-35 kg/m^2)[49,50].

At baseline and at the end of trial, participants attended the MU of the University of Navarra in 8-12 h fasting conditions. Body composition data were obtained by Dual X-Ray Absorptiometry (Lunar iDXA, encore 14.5, Madison, WI, USA). Fasting blood samples for biochemical analyses were collected by a nurse. Subcutaneous adipose tissue (SAT) biopsies were also obtained at the facilities of the Plastic and Reconstructive Surgery Department at the Clinic Universidad de Navarra. When baseline visit was completed, the corresponding capsules were given to each participant, training sessions were scheduled, and follow-up visits were established every 2 weeks.

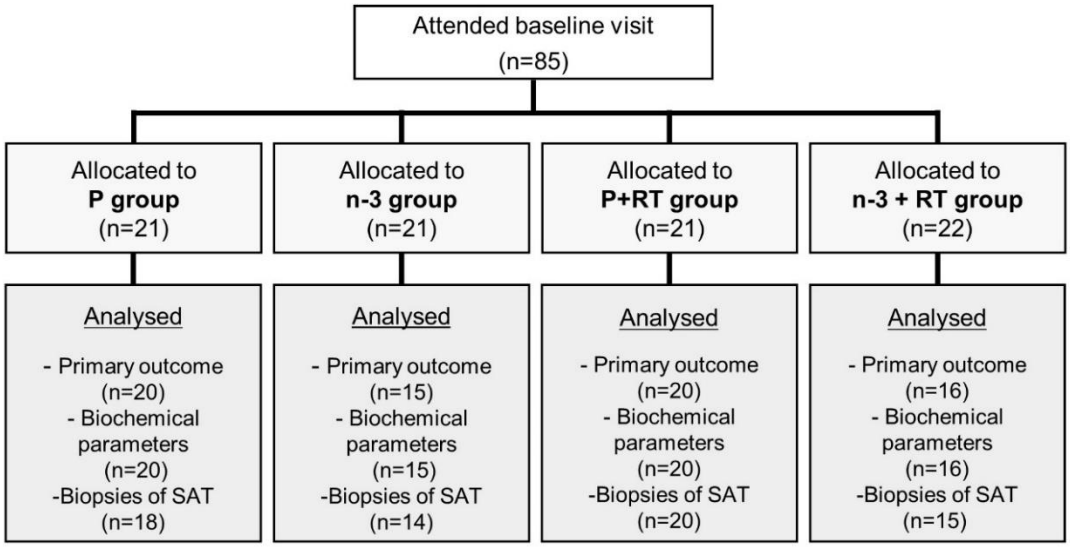

Figure 1. Flowchart of analyses. Out of 85 women who started the intervention, 14 did not complete the trial (16.5% drop out). Dropout rates were 4.8% for P and P+RT groups, 28.6% for n-3 group, and 27.3% for n-3+RT group. A total of 4 subjects rejected to give SAT samples at the end of the study for personal reasons. P: Placebo group, n-3: DHA-rich n-3 PUFA supplement group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplement + resistance training group; SAT: Subcutaneous adipose tissue.

Participants were instructed to not change their habitual physical activity (PA) during the 16 weeks of the trial. PA levels were assessed by filling a validated questionnaire[51] at baseline and end of the trial, and by one-week accelerometry recording (ActiGraph GT3X, Actigraph Corporation, Pensacola, FL, US). Dietary recommendations for a healthy diet based on the guidelines from the Spanish Society for Communitarian Nutrition (SENC, 2016)[52] were given to each participant. Dietary patterns were evaluated by a validated questionnaire of 14 items to assess Adherence to the Mediterranean Diet (p14)[53] at baseline and end of the trial. There were no differences between groups in p14 score changes, PA changes, or PA levels measured by accelerometry (Félix-Soriano *et al*., unpublished data).

2.2 Supplementation

Placebo and DHA-rich fish oil concentrate capsules (DHA^{scc} premium) were provided by Solutex® (Madrid, Spain). DHA premium is a 55% DHA concentrate in ethyl ester form. Participants consumed six capsules per day, two capsules with each meal (breakfast, lunch, and dinner). Placebo capsules contained 0.5 g of olive oil and the DHA dietary supplement capsules contained 0.5 g of a DHA-rich n-3 PUFA fish oil concentrate providing 1650 mg/day of DHA and 150 mg/day of EPA. Olive and DHA-rich oils were provided in hard gelatin transparent liquid fill capsules identical in shape and size (only a small difference in the oils' viscosity/color could be appreciated). To test the DHA-rich supplements oxidative status, peroxide and anisidine values were analyzed during the study. Levels were below maximum allowed. Dose of DHA-rich fish oil concentrate supplement was chosen based in previous studies[54–56] and according to the U.S. Food and Drug Administration (FDA) recommendations (3 g/day EPA and DHA maximum, up to 2 g/day from dietary supplements[57]). Fish consumption was controlled depending on their n-3 PUFA's content, according to EFSA daily recommendations for maintenance of a normal cardiac function (250 mg)[58] using food composition tables (Mataix-Verdú *et al.*[59], Easydiet®, Odimet®). n-3-PUFA enriched food and dietary supplements were not allowed during the study.

At every follow-up visit (2 weeks) and until the end of the study, participants received a total of 120 capsules and returned all blisters whether they were empty or not. Adherence to supplementation was assessed by leftover pill count and was above 95% in every group of study (Félix-Soriano *et al*., unpublished data).

2.3 Resistance training program

The strength training program used in this trial was previously described in detail (Félix-Soriano *et al*., unpublished data). Briefly, after three familiarization sessions, eight exercises for upper and lower main muscular groups were tested following the 1-RM testing procedure[60], and six exercises were included in each training session. Four exercises were maintained along the program and four changed in pairs every 2 weeks). Training progression was established using the pyramidal approach[61], so as 60% of maximum intensity was selected to start the RT program and a maximum of 80% was reached at week 10 and maintained until week 13. Subjects were re-tested at week 8 and at the end of the RT program to re-adjust training loads and to evaluate strength gains *vs*. baseline, respectively. In each session, one researcher was present to direct and assist each subject towards ensuring adequate performance in each exercise following resistance training guidelines for the older population[62]. The RT program effectiveness was assessed by un-trained groups also challenging the 1-RM test at baseline and the end of the trial (leg press and chest press). Mean attendance to training sessions was above 97% in trained groups (Félix-Soriano *et al*., unpublished data).

2.4 Blood cells count and serum inflammatory indexes and markers

Once basal blood samples were extracted, one sample was used for hematologic determinations (platelet, leukocyte, neutrophils, and lymphocytes count) on a Pentra 60 automated hematology analyzer (HORIBA ABX, Madrid, Spain). Afterwards, blood cell counts were used to calculate derived-inflammatory indexes: neutrophil (N$x10^9$/L)-to-lymphocyte (L$x10^9$/L) ratio (NLR), platelet (P$x10^9$/L)-to-lymphocyte ratio (PLR) and systemic Immune-Inflammation index (SII, PxN/L) as previously described[14]. The remaining blood samples were centrifuged (1500 *g*, 15 min, 4 ºC) to obtain serum and plasma aliquots, which were frozen at -80 ºC.

C Reactive Protein (CRP) was determined using a highly sensitive CRP ELISA (#DE740001, Demeditec Diagnostics GmbH, Kiel, Germany). The circulating levels of adipokines related with inflammation were determined in serum aliquots with ELISA kits following the manufacturer's instructions (Adiponectin human ELISA, #DY1065-05, R&D Systems, Inc., Minneapolis, MN, USA; Leptin human ELISA, #RD191001100 and Chemerin human ELISA, #RD191136200R, BioVendor R&D, Karasek, Czech Republic) on the autoanalyzer Triturus ELISA Instrument (Grifols Diagnostic Solutions, Barcelona, Spain). The Adiponectin/Leptin ratio was calculated as an index of adipose tissue-systemic inflammatory status[63].

2.5 Subcutaneous adipose tissue gene expression

At baseline and at the end of the intervention, biopsies of subcutaneous abdominal periumbilical adipose tissue (1–2 g) were obtained in the University of Navarra Clinic (Department of Plastic and Reconstructive Surgery) by liposuction under local anesthesia after an overnight fast. Once obtained, SAT biopsies were rapidly cleaned with PBS, aliquoted and frozen in liquid nitrogen, and later stored at -80 ºC until analyses. For gene expression assays, total RNA was extracted with QIAzol lysis reagent® protocol (Qiagen; Venlo, Limburg, The Netherlands) and eluted in RNase-free DEPC-treated water (Thermo Fisher Scientific, Waltham, MA, USA) using the RNeasy MinElute Cleanup Kit (Qiagen; Venlo, Limburg, The Netherlands). RNA quality and quantity were measured (Nanodrop Spectrophotometer ND1000; Thermo Fisher Scientific) and then incubated (2 μg) for 30 min at 37°C with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed to cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) following the manufacturers' instructions.

Real-time PCR was performed using the Touch Real-Time PCR System (C1000 + CFX384, BIO-RAD, Hercules, CA, USA) under Taqman Universal Master Mix (Applied Biosystems, CA, USA) methodology for predesigned Taqman Assays-on-Demand *LEPTIN* (Hs00174877_m1), *CHEMERIN (*Hs00161209_g1) and *IL6* (Hs00985639_m1). The expression of all other genes was analyzed under Power SYBR® Green PCR (Bio-Rad, München, Germany) methodology. Primers were designed with Primer-Blast software (National Center for Biotechnology Information, Bethesda, MD, USA; https://www.ncbi.nlm.nih.gov/tools/primer-blast): *ADIPONECTIN* (forward: 5'-AACATGCCCATTCGCTTTAC-3'; reverse: 5'- ATTACGCTCTCCTTCCCCAT-3'), *CCL2* (forward: 5'-GATCTCAGTGCAGAGGCTCG-3'; reverse: 5'-TGCTTGTCCAGGTGGTCCAT-3'), *CD11C* (forward: 5'-AGGTCCAAGGTGAATGCCC-3'; reverse: 5'-GCGGTGATTGTTCCCAGGA-3'), *FNDC5* (forward: 5'-ACGTCAGTACAAGGAGATGTTGGA-3'; reverse: 5'-TTCAGCCTCAGCCACTCC-3'), *TMEM26* (forward: 5'-ATGGAGGGACTGGTCTTCCTT-3'; reverse: 5'-CTTCACCTCGGTCACTCGC-3'); *UCP1* (forward: 5'-AGGTCCAAGGTGAATGCCC-3'; reverse: 5'-GCGGTGATTGTTCCCAGGA-3'); and *TBP* (forward: 5'-CCACTCACAGACTCTCACAAC-3'; reverse: 5'-ACTCGTCCACATACCCTGTCTGGT-3'). Relative expression was determined by the $2^{-\Delta\Delta Ct}$ method[64] using *TBP* as housekeeping gene.

2.6 Statistical analysis

Statistical analyses were performed using Stata Statistical Software, version 14. Differences were significant at 2-sided $P<0.05$ value. At baseline, differences between groups were evaluated by one-way ANOVA test or Kruskal-Wallis test after normal distribution assessment using Shapiro-Wilk test and Breusch-Pagan/Cook-Weisberg heteroscedasticity test. When significance was found, Tukey or Dunn's pot-hoc test were run to identify significantly different groups. Within-group effects were studied by paired Student's *t* test

or Wilcoxon signed-rank test according to the sample distribution. Between-groups effects were studied by two-way ANOVA of changes (endpoint – baseline values) to evaluate possible factor effects or interaction effects. When changes were significantly different at the interaction level, contrasts were performed to discriminate factor effects from individual group effects.

3. Results

3.1 Effects on body weight and body composition

After the intervention, all groups showed moderate but significant reductions in body weight and fat mass. However, no significant differences in changes between groups were observed when comparing changes by two-way ANOVA (**Figure 2**).

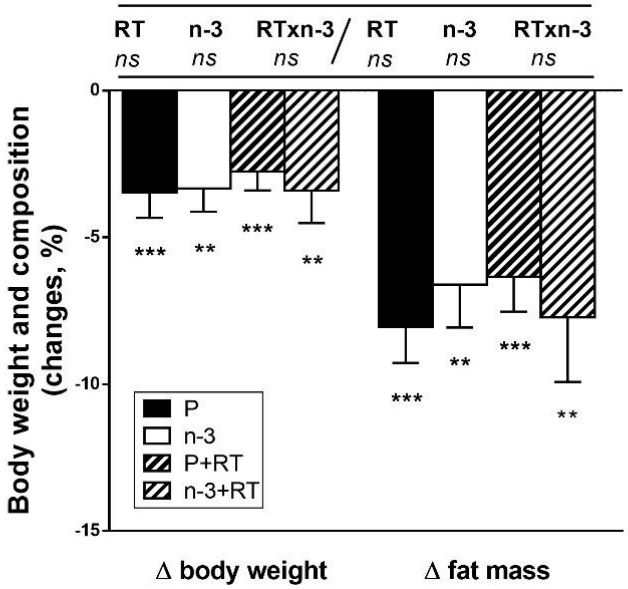

Figure 2. Changes (%) in body weight and fat mass studied by Dual X-ray Absorptiometry (DXA) in the four intervention groups. Differences in changes within groups were analyzed by paired Student's *t* test or Wilcoxon's signed-ranked after testing for normality; results are represented in the symbols below the SEM bar (***P*<0.01, ****P*<0.001). Differences in changes between groups were analyzed by two-way ANOVA and are represented in the rows above the bars (*ns*, non-significant, *P*>0.05). Data are mean ± SEM. P: Placebo group; n-3: DHA-rich n-3 PUFA supplementation group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplementation + resistance training group.

3.2 Effects on blood cells count and systemic inflammatory markers

Table 1 shows platelet, white blood cells counts and the derived inflammatory PLR, NLR and SII index values. Participants showed healthy values at baseline and at the end of the trial[14,65]. Platelet, leucocyte, and neutrophil counts were maintained in all groups. However, lymphocyte count decreased moderately but significantly in the P+RT group, and showed a trend (*P*=0.071) to be lower in the P group at the end of intervention, with no changes in the DHA-supplemented groups. Consequently, when differences in the

Table 1. Effects of 16 weeks of intervention with a DHA-rich n-3 PUFA supplement (n-3) and/or a RT program on blood cells count and inflammatory indexes in overweight /obese postmenopausal women.

	P	n-3	P+RT	n-3+RT	Two-way ANOVA[c]		
N	20	15	20	16	n-3	RT	n-3xRT
Platelet count (10^9/L)							
Baseline	250.63 ± 50.90	249.67 ± 67.37	240.95 ± 64.12	249.13 ± 43.07			
Change	-9.90 ± 27.24	-1.27 ± 29.08	-2.30 ± 29.07	-1.75 ± 23.51	*ns*	*ns*	*ns*
Adjusted change[d]	-9.83(6.34)	-1.32(7.50)	-2.08(6.42)	-2.06(5.61)	*ns*	*ns*	*ns*
Leucocyte count (10^9/L)							
Baseline	5.41± 0.93	5.56 ± 1.25	5.81 ± 1.74	5.31 ± 1.38			
Change	0.13 ± 1.00	0.15 ± 1.16	-0.21 ± 1.56	-0.16 ± 0.65	*ns*	*ns*	*ns*
Adjusted change[d]	0.07(0.25)	0.14(0.30)	-0.18(0.33)	-0.13(0.17)	*ns*	*ns*	*ns*
Lymphocyte count (10^9/L)							
Baseline	1.93 ± 0.34	1.92 ± 0.54	1.88 ± 0.59	1.64 ± 0.35			
Change	-0.14 ± 0.48	0.06 ± 0.31	-0.21 ± 0.99[b,**]	0.01 ± 0.27	0.009	*ns*	*ns*
Adjusted change[d]	-0.15(0.08)[a,†]	0.06(0.08)	-0.20(0.07)[b,*]	0.02(0.07)	0.008	*ns*	*ns*
Neutrophil count (10^9/L)							
Baseline	2.43 ± 0.65	2.48 ± 0.68	2.82 ± 1.02	2.15 ± 0.64			
Change	0.20 ± 0.80	-0.05 ± 0.76	-0.21 ± 0.99	0.03 ± 0.56	*ns*	*ns*	*ns*
Adjusted change[d]	0.18(0.21)	-0.07(0.22)	-0.18(0.26)	0.04(0.15)	*ns*	*ns*	*ns*
PLR							
Baseline	134.12 ± 36.80	134.06 ± 31.71	139.08 ± 50.71	159.72 ± 50.06			
Change	7.81 ± 27.59	-2.11 ± 17.88	11.58 ± 20.34[b,*]	-4.68 ± 29.69	0.029	*ns*	*ns*
Adjusted change[d]	8.43(6.21)	-1.98(4.63)	11.08(4.53)[b,*]	-4.91(7.53)	0.028	*ns*	*ns*
NLR							
Baseline	1.36 ± 0.48	1.39 ± 0.36	1.57 ± 0.56	1.39 ± 0.42			
Change	0.28 ± 0.66[a,†]	-0.03 ± 0.28	0.17 ± 0.78	0.03 ± 0.40	*ns*	*ns*	*ns*
Adjusted change[d]	0.35(0.16)[a,*]	-0.02(0.07)	0.12(0.21)	0.01(0.11)	*ns*	*ns*	*ns*
SII index							
Baseline	338.06 ± 136.08	330.22 ± 139.79	378.63 ± 188.92	349.21 ± 141.28			
Change	51.15 ± 132.54	-12.86 ± 92.63	14.43 ± 166.13	2.14 ± 133.10	*ns*	*ns*	*ns*
Adjusted change[d]	60.43(33.50)	-13.93(26.98)	7.85(44.35)	-0.49(33.84)	*ns*	*ns*	*ns*
CRP (µg/mL)							
Baseline	2.81 ± 1.70	5.52 ± 4.03	3.74 ± 2.98	3.42 ± 3.40			
Change	0.12 ± 1.90	-1.63 ± 2.19[a,**,1]	-0.55 ± 1.24[a,*]	-0.47 ± 0.96[1]	-	-	0.022
Adjusted change[d]	-0.14 (0.32)[1,2]	-1.44(0.57)[a,*,2]	-0.52(0.26)[1]	-0.27(0.23)	-	-	0.033

Note: P: Placebo group; n-3: DHA-rich n-3 PUFA supplementation group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplementation + resistance training group; NLR: Neutrophil-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; SII: Systemic immune-inflammation index; CRP: C-reactive protein. Data are mean ± SD.

[a]Wilcoxon's signed-rank test; [b]Paired Student's *t* test. [c]Differences in changes (16 weeks – baseline) between groups were evaluated by two-way ANOVA. When a significant interaction between factors was found (P<0.05 at n-3xRT), contrasts were performed to discriminate factor differences from group differences, expressed as means that share a common superscript number ([1]P<0.01, [2]P<0.001). [d]Means(SEM) adjusted by changes in body weight.

*P<0.05, **P<0.01, †P trend (P=0.064-0.079) *vs*. baseline; *ns*, nonsignificant (P>0.05).

changes between groups were studied by two-way ANOVA, a significant effect for the DHA-rich n-3 PUFA supplement on maintaining lymphocyte values compared to the decrease observed in the P-supplemented groups was found (**Table 1**).

Blood cell count-based inflammatory indexes NLR, PLR and SII are markers of inflammatory status in chronic inflammatory diseases including obesity[14]. Interestingly, values showed a pattern to remain similar (NLR) or decrease (PLR, SII index) in the n-3 supplemented groups compared to the trend to increase observed in P-supplemented groups (**Table 1**). However, these increases at the end of the intervention were only significant for NLR in the P group (after adjusting by changes in body weight) and for PLR in the P+RT group. Nonetheless, when differences in changes between groups were studied, only a significant differential effect of the DHA-rich supplementation on the PLR values was observed as compared to the P groups. The SII index, however, did not reveal significant changes neither for changes within group nor between groups (**Table 1**).

Finally, CRP circulating levels, the main marker of systemic inflammation, were also studied (**Table 1**). Levels were non significantly different at baseline despite being variable between groups. CRP levels were significantly reduced within the n-3 and P+RT groups at the end of the intervention. The analyses of the differences in changes between groups by two-way ANOVA revealed a significant interaction between DHA supplementation and RT program (**Table 1**). Contrasts analyses showed a lowering effect of CRP for RT compared to the untrained group when placebo supplementation was given. After values were adjusted by participants weight loss, lowering effects for the DHA-rich n-3 PUFA supplementation when groups were not trained also appeared. Hence, both interventions exerted lowering effects on circulating CRP; however, they were not observed when combined.

3.3 Effects on inflammation-related adipokines in serum

Adipose-related systemic inflammation was characterized by analyzing the circulating levels of adiponectin, leptin and chemerin (**Table 2**). At the end of the intervention, serum levels of the antiinflammatory adipokine adiponectin were significantly increased in the P group, as compared with baseline, while no significant changes were observed within the other intervention groups. On the other hand, the proinflammatory and adiposity marker leptin was significantly reduced in all intervention groups even after adjusting for fat mass loss, except for the n-3 group (P=0.063), which had the lowest values at baseline for this adipokine. Chemerin, another proinflammatory adipokine, was reduced in all groups at the end of the intervention. No differential effects were observed for the DHA-rich supplementation or the RT program on adiponectin, leptin and chemerin circulating levels when changes between groups were studied by two-way ANOVA (**Table 2**).

The adiponectin/leptin ratio was also analyzed, as it has been established to be a marker of metabolic inflammation[66]. Moderates, but significant increases were observed in all intervention groups, confirming an improvement in the systemic inflammatory state at the end of the intervention. However, the two-way ANOVA analysis of changes between groups revealed that the effects of the DHA-rich supplementation on increasing the adiponectin/leptin ratio were lower as compared to placebo supplemented groups. However, this lower effect disappeared after adjusting for fat mass losses, highlighting that the improvement on adiponectin/leptin ratio observed in the P groups seems to be mediated by fat mass loss and not by the supplementation itself (**Table 2**).

Table 2. Effects of 16 weeks of intervention with a DHA-rich n-3 PUFA supplement (n-3) and RT program on circulating levels of inflammation-related adipokines in overweight/obese postmenopausal women.

	P	n-3	P+RT	n-3+RT	Two-way ANOVA[c]		
N	20	15	20	16	n-3	RT	n-3xRT
Adiponectin (µg/mL)							
Baseline	43.38 ± 25.30	36.52 ± 19.97	48.03 ± 24.52	45.44 ± 23.68			
Change	6.74 ± 12.31[a,*]	2.36 ± 10.58	2.79 ± 15.18	-2.49 ± 13.26	*ns*	*ns*	*ns*
Adjusted change[d]	6.66(2.99)[b,*]	2.51(2.73)	3.26(3.58)	-2.31(3.36)	*ns*	*ns*	*ns*
Leptin (ng/mL)							
Baseline	30.51 ± 9.50	25.46 ± 10.49	32.32 ± 8.82	32.04 ± 9.82			
Change	-7.34 ± 8.04[b,***]	-2.99 ± 5.73[b,†]	-9.02 ± 7.98[b,***]	-7.56 ± 8.20[b,**]	*ns*	*ns*	*ns*
Adjusted change[d]	-6.62 (1.63)[b,***]	-3.51(1.75)[a,†]	-9.34(1.74)[b,***]	-7.49(1.76)[b,***]	*ns*	*ns*	*ns*
Chemerin (ng/mL)							
Baseline	273.20 ± 82.31	280.21 ± 87.69	298.68 ± 62.87	271.63 ± 95.65			
Change	-24.47 ± 66.90	-43.87 ± 69.30[b,*]	-55.95 ± 69.96[a,***]	-53.16 ± 51.40[b,***]	*ns*	*ns*	*ns*
Adjusted change[d]	-28.07(16.32)[a,*]	-41.46(17.79)[b,*]	-53.28(16.83)[a,**]	-51.88(12.27)[a,***]	*ns*	*ns*	*ns*
Adiponectin/Leptin							
Baseline	1.60 ± 1.08	1.63 ± 1.06	1.57 ± 0.91	1.52 ± 1.00			
Change	1.26 ± 1.94[a,***]	0.40 ± 0.80[a,*]	0.91 ± 0.91[a,***]	0.45 ± 1.18[a,*]	0.042	*ns*	*ns*
Adjusted change[d]	0.96(0.26)[a,***]	0.56(0.23)[b,*]	1.05(0.21)[a,***]	0.54(0.21)[a,**]	*ns*	*ns*	*ns*

Note: P: Placebo group; n-3: DHA-rich n-3 PUFA supplementation group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplementation + resistance training group. Data are mean ± SD.

[a]Wilcoxon's signed-rank test; [b]Paired Student's *t* test. [c]Differences in changes (16 weeks – baseline) between groups were evaluated by two-way ANOVA. [d]Means(SEM) adjusted by changes in body fat mass.

**P*<0.05, ***P*<0.01, ****P*<0.001, [†]*P* trend (*P*=0.063-0.071) *vs.* baseline; *ns*, nonsignificant (*P*>0.05).

3.4 Effects on gene expression of adipokines and browning markers in adipose tissue

To further characterize the contribution of adipose tissue to the changes in inflammatory status due to the DHA-rich n-3 PUFA supplementation and the RT program, the gene expression of adipokines were analyzed in SAT biopsies (**Figure 3**). mRNA levels of *ADIPONECTIN* were significantly increased in both trained groups at the end of the trial (**Figure 3A** top panel). Accordingly, the two-way ANOVA revealed significant differences in *ADIPONECTIN* changes between the RT program and the untrained groups (**Figure 3A**, bottom panel).

On the other hand, mRNA levels of *LEPTIN* and *CHEMERIN* were significantly decreased in all intervention groups except for the P group, which showed significant increases for both genes (**Figures 3B** and **3C**, top panels). The principal effects analyses by two-way ANOVA revealed a significant interaction for the DHA and RT interventions when analyzing changes between groups (**Figures 3B** and **3C**, bottom panels). Thus, a beneficial effect for the DHA-rich supplementation on reducing mRNA levels of both *LEPTIN* and *CHEMERIN* was found when compared to placebo supplementation in untrained groups, and similar effects were observed for the RT program compared to the untrained group in P-supplemented groups, with no synergic effects for the n-3+RT group.

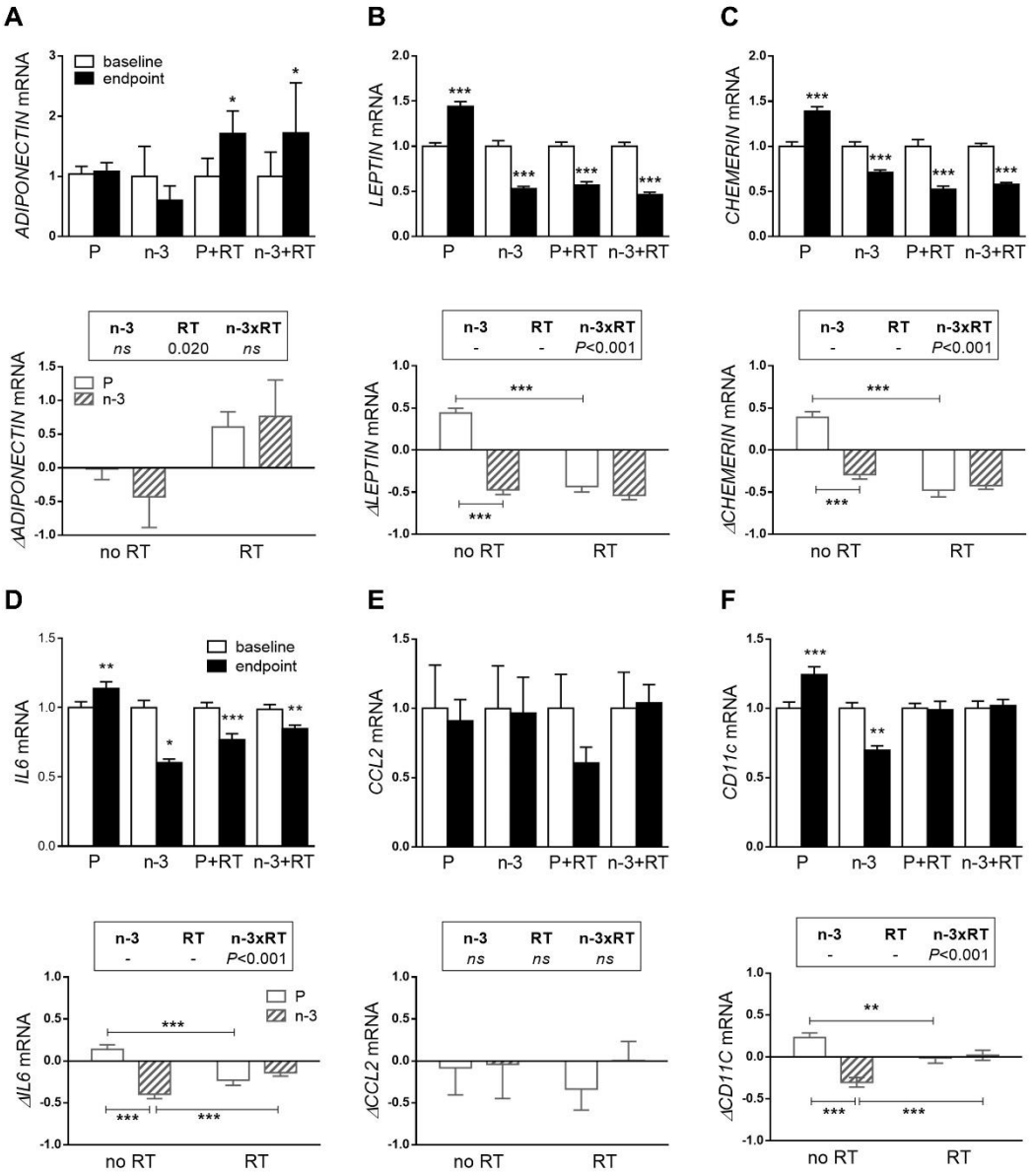

Figure 3. Gene expression levels of *ADIPONECTIN* (**A**), *LEPTIN* (**B**), *CHEMERIN* (**C**), *IL6* (**D**), *CCL2* (**E**) and *CD11c* (**F**) in subcutaneous abdominal adipose tissue from postmenopausal women after 16 weeks of DHA-rich n-3 PUFA supplementation (n-3) and/or a resistance training (RT) program. Top panels: Baseline *vs*. endpoint values were analyzed by paired Student's t-test or Wilcoxon's signed-ranked test after testing for normality (**P*<0.05, ***P*<0.01, ****P*<0.001 from baseline). Bottom panels: Differences in changes (endpoint – baseline) between groups were analyzed by two-way ANOVA (*ns*, nonsignificant, *P*>0.05). When a significant interaction between factors was found (*P*<0.05 at n-3xRT), contrasts were performed to discriminate factor differences from group differences (***P*<0.01, ****P*<0.001). Data are mean ± SEM. P: Placebo group; n-3: DHA-rich n-3 PUFA supplementation group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplementation + resistance training group.

The mRNA expression levels of the proinflammatory cytokine *IL6* were also reduced at the end of the intervention in the n-3 untrained group and in both placebo and n-3-supplemented RT groups (**Figure 3D**,

top panel). The analysis of differences between groups also showed an interaction between both interventions. The RT program reduced *IL6* in the P-supplemented group compared to the untrained P-supplemented group. However, the RT program had a negative effect on the reduction induced by n-3 compared to placebo supplementation in untrained groups. Thus, the reduction in IL6 was significantly lower in the n-3+RT group than in the n-3 alone group (**Figure 3D**, bottom panel).

Finally, we also analyzed the expression levels of *CCL2*, which encodes for MCP1, and *CD11c,* a marker of M1 proinflammatory macrophages (**Figure 3E-F**). While *CCL2* mRNA levels did not reveal any significant effects for any of the treatments neither within nor between groups, *CD11c* expression levels were moderately upregulated in the P group and significantly downregulated in the n-3 untrained group at the end of the trial, without significant changes in the other intervention groups (**Figure 3F**, top panel). However, analyses of differences between groups revealed a negative interaction between treatments, by which the lowering effect of the DHA-rich supplementation on *CD11c* was partly prevented in the n-3+RT group (**Figure 3F**, bottom panel).

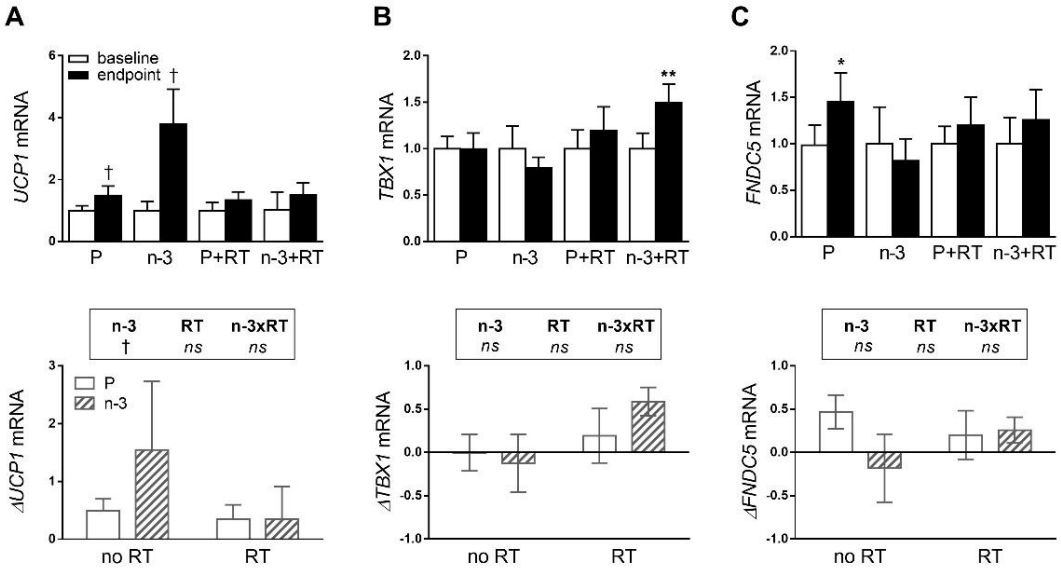

Figure 4. Gene expression levels of *UCP1* (**A**), *TBX1* (**B**) and *FNDC5* (**C**) in SAT from postmenopausal women before and after 16 weeks of DHA-rich n-3 PUFA supplementation (n-3) and/or a resistance training (RT) program. Baseline and endpoint values were analyzed by paired Student's *t* test for normally distributed samples or Wilcoxon's signed-ranked test for non-normally distributed samples. **P*<0.05, ***P*<0.01, †*P* trend (*P*=0.058-0.091). Differences in changes (endpoint – baseline) between groups were analyzed by two-way ANOVA (*ns*, nonsignificant, *P*>0.05). Data are mean ± SEM. P: Placebo group; n-3: DHA-rich n-3 PUFA supplementation group; P+RT: Placebo + resistance training group; n-3+RT: DHA-rich n-3 PUFA supplementation + resistance training group.

Finally, gene expression levels of specific markers of browning of WAT (*UCP1*, *TBX1* and *FNDC*5) were analyzed (**Figure 4**). Thus, *UCP1* showed a clear trend to increase in the n-3 untrained group, but this increase did not occur in trained groups. However, this effect did not reach statistical significance when changes between groups were compared (**Figure 4A**). By contrast, *TBX1* mRNA levels were upregulated

only in the RT+n-3 group, suggesting a synergic effect of both treatments on this specific marker of beige adipocytes (**Figure 4B**, top panel). Surprisingly, *FNDC5,* which encodes for the adipomyokine with browning properties irisin, was significantly increased in the P group, while non-significant increases were neither observed within the n-3 group not for both trained groups (**Figure 4C**, top panel). However, there were no significant differences in changes between groups for any of the three genes evaluated (**Figure 4A-C**, bottom panel).

4. Discussion

This study describes the effects of a 16-week RT program and a DHA-rich dietary supplement, alone or in combination, on systemic and adipose tissue inflammatory biomarkers. Our results revealed no synergistic effects on the pro- and antiinflammatory parameters studied, but beneficial effects were observed for the RT program and the DHA-rich n-3 PUFA supplementation separately.

With this regard, a decrease in the main marker of inflammation circulating CRP was observed with both treatments: due to the RT program in placebo-supplemented groups, and due to the DHA-rich supplementation in untrained groups. These results are in accordance to those described by the meta-analysis performed by Sardeli *et al.*[29], concluding that RT exerted lowering effects on circulating CRP in older adults. Similarly, lowering effects have been described also for DHA and EPA supplementation in the meta-analysis of Guo *et al.*[67]. However, when RT was combined with DHA-supplementation, the reducing effects on CRP levels were not potentiated. Few previous studies have analyzed the combined effects of both treatments in the inflammatory status of overweight/obese postmenopausal women. Among those who have, Strandberg *et al*.[68] showed that a diet rich in n-3 PUFA (n-6/n-3 ratio < 2) in combination with RT training for 18 weeks did not modify CRP levels. Moreover, Da Boit *et al*.[45] observed no effects on other inflammatory markers (IL-6 and TNF-α) in older women following a RT program and supplemented with n-3 PUFA (2.1 g/day EPA and 0.6 g/day DHA) for 18 weeks as compared to placebo (safflower oil) supplemented group, therefore revealing no synergistic effects for both treatments. No effects on other inflammatory markers (IL-6 and TNF-α) have been observed in older (>65) overweight and obese men after 12 weeks of n-3 PUFA supplementation (1.98 g/day EPA and DHA 0.99 g/day) and RT[70]. Nevertheless, the lack of beneficial synergistic effects of n-3 PUFA supplementation in young subjects is not as consistent. Indeed, young men following DHA supplementation and RT did not have effects on CRP or other inflammatory markers like IL-6[69]. However, EPA+DHA supplementation has been shown to decrease resting and acute aerobic exercise-induced inflammatory biomarkers (CRP)[71]. Thus, further investigations are warranted to unravel if age, sex, the type of training or its acute effects mediate the interaction between n-3 PUFA supplementation and exercise training.

The PLR, NLR and SII are novel inflammatory markers that could be useful in many diseases for predicting inflammation[14]. Our results revealed no principal effects for the RT program on changing platelets and white blood cell counts (leukocyte, lymphocytes, neutrophils) or their derived inflammatory indexes PLR, NLR and SII index. Although some studies have observed acute post-RT increases in total leukocyte and lymphocyte counts[27,72], those who found improved parameters of immune function in postmenopausal women[28,73] did not observe changes in white blood cells count. No changes in platelets have also been described in older obese men and women after a 16-week RT program[74]. On the other hand, beneficial immunomodulatory effects of n-3 PUFA on macrophages, neutrophils, T and B cells have been extensively reviewed[75]. Platelet

function has also been demonstrated to improve after only 400 mg/day DHA in type 2 diabetic postmenopausal women[76]. In healthy premenopausal women, DHA but not EPA-rich capsules improved platelet function[77]. Like our data, these effects occurred without affecting blood cell count. Nevertheless, our results revealed significant reductions on the PLR due to the DHA-rich dietary supplement. This finding adds beneficial effects for DHA, this time on a marker of platelet-immune/inflammation function. Interestingly, cardiovascular disease patients exhibited lower levels of PLR when the consumption of n-3 PUFA was high (>4.18 g/d)[78]. Thus, PLR index could be of special interest as a reliable marker of DHA antiinflammatory effects in healthy overweight/obese postmenopausal women who, importantly, exhibit a higher thrombosis risk than postmenopausal women with a normal weight BMI[79].

Adipose tissue derived adipokines contribute to the systemic inflammation observed in overweight/obese subjects. Among them, antiinflammatory adiponectin is decreased, while leptin and chemerin are increased in obesity, being these changes correlated with the main inflammatory marker CRP[80]. Our data revealed significant reductions in circulating levels of leptin and chemerin in the four groups at the end of the intervention, suggesting an improvement in adipose-related inflammation exerted by both DHA and olive oil supplementation as well as by the RT program. Adiponectin was maintained across the four experimental groups except for the placebo untrained group, which showed a significant increase. Importantly, the adiponectin/leptin ratio, which has been negatively correlated with markers of low-grade chronic inflammation[63], was increased in all experimental groups at the end of the intervention, confirming the improvement in adipose-systemic inflammatory status.

Few studies have investigated the effects of RT on circulating adipokines in postmenopausal women. Among those who have, the disparity of results and training protocols used, along with the unreported changes in body composition and the lack of differences between groups make it difficult to infer conclusions[30,81–83]. Thus, while Ward *et al.*[30] observed reductions in adiponectin and no changes in leptin after 15-weeks of RT with increasing resistance, Saeidi *et al.*[83] revealed increases in circulating adiponectin and decreases in leptin after only 8 weeks of RT at 55% of maximum strength (1-RM), both conducted in overweight and obese postmenopausal women. With regard to chemerin, several trials have demonstrated that aerobic exercise is effective in reducing chemerin circulating levels in obese, middle aged men[84], older adults[85], and young women[86]. However, few trials have been performed with RT. In agreement with our data, other studies have shown significant reductions in chemerin after an RT program (8-weeks, 3 days a week, 55-85% RM in obese, young men)[87] or after 6 months of combined aerobic and RT (2-3 sessions/week, increasing volume) in obese, middle aged subjects[88]. With respect to trials analyzing the effects of n-3 PUFA supplementation on circulating adipokines, meta-analyses have concluded that they exert moderate effects in increasing adiponectin[36] and decreasing leptin[37] in the general population. However, no effects on leptin or adiponectin were found for diabetic postmenopausal women after 2 months of fish oil supplementation (1.8 g n-3 PUFA: 1.08 g EPA and 0.72 g DHA)[89], or for overweight older adults with a high n-3 PUFA diet over 8 weeks[90], while increases in adiponectin were observed when studies included overweight and obese pre and postmenopausal women[91,92]. Not only age, but also the supplement formulation (EPA *vs.* DHA or EPA/DHA ratio) might be another important factor when considering the different outcomes obtained in circulating adipokines. Indeed, *in vitro* studies in cultured adipocytes have shown that DHA had antiinflammatory effects on adipokine's secretion, while results for EPA were controversial[93]. Regarding clinical trials analyzing the effects of n-3 PUFA on circulating chemerin, Huerta *et al.*[94] found a moderate but

non-significant reduction on circulating chemerin in overweight/obese premenopausal women after 10 weeks of hypocaloric diet supplemented with an EPA-rich oil (1.3 g/day).

The lack of principal effects for the RT program or the DHA-rich supplement might be also due to the finding that placebo supplementation (olive oil) also had beneficial actions on adiponectin, leptin and chemerin circulating levels in the untrained group. By contrast, the gene expression analyses showed that this group exhibited significant increases in *LEPTIN, CHEMERIN* and *IL6* mRNA in SAT. This contradictory finding between SAT gene expression and serum circulating levels suggests that other fat depots or tissues contributed to the systemic changes observed on these adipokines. It is true that monounsaturated fatty acids (MUFA, mainly oleic acid) and olive oil consumption have been associated with improvements of cardiovascular risk markers and reductions in circulating leptin[95]. Nevertheless, the effects of olive oil supplementation on leptin release are unclear; a recent study has found an increase in leptin circulating levels after olive oil supplementation in lean subjects but not in overweight/obese subjects[96]. However, habitual dietary intake of MUFA (mainly oleic acid) and n-3 PUFA has been negatively associated with *LEPTIN* gene expression in subcutaneous and visceral fat[97]. The increase in *CHEMERIN* mRNA levels that occurred in the placebo group agrees with the finding that oleic acid upregulates chemerin secretion in 3T3-L1 adipocytes[98]. Similarly, increased *IL6* mRNA levels has been observed also in cultured human visceral adipocytes from morbidly obese subjects treated with oleic acid[99].

Studies analyzing the effects of exercise on adipose tissue gene expression and protein profile are scarce. With this regard, our trial revealed that *ADIPONECTIN* gene expression in SAT biopsies was increased in both trained groups, reaching statistical significance for the RT program as compared to untrained groups. The RT program also caused a significant reduction in proinflammatory genes (*LEPTIN, CHEMERIN*, *IL6*), suggesting that the beneficial effects of exercise by ameliorating the inflammatory environment in SAT may occur even in absence of significant changes in body fat mass as compared to untrained groups. However, a trial investigated the possible impact of 12-week RT in SAT biopsies from overweight/obese postmenopausal women, finding no effects on *LEPTIN*, *ADIPONECTIN RECEPTOR*, nor in other inflammation related genes (*CCL2*, *TLR4*)[31]. Similarly, other trials combining 12 weeks of resistance and aerobic exercise, or dynamic resistance alone, did not find effects after training in SAT gene expression (*ADIPONECTIN*, *LEPTIN*, *IL6* and other tested genes) in middle-aged or young obese men[100,101], suggesting that prolonged and/or more intense training programs may be required to improve human adipose tissue function. In this way, the recent study of Čížková *et al.*[102] showed that 4 months of endurance training alleviated inflammation in SAT of older women, by decreasing gene expression of inflammatory markers and switching the infiltrated immune cells to a more antiinflammatory phenotype.

Interestingly, our study also found that proinflammatory genes *LEPTIN*, *CHEMERIN, IL6* and *CD11c* were decreased by the DHA-rich n-3 PUFA supplementation, without changes in *ADIPONECTIN* and *CCL2*. Despite Itariu *et al.*[103] found decreased *CCL2* and expression of CD40, an M1 macrophage marker, and trend to increased *ADIPONECTIN* in SAT of obese adults supplemented with n-3-PUFA as compared to those supplemented with placebo, it is worth of mention that doses were higher than ours (3.36 g EPA+DHA), and that placebo was butter fat. However, Hames et *al.*[104] found no changes in pro or antiinflammatory macrophages not in crown like structures in WAT from adult obese insulin-resistant men and women after 6 months of 3.9 g EPA+DHA supplementation. Other trial in postmenopausal women with type 2 diabetes showed that fish oil supplementation (1.08 g EPA, 0.72 g DHA) for 2 months did not modify *ADIPONECTIN, LEPTIN* or *CCL2* expression but downregulated the expression of other genes related with inflammation

(*MMP9* and *CD11b*) in SAT[89]. Nevertheless, previous *in vitro* studies have revealed the ability of DHA on reducing *CCL2, CHEMERIN or IL6,* in human SGBS[105] and primary adipocytes from overweight, obese and normal weight subjects[106–108] in the basal state and after inflammatory stimuli. In this way, adipose tissue RNASeq analysis has shown that a gene-nutrient interaction exists between inflammatory responses and dietary n-3 PUFA, suggesting that EPA/DHA may enhance the immune response to an inflammatory challenge[109].

Concerning WAT browning, our data revealed a trend for the DHA-rich supplementation to upregulate the thermogenic gene *UCP1*, however no significant changes on *TBX1*, a marker of beige adipocytes, or on *FNDC5* were observed in the DHA supplemented untrained group. Similarly, a recent study has described that dietary fish oil supplementation (1.32 g DHA and 1.86 g EPA) for 4 weeks has no effect on the recruitment of beige adipocytes in humans under thermoneutral conditions[110]. The RT intervention did not have any relevant effect *on UCP1, FNDC5*, or *TBX1*. This contrasts with the observations of Otero-Diaz *et al.*[48], who found that 12-week bicycle-training program (3 times per week, intensity 70–80% HRmax) promoted *UCP1* and *TBX1* expression in SAT of younger (20-40 years old) subjects. Our study also found that the combination of DHA+RT upregulated the expression of *TBX1* at the end of the intervention, suggesting that the combination of both interventions could be helpful to promote SAT beiging in humans, although no significant effects were observed on *UCP1* when combining both treatments. Interestingly, an upregulation of *FNDC5* was only observed in the placebo (olive oil) supplemented group, which also exhibited a trend for increased *UCP1*, suggesting that olive oil might promote SAT beiging, but probably higher doses or prolonged periods of intervention are required. This suggestion is in agreement with a recent manuscript describing that short dietary intervention with extra-virgin olive oil promotes BAT activity, but this effect was only observed in lean subjects[96].

5. Conclusions

In summary, our study suggests that DHA-rich n-3 PUFA supplementation and a 16-weeks RT program decreased circulating CRP levels. Importantly, the novel marker of immune-inflammation status PLR was reduced by the DHA-rich supplementation. Moreover, both interventions reduced the expression of inflammatory genes (*LEPTIN, CHEMERIN, IL6, CD11c*) in SAT, and RT upregulated *ADIPONECTIN*, supporting their effectiveness in reducing WAT inflammation. Altogether, our data support the recommendation of enrolling RT programs and promoting n-3 PUFA consumption as a good strategy to ameliorate inflammation and improve metabolic health in overweight/obese postmenopausal women. However, the combination of the DHA-rich supplement and the RT program did not seem have relevant synergistic beneficial effects.

References

1. Kelly T, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. *Int J Obes*. 2008;32:1431-1437. doi:10.1038/ijo.2008.102
2. United Nations Department of Economic and Social Affairs. World population prospects 2019: highlights.
3. Levine ME, Lu AT, Chen BH, et al. Menopause accelerates biological aging. *Proc Natl Acad Sci U S A*. 2016;113:9327-9332. doi:10.1073/pnas.1604558113

4. Ambikairajah A, Walsh E, Tabatabaei-Jafari H, Cherbuin N. Fat mass changes during menopause: a metaanalysis. *Am J Obstet Gynecol*. 2019;221:393-409.e50. doi:10.1016/j.ajog.2019.04.023

5. Fulop T, Larbi A, Dupuis G, et al. Immunosenescence and inflamm-aging as two sides of the same coin: Friends or Foes? *Front Immunol*. 2018;8. doi:10.3389/fimmu.2017.01960

6. Abildgaard J, Tingstedt J, Zhao Y, et al. Increased systemic inflammation and altered distribution of T-cell subsets in postmenopausal women. Barreto GE, ed. *PLoS One*. 2020;15:e0235174. doi:10.1371/journal.pone.0235174

7. Xu F, Zheng X, Lin B, et al. Diet-induced obesity and insulin resistance are associated with brown fat degeneration in SIRT1-deficient mice. *Obesity*. 2016;24:634-642. doi:10.1002/oby.21393

8. Maurizi G, Della L, Angela G, Antonella M. Adipocytes properties and crosstalk with immune system in obesity-related inflammation. 2018:88-97. doi:10.1002/jcp.25855

9. Rodríguez A, Ezquerro S, Méndez-Giménez L, Becerril S, Frühbeck G. Revisiting the adipocyte: A model for integration of cytokine signaling in the regulation of energy metabolism. *Am J Physiol - Endocrinol Metab*. 2015;309:E691-E714. doi:10.1152/ajpendo.00297.2015

10. Graja A, Gohlke S, Schulz TJ. Aging of brown and beige/brite adipose tissue. *Handb Exp Pharmacol*. 2019;251:55-72. doi:10.1007/164_2018_151.

11. Villarroya F, Cereijo R, Gavaldà-Navarro A, Villarroya J, Giralt M. Inflammation of brown/beige adipose tissues in obesity and metabolic disease. *J Intern Med*. 2018;284:492-504. doi:10.1111/joim.12803

12. Laurson KR, McCann DA, Senchina DS. Age, sex, and ethnicity may modify the influence of obesity on inflammation. *J Investig Med*. 2011;59:27-31. doi:10.2310/JIM.0b013e318200151a

13. Vuong J, Qiu Y, La M, Clarke G, Swinkels DW, Cembrowski G. Reference intervals of complete blood count constituents are highly correlated to waist circumference: Should obese patients have their own “normal values?” *Am J Hematol*. 2014;89:671-677. doi:10.1002/ajh.23713

14. Fest J, Ruiter R, Ikram MA, Voortman T, Van Eijck CHJ, Stricker BH. Reference values for white blood-cell-based inflammatory markers in the Rotterdam Study: A population-based prospective cohort study. *Sci Rep*. 2018;8:1-7. doi:10.1038/s41598-018-28646-w

15. Trellakis S, Rydleuskaya A, Fischer C, et al. Low adiponectin, high levels of apoptosis and increased peripheral blood neutrophil activity in healthy obese subjects. *Obes Facts*. 2012;5:305-318. doi:10.1159/000339452

16. Erdal -2019-Platelet-to-lymphocyte ratio (PLR) and Plateletcrit (PCT) in young patients with morbid obesity.pdf.

17. Furuncuoğlu Y, Tulgar S, Dogan AN, Cakar S, Tulgar YK, Cakiroglu B. How obesity affects the neutrophil/lymphocyte and platelet/lymphocyte ratio, systemic immune-inflammatory index and platelet indices: A retrospective study. *Eur Rev Med Pharmacol Sci*. 2016;20:1300-1306.

18. Osadnik T, Bujak K, Osadnik K, et al. Novel inflammatory biomarkers may reflect subclinical inflammation in young healthy adults with obesity. *Endokrynol Pol*. 2019;70:135-142. doi:10.5603/EP.a2019.0002

19. Gupta V, Mishra S, Mishra S, Kumar S, Gupta V. Association of Leptin: Adiponectin ratio and metabolic risk markers in postmenopausal women. *Immunol Lett*. 2018;196:63-67. doi:10.1016/j.imlet.2018.01.008

20. Ahn SV, Jung DH, Yadav D, Kim JY, Koh SB. Relative contribution of obesity and menopause to the association between serum adiponectin and incident metabolic syndrome. *Menopause*. 2018;25:154-159. doi:10.1097/GME.0000000000000960

21. Coimbra S, Brandão Proença J, Santos-Silva A, Neuparth MJ. Adiponectin, leptin, and chemerin in elderly patients with type 2 diabetes mellitus: A close linkage with obesity and length of the disease. *Biomed Res Int*. 2014;2014. doi:10.1155/2014/701915

22. Elhakeem A, Cooper R, Whincup P, Brage S, Kuh D, Hardy R. Physical Activity, Sedentary Time, and Cardiovascular Disease Biomarkers at Age 60 to 64 Years. *J Am Heart Assoc*. 2018;7:1-14. doi:10.1161/JAHA.117.007459

23. Johannsen NM, Priest EL, Dixit VD, Earnest CP, Blair SN, Church TS. Association of white blood cell subfraction concentration with fitness and fatness. *Br J Sports Med*. 2010;44:588-593. doi:10.1136/bjsm.2008.050682

24. Peterson MD, Sen A, Gordon PM. Influence of Resistance Exercise on Lean Body Mass in Aging Adults: A Meta-Analysis. *Med Sci Sports Exerc*. 2011;43:249-258. doi:10.1249/MSS.0b013e3181eb6265.

25. Guizelini PC, de Aguiar RA, Denadai BS, Caputo F, Greco CC. Effect of resistance training on muscle strength and rate of force development in healthy older adults: A systematic review and meta-analysis. *Exp Gerontol*. 2018;102:51-58. doi:10.1016/j.exger.2017.11.020

26. Bartholomeu-Neto J, Brito CJ, Nóbrega OT, et al. Adaptation to Resistance Training Is Associated with Higher Phagocytic (but Not Oxidative) Activity in Neutrophils of Older Women. *J Immunol Res*. 2015;2015. doi:10.1155/2015/724982

27. Mcfarlin BK, Flynn MG, Phillips MD, Stewart LK, Timmerman KL. Killer Cell Activity in Older Women. *America (NY)*. 2005;60:1315-1318.

28. Cao Dinh H, Njemini R, Onyema OO, et al. Strength Endurance Training but Not Intensive Strength Training Reduces Senescence-Prone T Cells in Peripheral Blood in Community-Dwelling Elderly Women. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2019;74:1870-1878. doi:10.1093/gerona/gly229

29. Sardeli AV, Tomeleri CM, Cyrino ES, Fernhall B, Cavaglieri CR, Chacon-Mikahil MPT. Effect of resistance training on inflammatory markers of older adults: A meta-analysis. *Exp Gerontol*. 2018;111:188-196. doi:10.1016/j.exger.2018.07.021

30. Ward LJ, Nilsson S, Hammar M, et al. Resistance training decreases plasma levels of adipokines in postmenopausal women. *Sci Rep*. 2020;10:1-9. doi:10.1038/s41598-020-76901-w

31. Phillips MD, Patrizi RM, Cheek DJ, Wooten JS, Barbee JJ, Mitchell JB. Resistance training reduces subclinical inflammation in obese, postmenopausal women. *Med Sci Sports Exerc*. 2012;44:2099-2110. doi:10.1249/MSS.0b013e3182644984

32. Phillips CM, Tierney AC, Perez-Martinez P, et al. Obesity and body fat classification in the metabolic syndrome: Impact on cardiometabolic risk metabotype. *Obesity*. 2013;21:154-161. doi:10.1038/oby.2012.188

33. Wang Y, Huang F. N-3 polyunsaturated fatty acids and inflammation in obesity: Local effect and systemic benefit. *Biomed Res Int*. 2015;2015. doi:10.1155/2015/581469

34. Kopecky J, Rossmeisl M, Flachs P, et al. N-3 PUFA: Bioavailability and modulation of adipose tissue function. *Proc Nutr Soc*. 2009;68:361-369. doi:10.1017/S0029665109990231

35. AbuMweis S, Jew S, Tayyem R, Agraib L. Eicosapentaenoic acid and docosahexaenoic acid containing supplements modulate risk factors for cardiovascular disease: a meta-analysis of randomised placebo-control human clinical trials. *J Hum Nutr Diet*. 2018;31:67-84. doi:10.1111/jhn.12493

36. Wu JHY, Cahill LE, Mozaffarian D. Effect of fish oil on circulating adiponectin: A systematic review and meta-analysis of randomized controlled trials. *J Clin Endocrinol Metab*. 2013;98:2451-2459. doi:10.1210/jc.2012-3899

37. Hariri M, Ghiasvand R, Shiranian A, Askari G, Iraj B, Salehi-Abargouei A. Does omega-3 fatty acids supplementation affect circulating leptin levels? A systematic review and meta-analysis on randomized controlled clinical trials. *Clin Endocrinol (Oxf)*. 2015;82:221-228. doi:10.1111/cen.12508

38. Caspar-Bauguil S, Fioroni A, Galinier A, et al. Pro-inflammatory phospholipid arachidonic acid/eicosapentaenoic acid ratio of dysmetabolic severely obese women. *Obes Surg*. 2012;22:935-944. doi:10.1007/s11695-012-0633-0

39. Zhuang P, Lu Y, Shou Q, et al. Differential anti-adipogenic effects of eicosapentaenoic and docosahexaenoic acids in obesity. *Mol Nutr Food Res*. 2019;63:e1801135. doi:10.1002/mnfr.201801135

40. Innes JK, Calder PC. The differential effects of eicosapentaenoic acid and docosahexaenoic acid on cardiometabolic risk factors: A systematic review. *Int J Mol Sci*. 2018;19. doi:10.3390/ijms19020532

41. Shin G, Jang K, Kim M, Lee JH, Yoo HJ. Inflammatory Markers and Plasma Fatty Acids in Predicting WBC Level Alterations in Association With Glucose-Related Markers: A Cross-Sectional Study. *Front Immunol*. 2020;11. doi:10.3389/fimmu.2020.00629

42. Allaire J, Vors C, Tremblay AJ, et al. High-dose DHA has more profound effects on LDL-related features than high-dose EPA: The ComparED study. *J Clin Endocrinol Metab*. 2018;103:2909-2917. doi:10.1210/jc.2017-02745

43. Guo XF, Li X, Shi M, Li D. N-3 polyunsaturated fatty acids and metabolic syndrome risk: A meta-analysis. *Nutrients*. 2017;9:1-14. doi:10.3390/nu9070703

44. Howe PRC, Buckley JD, Murphy KJ, Pettman T, Milte C, Coates AM. Relationship between erythrocyte omega-3 content and obesity is gender dependent. *Nutrients*. 2014;6:1850-1860. doi:10.3390/nu6051850

45. Da Boit M, Sibson R, Sivasubramaniam S, et al. Sex differences in the effect of fish-oil supplementation on the adaptive response to resistance exercise training in older people: A randomized controlled trial. *Am J Clin Nutr*. 2017;105:151-158. doi:10.3945/ajcn.116.140780

46. Vidal P, Stanford KI. Exercise-Induced Adaptations to Adipose Tissue Thermogenesis. *Front Endocrinol (Lausanne)*. 2020;11:270. doi:10.3389/fendo.2020.00270

47. Fernández-Galilea M, Félix-Soriano E, Colón-Mesa I, Escoté X, Moreno-Aliaga MJ. Omega-3 fatty acids as regulators of brown/beige adipose tissue: from mechanisms to therapeutic potential. *J Physiol Biochem*. 2019;76:251-257. doi:10.1007/s13105-019-00720-5

48. Otero-Díaz B, Rodríguez-Flores M, Sánchez-Muñoz V, et al. Exercise Induces White Adipose Tissue Browning Across the Weight Spectrum in Humans. *Front Physiol*. 2018;9. doi:10.3389/fphys.2018.01781

49. WHO Technical Report. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. 2000;894.

50. WHO. *Men Ageing And Health*.; 2001.

51. Martínez-González MA, López-Fontana C, Varo JJ, Sánchez-Villegas A, Martinez JA. Validation of the Spanish version of the physical activity questionnaire used in the Nurses' Health Study and the Health Professionals' Follow-up Study. *Public Health Nutr*. 2005;8:920-927. doi:10.1079/phn2005745

52. Aranceta Bartrina J, Arija Val V V., Maíz Aldalur E, et al. Dietary Guidelines for the Spanish population (SENC, diciembre 2016); the new graphic icon of healthy food. *Nutr Hosp*. 2016;33:1-48. doi:10.20960/nh.827

53. Schröder H, Fitó M, Estruch R, et al. A Short Screener Is Valid for Assessing Mediterranean Diet Adherence among Older Spanish Men and Women. *J Nutr*. 2011;141:1140-1145. doi:10.3945/jn.110.135566

54. Munro IA, Garg ML. Prior supplementation with long chain omega-3 polyunsaturated fatty acids promotes weight loss in obese adults: A double-blinded randomised controlled trial. *Food Funct*. 2013;4:650-658. doi:10.1039/c3fo60038f

55. Munro IA, Garg ML. Dietary supplementation with long chain omega-3 polyunsaturated fatty acids and weight loss in obese adults. *Obes Res Clin Pract*. 2013;7:e173-e181. doi:10.1016/j.orcp.2011.11.001

56. Munro IA, Garg ML. Dietary supplementation with n-3 PUFA does not promote weight loss when combined with a very-low-energy diet. *Br J Nutr*. 2012;108:1466-1474. doi:10.1017/S0007114511006817

57. U.S. Food and Drug Administration. Qualified Health Claims: Letters of Enforcement Discretion | FDA. Accessed November 23, 2020. https://www.fda.gov/food/food-labeling-nutrition/qualified-health-claims-letters-enforcement-discretion

58. Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). *EFSA J*. 2012;10. doi:10.2903/j.efsa.2012.2815

59. Mataix-Verdú J, Mañas Almendros M, Llopis González J, Martínez de Victoria E. *Tablas de Composición de Alimentos*. Universidad de Granada; 1993.

60. Cuff DJ, Meneilly GS, Martin A, Ignaszewski A, Tildesley HD, Frohlich JJ. Effective Exercise Modality to Reduce Insulin Resistance in Women With Type 2 Diabetes. *Diabetes Care*. 2003;26:2977-2982. doi:10.2337/diacare.26.11.2977

61. American College of Sports Medicine. Progression models in resistance training for healthy adults. *Med Sci Sports Exerc*. 2009;41:687-708. doi:10.1249/MSS.0b013e3181915670

62. Baechle T, Earle R. Resistance Training. In: *Essentials of Strength Training and Conditioning*. National Strength & COnditioning Association; 2008:318-412.

63. Frühbeck G, Catalán V, Rodríguez A, Gómez-Ambrosi J. Adiponectin-leptin ratio: A promising index to estimate adipose tissue dysfunction. Relation with obesity-associated cardiometabolic risk. *Adipocyte*. 2018;7:57-62. doi:10.1080/21623945.2017.1402151

64. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402-408. doi:10.1006/meth.2001.1262

65. Pekelharing JM, Hauss O, De Jonge R, et al. *Haematology Reference Intervals for Established and Novel Parameters in Healthy Adults*. Vol 1.

66. Frühbeck G, Catalán V, Rodríguez A, Gómez-Ambrosi J. Adiponectin-leptin ratio: A promising index to estimate adipose tissue dysfunction. Relation with obesity-associated cardiometabolic risk. *Adipocyte*. 2018;7:57-62. doi:10.1080/21623945.2017.1402151

67. Guo X fei, Li K lei, Li J mei, Li D. Effects of EPA and DHA on blood pressure and inflammatory factors: a meta-analysis of randomized controlled trials. *Crit Rev Food Sci Nutr*. 2019;59:3380-3393. doi:10.1080/10408398.2018.1492901

68. Strandberg E, Edholm P, Ponsot E, et al. Influence of combined resistance training and healthy diet on muscle mass in healthy elderly women: A randomized controlled trial. *J Appl Physiol*. 2015;119:918-925. doi:10.1152/japplphysiol.00066.2015

69. Di Lorenzo FM, Drager CJ, Rankin JW. Docosahexaenoic acid affects markers of inflammation and muscle damage after eccentric exercise. *J strength*. 2014;28:2768-2774.

70. Cornish SM, Myrie SB, Bugera EM, Chase JE, Turczyn D, Pinder M. Omega-3 supplementation with resistance training does not improve body composition or lower biomarkers of inflammation more so than resistance training alone in older men. *Nutr Res*. 2018;60:87-95. doi:10.1016/j.nutres.2018.09.005

71. Bloomer RJ, Larson DE, Fisher-Wellman KH, Galpin AJ, Schilling BK. Effect of eicosapentaenoic and docosahexaenoic acid on resting and exercise-induced inflammatory and oxidative stress biomarkers: A randomized, placebo controlled, cross-over study. *Lipids Health Dis*. 2009;8:1-12. doi:10.1186/1476-511X-8-36

72. Neves S da C, Lima RM, Simões HG, Marques MC, Reis VM, de Oliveira RJ. Resistance exercise sessions do not provoke acute immunosuppression in older women. *J Strength Cond Res*. 2009;23:259-265.

73. Bartholomeu-Neto J, Brito CJ, Nóbrega OT, et al. Adaptation to Resistance Training Is Associated with Higher Phagocytic (but Not Oxidative) Activity in Neutrophils of Older Women. *J Immunol Res*. 2015;2015. doi:10.1155/2015/724982

74. Bobeuf F, Labonté M, Khalil A, Dionne IJ. Effect of Resistance Training on Hematological Blood Markers in Older Men and Women: A Pilot Study. *Curr Gerontol Geriatr Res*. 2009;2009:1-4. doi:10.1155/2009/156820

75. Gutiérrez S, Svahn SL, Johansson ME. Effects of omega-3 fatty acids on immune cells. *Int J Mol Sci*. 2019;20. doi:10.3390/ijms20205028

76. Véricel E, Colas R, Calzada C, et al. Moderate oral supplementation with docosahexaenoic acid improves platelet function and oxidative stress in type 2 diabetic patients. *Thromb Haemost*. 2015;114:289-296. doi:10.1160/TH14-12-1003

77. Phang M, Lincz LF, Garg ML. Eicosapentaenoic and Docosahexaenoic Acid Supplementations Reduce Platelet Aggregation and Hemostatic Markers Differentially in Men and Women. *J Nutr*. 2013;143:457-463. doi:10.3945/jn.112.171249

78. Sut A, Chizyński K, Rózalski M, Golański J. Dietary intake of omega fatty acids and polyphenols and its relationship with the levels of inflammatory markers in men with chronic coronary syndrome after percutaneous coronary intervention. *Kardiol Pol*. 2020;78:117-123. doi:10.33963/KP.15078

79. Oren E, Smith NL, Doggen CJM, Heckbert SR, Lemaitre RN. Body mass index and the risk of venous thrombosis among postmenopausal women [3]. *J Thromb Haemost*. 2006;4:2273-2275. doi:10.1111/j.1538-7836.2006.02119.x

80. Zylla S, Pietzner M, Kühn JP, et al. Serum chemerin is associated with inflammatory and metabolic parameters—results of a population-based study. *Obesity*. 2017;25:468-475. doi:10.1002/oby.21735

81. Prestes J, Shiguemoto G, Botero JP, et al. Effects of resistance training on resistin, leptin, cytokines, and muscle force in elderly post-menopausal women. *J Sports Sci*. 2009;27:1607-1615. doi:10.1080/02640410903352923

82. Botero JP, Shiguemoto GE, Prestes J, et al. Effects of long-term periodized resistance training on body composition, leptin, resistin and muscle strength in elderly post-menopausal women. *J Sports Med Phys Fitness*. 2013;53:289-294.

83. Saeidi A, Jabbour G, Ahmadian M, et al. Independent and combined effects of antioxidant supplementation and circuit resistance training on selected adipokines in postmenopausal women. *Front Physiol*. 2019;10:1-10. doi:10.3389/fphys.2019.00484

84. Khoo J, Dhamodaran S, Chen DD, Yap SY, Chen RYT, Tian RHH. Exercise-Induced weight loss is more effective than dieting for improving adipokine lle, Insulin resistance, And inflammation in obese men. *Int J Sport Nutr Exerc Metab*. 2015;25:566-575. doi:10.1123/ijsnem.2015-0025

85. Malin S, Navaneethan S, Mulya A, Huang H, Kirwan J. Exercise-induced lowering of chemerin is associated with reduced cardiometabolic risk and glucose-stimulated insulin secretion in older adults. *J Nutr Heal Aging*. 2014;18:608-615. doi:10.1007/s12603-014-0459-7

86. Lakhdar N, Denguezli M, Zaouali M, Zbidi A, Tabka Z, Bouassida A. Diet and diet combined with chronic aerobic exercise decreases body fat mass and alters plasma and adipose tissue inflammatory markers in obese women. *Inflammation*. 2013;36:1239-1247. doi:10.1007/s10753-013-9661-8

87. Kolahdouzi S, Baghadam M, Kani-Golzar FA, et al. Progressive circuit resistance training improves inflammatory biomarkers and insulin resistance in obese men. *Physiol Behav*. 2019;205:15-21. doi:10.1016/j.physbeh.2018.11.033

88. Stefanov T, Blüher M, Vekova A, et al. Circulating chemerin decreases in response to a combined strength and endurance training. *Endocrine*. 2014;45:382-391. doi:10.1007/s12020-013-0003-2

89. Kabir M, Skurnik G, Naour N, et al. Treatment for 2 mo with n-3 polyunsaturated fatty acids reduces adiposity and some atherogenic factors but does not improve insulin sensitivity in women with type 2 diabetes: A randomized controlled study. *Am J Clin Nutr*. 2007;86:1670-1679. doi:10.1093/ajcn/86.6.1670

90. Tsitouras PD, Gucciardo F, Salbe AD, Heward C, Harman SM. High omega-3 fat intake improves insulin sensitivity and reduces CRP and IL6, but does not affect other endocrine axes in healthy older adults. *Horm Metab Res*. 2008;40:199-205. doi:10.1055/s-2008-1046759

91. Simão ANC, Lozovoy MAB, Bahls LD, et al. Blood pressure decrease with ingestion of a soya product (kinako) or fish oil in women with the metabolic syndrome: Role of adiponectin and nitric oxide. *Br J Nutr*. 2012;108:1435-1442. doi:10.1017/S0007114511006921

92. Sedláček P, Plavinová I, Langmajerová J, et al. Effect of N-3 fatty acids supplementation during life style modification in women with overweight. *Cent Eur J Public Health*. 2018;26:265-271. doi:10.21101/cejph.a5259

93. Prostek A, Gajewska M, Kamola D, Bałasińska B. The influence of EPA and DHA on markers of inflammation in 3T3-L1 cells at different stages of cellular maturation. *Lipids Health Dis*. 2014;13:1-9. doi:10.1186/1476-511X-13-3

94. Huerta AE, Prieto-Hontoria PL, Sáinz N, Martínez JA, Moreno-Aliaga MJ. Supplementation with α-lipoic acid alone or in combination with eicosapentaenoic acid modulates the inflammatory status of healthy overweight or obese women consuming an energy-restricted diet. *J Nutr*. 2016;146:889S-896S. doi:10.3945/jn.115.224105

95. Gillingham LG, Harris-Janz S, Jones PJH. Dietary monounsaturated fatty acids are protective against metabolic syndrome and cardiovascular disease risk factors. *Lipids*. 2011;46:209-228. doi:10.1007/s11745-010-3524-y

96. Monfort-Pires M, U-Din M, Nogueira GA, et al. Short dietary intervention with olive oil increases brown adipose tissue activity in lean but not overweight subjects. *J Clin Endocrinol Metab*. 2020;dgaa824:1-27. doi:10.1210/clinem/dgaa824.

97. Rostami H, Samadi M, Yuzbashian E, et al. Habitual dietary intake of fatty acids are associated with leptin gene expression in subcutaneous and visceral adipose tissue of patients without diabetes. *Prostaglandins Leukot Essent Fat Acids*. 2017;126:49-54. doi:10.1016/j.plefa.2017.09.010

98. Schmid A, Bala M, Leszczak S, Ober I, Buechler C, Karrasch T. Pro-inflammatory chemokines CCL2, chemerin, IP-10 and RANTES in human serum during an oral lipid tolerance test. *Cytokine*. 2016;80:56-63. doi:10.1016/j.cyto.2016.02.010

99. Rodriguez-Pacheco F, Gutierrez-Repiso C, Garcia-Serrano S, et al. The pro-/anti-inflammatory effects of different fatty acids on visceral adipocytes are partially mediated by GPR120. *Eur J Nutr*. 2017;56:1743-1752. doi:10.1007/s00394-016-1222-0

100. Stinkens R, Brouwers B, Jocken JW, et al. Exercise training-induced effects on the abdominal subcutaneous adipose tissue phenotype in humans with obesity. *J Appl Physiol*. 2018;125:1585-1593. doi:10.1152/japplphysiol.00496.2018

101. Klimcakova E, Polak J, Moro C, et al. Dynamic strength training improves insulin sensitivity without altering plasma levels and gene expression of adipokines in subcutaneous adipose tissue in obese men. *J Clin Endocrinol Metab*. 2006;91:5107-5112. doi:10.1210/jc.2006-0382

102. Čížková T, Štěpán M, Daďová K, et al. Exercise training reduces inflammation of adipose tissue in the elderly: cross-sectional and randomized interventional trial. *J Clin Endocrinol Metab*. 2020;105:e4510–e4526. doi:10.1210/clinem/dgaa630

103. Itariu BK, Zeyda M, Hochbrugger EE, et al. Long-chain n-3 PUFAs reduce adipose tissue and systemic inflammation in severely obese nondiabetic patients: A randomized controlled trial. *Am J Clin Nutr*. 2012;96:1137-1149. doi:10.3945/ajcn.112.037432

104. Hames KC, Morgan-Bathke M, Harteneck DA, et al. Very-long-chain ω-3 fatty acid supplements and adipose tissue functions: A randomized controlled trial. *Am J Clin Nutr*. 2017;105:1552-1558. doi:10.3945/ajcn.116.148114

105. Han CY, Kargi AY, Omer M, et al. Differential effect of saturated and unsaturated free fatty acids on the generation of monocyte adhesion and chemotactic factors by adipocytes: Dissociation of adipocyte hypertrophy from inflammation. *Diabetes*. 2010;59:386-396. doi:10.2337/db09-0925

106. Björk C, Wilhelm U, Mandrup S, et al. Effects of selected bioactive food compounds on human white adipocyte function. *Nutr Metab*. 2016;13:1-10. doi:10.1186/s12986-016-0064-3

107. Saínz N, Fernández-Galilea M, Costa AGV, Prieto-Hontoria PL, Barraco GM, Moreno-Aliaga MJ. N-3 polyunsaturated fatty acids regulate chemerin in cultured adipocytes: role of GPR120 and derived lipid mediators. *Food Funct*. 2020;11:9057-9066. doi:10.1039/d0fo01445a

108. Murumalla RK, Gunasekaran MK, Padhan JK, et al. Fatty acids do not pay the toll: Effect of SFA and PUFA on human adipose tissue and mature adipocytes inflammation. *Lipids Health Dis*. 2012;11:1-9. doi:10.1186/1476-511X-11-175

109. Ferguson JF, Xue C, Hu Y, Li M, Reilly MP. Adipose tissue RNASeq reveals novel gene-nutrient interactions following n-3 PUFA supplementation and evoked inflammation in humans. *J Nutr Biochem*. 2017;April:126-132. doi:10.1016/j.jnutbio.2015.12.010.Adipose

110. Maurer SF, Dieckmann S, Lund J, et al. No Effect of Dietary Fish oil Supplementation on the Recruitment of Brown and Brite Adipocytes in Mice or Humans Under Thermoneutral Conditions. *Mol Nutr Food Res*. 2020;2000681:2000681. doi:10.1002/mnfr.202000681

General Discussion

The present work is based on the premise that adipose tissue is one of the main contributors to the chronic, low grade inflammation that characterizes the concurrence of obesity and aging. Under both conditions, white and brown adipose tissues and their constituting adipocytes (white, brown and beige) experience a dysregulation and loss of function[49,95,361,451,452], together with the development of a local proinflammatory status[31,48]. The adipose organ is a key regulator of energy metabolism with relevant roles in glucose and triglyceride clearance[49,93]. Moreover, it secretes adipose-specific adipokines/batokines that have actions on other metabolic organs[53,71,265]. Therefore, reducing inflammation in adipose tissue would improve its functionality and the adipose-systemic metainflammation[99,453], hence preventing or reducing the progression and establishment of the well-known obesity and aging comorbidities[11].

Due to the increasing obese and aged populations[6,454], current investigations are elucidating potential treatments for the prevention of their associated diseases[455,456]. In this study, docosahexaenoic acid (DHA) supplementation and exercise training were proposed as effective approaches to tackle the adipose-systemic inflammatory axis. Noteworthy, DHA and exercise have proven beneficial effects on adipose tissue remodeling towards an antiinflammatory, substrate oxidative and thermogenic profile[128,129,133,224]. In fact, DHA and physical exercise have been recommended for the prevention and concomitant treatment of obesity and aging-related disturbances including cardiovascular disease[216,328], hyperlipidaemia[457,458], weight gain[327], type 2 diabetes[212,329,330], musculoskeletal disorders and frailty[459,460].

The growing evidence showing the need for population-based[461,462] and sex-specific preventive interventions[463,464], together with the still reduced number of studies in female animals and women[465,466], led us to focus our investigation in old female mice (18 months-old) and postmenopausal women. In this sense, it is worth mentioning that postmenopausal women are an ideal target for lifestyle therapies for a healthy aging, as women live more but live worse[19,29]. Indeed, menopause leads to changes in body composition and physiology that make obesity and aging-associated comorbidities more prevalent among post than premenopausal women, and for some cases, than age-matched men[22,467].

Thus, we developed an animal study in aged obese female mice and later, an intervention in postmenopausal women with overweight or obesity, to characterize the potential benefits of DHA supplementation and physical exercise in this population of high sociosanitary interest. The effects on two main adipose depots, subcutaneous white adipose tissue (scWAT) and interscapular brown adipose tissue (iBAT) inflammation and function were analyzed in the murine model of obesity and aging. On the other hand, the nutritional and physical exercise intervention provided evidence for the recommendation of both therapies to improve body composition and adipose-systemic metabolic inflammation in postmenopausal women with overweight or obesity.

Chronic DHA supplementation reduces inflammation and induces metabolic remodeling of scWAT and iBAT in aged obese female mice

The studies in diet-induced obese (DIO) female mice fed with a DHA-enriched HFD were conducted from adulthood until they reached the late middle-age (from 6 to 18 months of age) (*Chapter 1* and *Chapter 2*), at which the mouse is already considered old[468]. The effects of DHA supplementation were compared in the background of a chronic feeding with a high-fat diet (HFD) rich in saturated fat, by means of replacing 15% of dietary saturated fat (wt/wt) with the DHA-rich *n*-3 PUFA concentrate. We used this method as an

approach to study if the fat quality, and not the quantity, had the expected beneficial impacts on scWAT and iBAT, thus mediating the improvements observed in metabolic health biomarkers and body composition, as previously reported in DHA-supplemented young obese mice[132].

As expected, aged obese female animals revealed an unhealthier body composition than aged lean animals, characterized by higher body weight gain and fat mass, and heavier scWAT, visceral WAT (vWAT), and iBAT depots[469]. Importantly, the DHA-enriched diet induced a moderate but non-significant reduction in body weight and fat mass, as well as in scWAT weight (*Chapter 1*). Previous studies in younger adult animals have revealed effects for DHA and EPA on reducing scWAT mass and other WAT depots (gonadal), when given as dietary treatment with the HFD[132,249,261,470] or as intraperitoneal injections[245,246] for shorter periods. Therefore, the current DHA treatment may have not been enough to counteract our experimental life lasting (16 months) high-fat feeding, which started at 2 months of age. Nevertheless, the DHA-treated DIO mice showed smaller adipocytes than the DIO mice (*Chapter 1*). This result is of high relevance, since smaller adipocytes exhibit a less proinflammatory secretory pattern, as well as higher insulin sensitivity[471].

Importantly, the reduction caused by the DHA-enriched diet on adipocyte size seemed to be mainly mediated by an inhibition of triglycerides' accumulation in scWAT, as confirmed by the significant downregulation observed in main genes involved in these metabolic pathways (*Lpl, Fasn*, *Dgat1, Scd1*). In fact, these effects have been previously observed in young rodents treated with *n*-3 PUFA[472–474] and in cultured adipocytes[60]. In the aged obese mice, DHA supplementation also prevented the paradoxical upregulation of lipolytic and fatty oxidation genes observed in the aged DIO mice, which could be an adaptive mechanism to reduce the fatty acid overload in scWAT. Noteworthy, the DHA-enriched HFD also promoted an increase in *Cpt1a*, a fatty acid oxidation gene, as compared to aged CT mice, which agrees with previous studies in younger mice[232]. Collectively, the integration of the gene expression studies suggest that DHA-supplementation could promote an increased β-oxidation in DIOMEG mice, with lower lipogenesis rates.

Moreover, scWAT of the DHA-treated mice exhibited an improved local inflammatory status, as revealed by a reduction in the expression of proinflammatory genes (*Tlr4*, *Ccl2* and *Lep)* even in the context of the HFD. Interestingly, the expression of *Adipoq* was decreased by DHA, supporting the "adiponectin paradox" that occurs in aging. Furthermore, this effect could be a consequence of the reduced inflammation observed in scWAT of these aged mice[162,475]. Although DHA supplementation did not modify the total macrophages ($F4/80^+/CD11b^+$) content, the M1 marker *Cd11c* was decreased and the M2 macrophages marker CD206 was dramatically increased in DIOMEG animals. These data suggest that chronic DHA supplementation prompted a switch in macrophages phenotype from M1 (proinflammatory) to M2 (antiinflammatory), without altering the total macrophages pool.

In parallel, the scWAT of DHA-supplemented mice exhibited an increased expression of specific beige adipocytes gene markers (*Cd137*, *Tmem26*, *Tbx1),* with the recovery of the main thermogenic protein UCP1, suggesting that DHA was able to prevent/restore the drop of beiging properties that accompanies obesity and aging. Interestingly, previous studies have shown that the recruitment of M2 macrophages plays a key role in the browning process of WAT[124,476]. Hence, our data seem to indicate that DHA could preserve the browning capacity of the aged scWAT by promoting M2 macrophages. It is worth of mention that, while the adipose tissue browning properties of *n*-3 PUFA (mainly EPA), have been described in previous studies in young mice and in cultured adipocytes[131,133,235,477,478], we here report for the first time this phenomena in older animals supplemented with DHA.

Previous studies have suggested that DHA could exert its anti-inflammatory actions through the physiological production of its derived specialized proresolving lipid mediators (SPMs). SPMs were first described by Serhan *et al*.[479] as potent lipid mediators produced from *n*-3 PUFA to actively resolve the inflammatory process. SPMs effects have been proven, among other inflammatory diseases, in the inflamed obese WAT[267]. DHA-derived MaR1, 17-HDHA, RvD1 and RvD2 have shown therapeutic actions in ameliorating WAT inflammation under obesogenic conditions *via* macrophage polarization towards an M2 phenotype, increased adiponectin secretion, and induction of antiinflammatory signaling in animal models of obesity[245,246,271,291,480]. Interestingly, an impaired local production of some SPMs precedes the development of obesity-associated inflammation and insulin resistance in WAT[271]. Moreover, increments in SPMs and lipid intermediates (PD1, RvD1 and 17-HDHA) have been observed after partial dietary fat replacement with *n*-3 PUFA, in both scWAT and vWAT[271,284].

However, the lipid mediator profile of iBAT is poorly understood. Some recent studies have shown that iBAT secretes lipid metabolites derived from the same pathways as SPMs (LOX enzyme) after cold and physical exercise stimuli in mice[78,83,84], in parallel to a promotion in cold adaptive thermogenesis and glucose uptake in iBAT. We decided to analyze the SPMs signature in iBAT from our aged, obese mice treated or not with DHA. Due to the novelty and relevance of these data, a 2-month-old lean group (CT) was included in this study (*Chapter 2*), to characterize if the "inflammaging" of iBAT could be also associated to decreased production of SPMs.

The lipid mediator profiling of iBAT revealed meaningful drops in the sum of *n*-3 DPA and DHA-derived protectins (PDs), and in arachidonic acid (AA)-derived lipoxins (LXs) in aged obese animals compared to young lean mice. This drop was also observed in the concentration of several individual SPMs, including D-series and T-series resolvins (RvDs: RvD6, RvT1, $RvD5_{n\text{-}3\ DPA}$), protectins (PDs: 17R-PD1, PDX, $PD2_{n\text{-}3\ DPA}$, 10S,17S-diHDPA) and maresins (MaRs: MaR2, 4,14-diHDHA). It is worth mentioning that AA-derived LXA_4, LXB_4, $PGF_{2\alpha}$ and 15-epi-LXA_4 were also decreased by aging and obesity in the aged DIO group. As expected, dietary supplementation with the DHA-rich *n*-3 PUFA oil increased the levels of all *n*-3 PUFA derived SPMs (DPA, DHA and EPA-derived MaRs, PDs, Rvs), regardless of their drop or maintenance in aged obese animals.

Importantly, LXs and MaRs also the most abundant SPMs in young CT (2 months old) iBAT compared with the rest of SPMs classes. Because LXs were not recovered with the DHA dietary treatment, we hypothesize that these AA-derived SPMs may have a determinant role in iBAT function. In fact, when the associations of SPMs and the genes studied for iBAT function were analyzed, the sum of LXs revealed significant positive correlations with *Prdm16*, and negative correlations with *Ccl2*. These data support the previous findings that both COX and LOX derived lipid mediators play a role in browning and inflammation in WAT[294,296,297], and suggest that the resolution of inflammation exerted by means of increasing SPMs in iBAT might be an effective therapy to restore iBAT function.

Interestingly, the drop in SMPs occurred in parallel to the aging of iBAT and especially in obesity, since aged obese animals displayed reductions in *Prdm16* and UCP1. Because these drops were partly reverted by DHA supplementation, it is likely that the recovery of *Prdm16* and UCP1 took place in association with the recovery of *n*-3 PUFA derived SPMs. However, besides the marked increment in all *n*-3 PUFA-derived SPMs, DHA was not able to restore the reduced iBAT cold-induced activity observed in aged DIO mice. This result suggests that aging might also alter the expression or activity of the main SPMs receptors, or that the unrecovered LXs concentrations could play an important role in this process. Therefore, future studies

aiming to explore these possibilities are likely to provide novel insights in the role of specialized SPMs in iBAT function in aging and in obesity.

On the other hand, several investigations in the past years have pointed a role for GPR120 and FGF21 in the maintenance of iBAT and WAT thermogenic function[131,481]. However, our current data suggest that neither *Gpr120* nor *Fgf21* were mediating the stimulatory effects of DHA on genes and proteins involved in browning of scWAT. In this regard, it is important to note that previous studies have shown differential effects for EPA and DHA treatments, being EPA a clear inducer of *Gpr120* and of iBAT and WAT thermogenic response, while DHA had barely significant effects[131]. Here, we show that the DHA-enriched diet clearly downregulated the increase of *Fgf21* observed in aged DIO mice. Moreover, it also inhibited the expression of the FGF21 receptor components (*Fgfr1*, *β-klotho*) and the downstream signaling marker *Egr1* (scWAT). Furthermore, the dual role of this signaling pathway on thermogenesis warrants further research, since the inhibition of *Egr1* has been proven to cause browning of scWAT[482,483]. The role of *Gpr120* is also controversial, as it acts as an *n*-3 PUFA receptor and mediates insulin sensitizing processes[247,484], but also inflammatory responses[485]. In summary, our data suggests that the beneficial actions of *n*-3 PUFA in aging and in obesity are not mediated by GPR120 nor by FGF21, as previously demonstrated in other studies in younger mice[254].

In addition to the effects exerted in scWAT and iBAT of the aged obese female animals, it is important to mention that the long-term feeding with the DHA-enriched HFD induced a decrease in total cholesterol, LDL-cholesterol and in the atherogenic indexes (total chol/HDL-cholesterol and LDL-cholesterol/HDL-cholesterol, *Chapter 1* and *Chapter 2*). These results are in line with others observing an amelioration in the blood lipid profile of HFD-fed mice treated with DHA[132]. However, it is worth mentioning that serum triglycerides did not present any change due to aging or obesity, as their levels were similar in the four experimental groups (young CT and aged CT, DIO, and DIOMEG, *Chapter 2*). This result might be due to the fact that aging reduces triglycerides in mice[486], and thus this process may have interfered with the HFD-induced increase in triglycerides levels.

It is worth mentioning that long-term feeding with the DA-enriched HFD led to statistical trends towards a reduction in both scWAT and vWAT mass. These results contrast with those observed previously in younger rodents (14 months[249] and 7 months of age[232,470]), with effects for DHA-enriched diets on reducing epididymal WAT mass of DIO rodents. According to the well-established relationship between insulin resistance and visceral fat[58], the HOMA-IR index was non-significantly decreased compared to the DIO group. Several studies have found improved fasting glucose, insulin, and/or insulin resistance in high-fat fed mice supplemented with fish oil[254,256,470,487], or treated with DHA[132,245] in younger animas (5-7 months old). However, in our study, aging may have spoiled the effects of the DHA-rich *n*-3 PUFA diet on insulin resistance. Indeed, the impairment of insulin sensitivity has been described to be the one of the main alterations of the different WAT depots (inguinal, epididymal and perirenal) in aging. These effects were revealed by the lower abundance and phosphorylation of insulin receptors already in 8 months old mice, an observation that was maintained in 24 months old animals[361].

While vWAT was not the focus of this investigation, a reduction in visceral adiposity would be of special relevance in our study population, since visceral adipose tissue has been established to predict mortality in animal studies[488] and in observational studies in humans[489]. Because of the observed trend towards a reduced vWAT, the direct association of this depot with HOMA-IR and proatherogenic profiles[490], and

considering some studies have found deeper effects on vWAT than scWAT mass[232,491], future studies assessing the contribution of vWAT to the beneficial metabolic effects of DHA are of special interest.

Physical exercise induces differential adaptations in scWAT and iBAT in aged obese female mice

Exercise training has been revealed to exert beneficial systemic effects mediated, in part, by its actions on white and brown adipose tissues[78,130]. Hence, our second experimental approach in obese and aged animals consisted of a long-term exercise training protocol. In this experiment, the trained group was submitted to a treadmill running exercise protocol based on a mild intensity training program maintained from 6 months up to 18 months of age. The training program was conducted concomitantly to the high-fat feeding, in order to elucidate the effects of exercise *per se* on aged DIO mice.

The exercise program did not have any effect on the weight of DIO animals nor in their fat depots weight, including scWAT, vWAT and iBAT depots. No effects were neither observed in the blood lipid levels of exercised mice. However, the long-term trained DIO animals did show a lowering trend on fasting glucose and insulin levels that, moreover, resulted in a decreased HOMA-IR index compared to the untrained obese animals. Furthermore, the GTT excursion curve of exercised animals showed a smaller area than the one observed in untrained animals, revealing lower circulating glucose levels at 30 min post intraperitoneal glucose administration. In this context, the study performed by Bae *et al*.[353] described beneficial actions for exercise on 16 months old obese animals, including lower body weight and visceral adiposity, lower LDL-cholesterol, as well as higher HDL-cholesterol, without reporting effects on glucose metabolism. However, the HFD feeding period of this experiment began at 12 months of age and was prolonged for only for 4 months, which is a short period compared to the 16 months of high-fat feeding performed in our aged animals. From this point of view, our data are relevant since they indicate that lifelong exercise has the ability to partially reduce the appearance of glucose intolerance and insulin resistance that occurs in aged animals[361] even when chronically fed with a HFD, and supports the observed effects for exercise at younger life stages in DIO animals[130].

Regarding the adipose depots, scWAT revealed a markedly higher response to exercise as compared to iBAT. Indeed, *de novo* lipogenesis (*Fasn*) and fatty acid oxidation (*Cpt1a*, *Acox1*) genes were down and upregulated, respectively, in scWAT of trained mice. Unexpectedly, *Dgat1* mRNA was increased, which suggests a promotion of fatty acid esterification and accumulation as triglycerides in scWAT. In the context of a chronic HFD, the promotion of fat deposition in scWAT, which is known to be reduced by aging, could be a beneficial effect to buffer the fat excess and prevent its ectopic accumulation in other metabolic organs like the liver, as we have recently reported in these animals[492]. Altogether, our data suggest an increased fatty acid traffic towards higher rates of fatty acid oxidation in scWAT adipocytes, which has been described previously in young DIO rodents after exercise training programs[128].

Similar to the effects observed on fatty acid/triglyceride metabolism, exercise was able to induce a decrease in scWAT inflammation mediated by downregulation of some proinflammatory cytokines genes (*Tnf*, *Il6)* and upregulation of the anti-inflammatory ones (*Adipoq*, *Il4*). Moreover, exercise induced a significant decrease in total macrophages content ($F4/80^+/CD11b^+$), in parallel with a reduced expression of both M1 (*Cd11c*) and M2 (*Cd206*) macrophages markers. These reductions may have accounted for the downregulation of

the classic aging and obesity proinflammatory signals (*Tnf*, *Il6).* It is worth mentioning that, in the context of the reduced inflammatory markers in scWAT after exercise, an increased infiltration of B lymphocytes was also observed. However, the actions of B lymphocytes are subset dependent (B1-antiinflammatory or B2-proinflammatory), and the subset abundance in scWAT has not been well characterized yet[493]. Therefore, further research about the effects of exercise on B lymphocytes-mediated antiinflammatory actions on adipose tissue is needed to clarify their role also in the aged and obese WAT.

Regarding the effects of exercise on fatty acid pathways and on the inflammatory status of iBAT, only *Il4* transcript was significantly changed after the chronic exercise program, coinciding with the elevation of *Il4* in scWAT. Interestingly, IL-4 signaling has been described to play a major role in development of functional beige fat[124]. Indeed, genetic loss of IL-4/13 signaling impairs biogenesis of beige fat in response to cold. On the contrary, administration of IL-4 increases beige fat mass and thermogenic capacity in obese mice[124]. Therefore, the upregulation observed on *Il4* could be also underlying the browning properties of long-term exercise both in scWAT and iBAT. Indeed, the trained DIO mice showed an upregulation of beige adipocytes-specific genes in scWAT (*Cd137*, *Tbx1*), together with an increase in genes/proteins involved in mitochondrial biogenesis and thermogenesis (*Pgc1a*, *Nrf1*, *Tfam*, *Ucp1*). However, *Prdm16* was not enhanced, but decreased, in scWAT after exercise training. iBAT also showed an increased expression of *Pgc1a* and UCP1, together with an upregulation of *Prdm16*, although *Nrf1* and *Tfam* levels were unchanged. This differential effects of exercise may be due to the fact that PRDM16 expression is higher in brown compared to white/beige adipocytes, as it is in charge of development and maintenance of brown fat cell identity[494,495]. Therefore, PRDM16 upregulation promotes iBAT recruitment and prevents the loss of brown fat characteristics that occurs during obesity and aging, as recently reported by our group[496]. The changes observed in scWAT were similar to those reported by Lehnig *et al*.[349], revealing higher mitochondrial function and thermogenic genes together with unaltered levels of *Prdm16*. However, in contrast to our study, they did not find any changes on iBAT UCP1 or *Prdm16* expression.

Importantly, the actions of exercise on scWAT and iBAT were accompanied by a dramatically depot-differential regulation of the *Fgf21* signaling pathway. Indeed, while apparently the signal was unaltered in scWAT (*Fgf21*, *Fgfr1*, *β-klotho*) its metabolic downstream signal, *Egr1*, was increased by the exercise program. Conversely, *Fgf21* mRNA levels were upregulated in iBAT, but its receptor components were unaltered, and *Egr1* was downregulated. The increase in *Egr1* in scWAT might suggest an increased sensibility to FGF21, which has been proposed for the exercise-induced metabolic beneficial effects in the different adipose tissues[497]. On the other hand, the increase in *Fgf21* levels in iBAT could be associated with an increase in its secretion, as demonstrated previously in animal models of obesity after different stimuli[265]. Therefore, a potential cross talk between both adipose tissues in response to exercise should not be discarded. Nevertheless, to our knowledge there are no studies analyzing the changes in EGR1 in response to exercise in WAT or iBAT. Hence, studies are needed to characterize the role of FGF21 signaling, and specifically of EGR1 in iBAT function, in response to exercise.

In summary, our data reinforce the recent results reported by previous studies observing differential effects for exercise in the distinct adipose tissue locations[349]. In the current study, long-term exercise promoted a beneficial phenotype in scWAT of aged obese mice, characterized by the modulation of pro and antiinflammatory signaling genes towards an antiinflammatory profile, a reduction in macrophage infiltration to scWAT, and the upregulation of genes involved in fatty acid oxidation, mitochondrial biogenesis, and adipose tissue beiging. Although the upregulation of thermogenic genes and protein markers was also

observed in iBAT, this depot seemed to be less responsive to exercise than scWAT in aged DIO animals. Altogether, our data indicate that these adaptations may contribute to the improvements observed in glucose tolerance and insulin resistance in aged obese animals, as previously observed in young DIO animals[130].

Physical exercise and DHA supplementation also have differential health benefits in overweight/obese postmenopausal women

Finally, we studied if the responses observed in aged animals to the long-term moderate exercise or the DHA dietary supplementation at the systemic and adipose tissue level could be translated to health benefits also in postmenopausal women with overweight and/or obesity.

The analysis of the mRNA levels of pro and anti-inflammatory genes in the subcutaneous adipose tissue (SAT) biopsies showed that both interventions (DHA supplementation and resistance training-RT) had lowering effects on *LEPTIN*, *CHEMERIN*, *CD11c* and *IL6* compared to the placebo group. However, this effect was not observed in the group combining both interventions, which exhibited changes that were similar or lower to that displayed by the DHA or the placebo supplemented + exercised group.

On the other hand, the RT program was able to induce an increase in the expression of the antiinflammatory *ADIPONECTIN* in SAT biopsies of the trained volunteers, regardless of placebo or DHA-rich *n*-3 PUFA supplementation. This increase may have contributed to the improvement of glucose tolerance induced by the RT program, due to the close association between adiponectin levels with glucose homeostasis and insulin sensitivity in lean and obese individuals[475]. However, it is important to note that serum adiponectin levels did not increase significantly in the trained participants, suggesting that other subcutaneous or visceral fat depots that also contribute to its circulating levels could be differentially affected by the RT program. In this regard, differential correlations have been described between circulating adiponectin and subcutaneous adipose tissue (positive association) and visceral adipose tissue (neutral association) in humans[498].

Indeed, our data suggest that the significant changes observed the expression of adipocytokines in adipose tissue by RT or DHA could not be translated to similar significant changes in their circulatory levels (leptin, chemerin, adiponectin). This local effect exerted in SAT biopsies but not extended to systemic effects could be due to the need of a more prolonged treatment to observe changes in circulating levels of adipokines, or to the fact that adipokines interact with several metabolic organs in a complex crosstalk that we cannot rule out with the present data. However, the study of blood cell counts revealed antiinflammatory effects exerted by the DHA-rich *n*-3 PUFA supplement, regardless of sedentarism or RT, both in lymphocytes count and in the PLR index. These results are of high clinical interest, as both parameters can be obtained in routine hemograms. With this in mind, the PLR index was recently described as an inflammatory index with the ability to predict chronic disease in aging subjects[450].

Finally, the metabolic effects of RT included an improved glucose tolerance, as mentioned above. This was observed in the glucose AUC resulting from the OGTT glucose excursion curve. However, no improvements were observed in fasting glucose or insulin levels, nor in insulin resistance indexes or in the blood lipid profile (*Chapter 4*). It is important to recall that the *n*-3+RT group displayed a high variability that may have blunted the statistical significance for these data, as most of these parameters were also significantly improved in this group. Moreover, the high variability of RT cardiometabolic effects has been recently reported in a large population study collecting biochemical data resulting from the same RT protocol, which was conducted

across several population groups[499]. Furthermore, the lack of significant effects in whole-body fat mass may have also impeded the decrease in the associated proatherogenic profile. Nevertheless, our data suggest that both increments in muscle quality and strength, as well as the adipose tissue remodeling, may have contributed to the improvements induced by the RT program on glucose tolerance.

One of the main results expected to occur after the RT program was the improve in body composition, especially with an increase in lean mass[390]. As discussed in *Chapter 4*, current investigations about RT effects in middle-aged men and women are trying to unravel the best RT approach to induce a decrease in fat mass and an increase in lean/muscle mass. With this regard, research has been conducted to elucidate the training frequency[500,501] and volume[502], with inconclusive results. On the other hand, it has to be noted that most of the RT programs exerting beneficial effects on muscle mass and fat mass in postmenopausal women were much more prolonged or intense than ours[414]. However, the intensity and duration of an RT program are also crucial determinators of the stress markers levels, which are likely to increase in previously untrained obese subjects[503]. Although the controversy of the so called non-responders to exercise was terminated a while ago[504], these stress markers can halt the beneficial effects of exercise, increasing inflammation and fat deposition[503], as well as incrementing the likelihood to induce low adherence rates in overweight/obese women[505].

Indeed, similar BMI and fat losses were observed in the four experimental groups. It has to be noted that several well conducted investigations have established that, just by participating in a lifestyle intervention, subjects improve their behavior only by allocation to a group[506]. In fact, the four groups revealed a certain increase in physical activity and in the adherence to Mediterranean Diet after the intervention (*Chapter 4*), despite there were no significant differences between their values. The lack of effects at whole body composition after RT resulted, however, in upper body muscle mass increments, that occurred together with muscle strength and quality gains. Moreover, these local effects were also observed in lower limbs, including a decrease in fat mass and higher strength and muscle quality. Arms muscle mass gains and legs fat mass losses are expected after hypertrophic RT due to the limbs' intrinsic anatomy and function, which leads to greater responses in smaller and weaker muscle groups[507,508]. Moreover, since the training program did not include abdominal exercises and consequently, no changes were observed in trunk segmented composition, we can conclude that the program was effective in the studied population. In summary, these data, together with the finding that the RT maintained bone mineral content (BMC) compared to untrained groups, highlight the beneficial effects of this training approach in the postmenopausal population with overweight or obesity.

Regarding the DHA-enriched *n*-3 PUFA supplement, however, the main effects were coherent with the reduction observed in metabolic inflammation *via* reducing CRP. Indeed, the DHA-rich *n*-3 PUFA supplement did not induce any change on body composition beyond a lower decrease in arms fat mass compared to placebo supplemented groups. These data might reflect an increased triglyceride deposition in muscle after an *n*-3 PUFA supplement, since intramuscular triglyceride synthesis has been related to insulin sensitivity in obese subjects[509]. Because the effect was smaller in the *n*-3+RT group, this might reflect an increase in muscle triglyceride utilization during exercise[510], which has been already shown in exercised obese subjects even in the absence of changes in intramuscular concentrations. Nevertheless, the DHA-rich supplement was able to decrease two main markers of metabolic inflammation and metabolic syndrome, diastolic blood pressure and circulating triglycerides. These data support the DHA beneficial effects on blood pressure and circulating inflammatory factors, as observed in the lowering effect of CRP, both observed previously in several trials and stated by a recent meta-analysis performed by Guo *et al*.[314]. Similarly, the lowering

triglyceride effect is in accordance to the recommendations stated by the AHA, regarding the preventive use of *n*-3 PUFA in normo and hypertriglyceridemic women[511].

To finish, a remarkable result revealed by the RT program in postmenopausal women was, as mentioned above, the maintenance of bone mineral content (BMC). The relevance of this results is due to the fact that BMC is as a marker of fracture risk[512]. In fact, recent studies for the effects of resistance training in postmenopausal women revealed local increments in femoral BMC that occurred together with an increase in bone thickness[513]. In turn, bone thickness is strongly related to an increment in the femoral neck fracture load[514], which is defined as the load which is great enough to break the bone, and thus could be understood as bone resistance to fracture. Furthermore, BMC has even been proposed for the clinical diagnose of osteoporosis besides bone mineral density (BMD)[515], being the first a proxy of bone geometry and the latter of bone quality, both relevant for the maintenance of a healthy bone structure.

To summarize, RT and DHA-supplementation resulted in some distinct effects in adipose tissue, and in differential outcomes at the systemic level. These effects can be summarized as RT having greater systemic impact in glucose metabolism and body composition, with DHA lowering metabolic inflammation *via* PLR inflammatory index, blood lymphocytes and triglycerides levels, as well as diastolic blood pressure. Importantly, the combination of both treatments did not have any synergistic effect and, in some parameters, resulted in negative interactions that led to no effects in the group combining both treatments as compared to the DHA-supplemented group and to the placebo supplemented, trained group.

Summary, limitations, and future perspectives

In summary, the current investigation in rodents demonstrates the relevance of replacing saturated fat in the diet by *n*-3 PUFA, especially DHA. Indeed, the increased content of dietary *n*-3 PUFA, even in the context of a HFD maintained throughout life, induced a healthier phenotype in scWAT and iBAT in aged obese female mice. Since our data reveal that the induction of this phenotype was accompanied by a dramatic increase in *n*-3 PUFA derived SPMs in iBAT, it is likely that the results observed in scWAT are also partially mediated by these *n*-3 PUFA-derived lipid mediators that effectively resolve inflammation.

On the other hand, our research in mice also reveals the beneficial effects of maintaining physical activity levels during the adulthood and middle age by performing moderate aerobic exercise training, which is apparently more impactful on scWAT than in iBAT, also in the context of a sustained HFD consumption. Importantly, the local effects exerted by chronic feeding with the DHA-enriched HFD and the long-term moderate exercise training program on the different adipose tissues, may contribute to the actions observed at the systemic level. These effects include a less proatherogenic lipid profile and an improve in glucose tolerance and amelioration of insulin resistance, respectively. In general, our data provide evidence on the significance of replacing dietary saturated fat with high-quality *n*-3 PUFA, and of performing moderate exercise training, to achieve a healthy aging, even in the background of a HFD feeding maintained from youth to the old age.

Moreover, the results of the clinical trial conducted in postmenopausal women with overweight/obesity corroborate the potential of DHA-rich *n*-3 PUFA supplementation and RT to induce a remodeling in subcutaneous abdominal adipose tissue with concomitant systemic improvements in inflammatory and metabolic biomarkers, glucose tolerance and muscle quality/strength. In the human study, these results can

be observed in the context of a previously existing overweight/obesity, and in parallel to a nutritional advice for a healthy diet. Furthermore, since our study was designed with parallel groups allocated either to DHA/Placebo supplementation together with RT/sedentarism, we can conclude that the combination of both interventions does not lead to any relevant synergistic effect in postmenopausal women with overweight/obesity.

Hence, these actions on overweight/obese postmenopausal women are partially supported by the actions observed in aged female mice with diet-induced obesity. However, it must be recalled that the designs of the animal study and the clinical trial have several differences that limit the possibility of making direct comparison between the outcomes observed, in addition to the well-established limitations of comparing studies in mice to those in humans. To begin with, female mice do not experience a similar menopause that women[516], and thus do not undergo the same dramatic hormonal changes described in menopausal women, which limits the translation of results from aged female mice to postmenopausal women. Moreover, the experimental consumption of a DHA-enriched HFD, as well as the aerobic exercise training interventions in female mice, were conducted during 12 months, which represents a period of almost half-life of a mice, from the adulthood (6 months) to the early old age (18 months)[468]. By contrast, the clinical trial in overweight/obese postmenopausal women was conducted for a period of 16 weeks.

On the other hand, the exercise programs conducted were also different, since women underwent a RT protocol and mice performed a treadmill running program (aerobic exercise). The most popular RT protocol in mice consists of adding weight to wheels in order to create resistance[517]. However, this training approach is, in turn, more similar to a combined aerobic+RT. Moreover, the rest of the available RT protocols in mice comprise treadmill running, but adding a dramatic slope (from 80 to 90º angulation)[517] which would entail huge difficulty for obese mice, even more during aging. Therefore, and since animals are less likely to voluntary exercise in the late adulthood[518], we decided to conduct a treadmill running program.

Nevertheless, the studies in animals and the clinical trial revealed some interesting similarities regarding the results observed in both models. Indeed, both humans and animals experimented an improvement in glucose tolerance with exercise and an improved lipid profile after the DHA-rich *n*-3 PUFA supplementation. At the adipose tissue level, the lowering effect of both exercise training and DHA supplementation on the inflammatory status was observed in macrophage markers and on adipokines in both aged obese female mice and postmenopausal women.

However, more studies would be necessary in other to establish if the principal results observed in the animal and human studies were similar in both models. First, it would be of high relevance to confirm that the DHA-enriched HFD also led to an increase in the *n*-3-derived SPMs in the rest of the adipose tissues, or at least, in the main ones (scWAT and vWAT) in aged obese female mice. In turn, the ability of *n*-3 PUFA (DHA and EPA) to induce an increment in the abundance of several SPMs in the different WAT depots (scWAT, gonadal WAT) has been demonstrated, either when given with a chow or a HFD*,* but only in young and adult rodents[227,271,519]. Similarly, it would be highly significant to study the changes in the SPMs profile both in plasma and adipose tissue, from the volunteers supplemented with the DHA-rich fish oil concentrate. In fact, it has already reported that only 1 g/day of DHA plus 1 g/day EPA leads to increases in plasma RvD1,RvD2, 17S-DHA, 17R-RvD1, PD1 and PDX, that positively correlate with plasma DHA in healthy subjects[520]. Since increments in serum SPMs have been described after *n*-3 PUFA supplementation in obese middle-aged women[521] and middle-aged subjects with metabolic syndrome[522], such increments could be expected in our study population.

Moreover, as dose-dependent increments in *n*-3 PUFA derived oxylipins, including SPMs precursors, have been described after *n*-3 PUFA supplementation[288], another relevant issue is to confirm the threshold *n*-3 PUFA consumption for achieving an increment in plasma SPMs. This threshold was recently proposed by Calder *et al*.[285], and could be determinant for establishing the needed *n*-3 PUFA status for a healthy aging.

Finally, a lipidomic study of the SPMs signatures of the overweight/obese SAT biopsies of the volunteers would complete this study. Since increments in SPMs have been described after *n*-3 PUFA supplementation in severely obese diabetic subjects[286], and the incorporation of DHA and EPA to adipose tissue of supplemented subjects has been also described to decrease with age[523]

On the other hand, it would be interesting to characterize the effects of long-term DHA supplementation and exercise training on vWAT in the animal model of obesity and aging, as these effects could be also contributing to the improvements observed in lipid and glucose metabolism, respectively induced by each treatment. Moreover, in order to understand the mechanisms involved in the systemic effects, the contribution of the other main metabolic organs (muscle, liver) is currently being studied in our research group. Hopefully, the results will help to better unravel the contributions of the different adipose tissue depots and other tissues (interorgan crosstalk) to the actions observed at the systemic level.

Unfortunately, conducting such studies in human clinical trials is highly complex due to the difficulty of obtaining biopsies of vWAT and BAT before and after nutritional or exercise interventions. In order to overcome this limitation, PET/CT studies have been used to estimate BAT activity also in humans[289] . Thus, it would be of significant interest to characterize if the response to DHA supplementation and/or to the RT program could be different in BAT positive and BAT negative subjects in response to cold exposure. In this sense, it would be also of notable significance to investigate the changes in plasma SPMs after a cold challenge, and to study whether the DHA-rich supplement or the exercise program could promote the secretion of SPMs by brown fat. This experimental approach has been previously demonstrated for some oxylipins in humans, that are secreted by BAT after cold stimuli[289].

The interesting outcomes of our study in postmenopausal women with overweight/obesity highlight the relevance of performing larger trials with longer intervention periods and a higher number of participants to better establish the more efficient and safe doses of n-3 PUFA, as well as the more appropriate exercise program, in order to reduce overweight/obesity, muscle and bone frailty, inflammation, and metabolic disorders. These studies would also help to better characterize the mechanisms underlying these processes.

Finally, considering the evidence of sex-differential responses in adipose tissue and metabolic health biomarkers, both in rodents and humans[524–526], it would be necessary to perform similar preclinical and clinical studies including both males and females. This would help to better characterize what intervention is best for each type of patient, in order to continue developing personalized treatments and achieve a healthy aging even in conditions of overweight and obesity.

Conclusions

1. Chronic DHA supplementation induced a healthier phenotype in subcutaneous white adipose tissue of aged obese female mice, characterized by: i) a reduction in adipocytes' size and a downregulation of main lipogenic genes' expression (*Dgat1, Fasn, Scd1*); ii) the reduction of the local inflammatory status, revealed by the decreased expression of proinflammatory genes (*Tlr4, Ccl2; Lep*) and M1 macrophage markers (*Cd11c*), together with the increase in M2 macrophage levels (CD206), and iii) the increment in mRNA levels of beige adipocytes gene markers (*Cd137, Tbx1, Tmem26*), as well as the prevention of the drop in the thermogenic protein UCP1 levels observed in diet-induced obese mice.

2. In brown adipose tissue, chronic high-fat feeding-induced obesity aggravated the inflammation and loss of function and activity that occurred during aging in female mice. This was revealed by the downregulation in the expression of master regulators of brown adipose tissue development and function (*Prdm16* and UCP1), and by the upregulation of *Ccl2*, a proinflammatory chemokine. Chronic DHA supplementation, in the context of a high-fat diet, partially restored these alterations on inflammatory and thermogenic regulators, even without major morphological changes. Altogether, the effects induced by the long-term DHA-enriched diet on subcutaneous white and in brown adipose tissues may have contributed to the improvements observed in total and LDL-cholesterol, and in the atherogenic indexes.

3. Lipidomic analyses of brown adipose tissue revealed that arachidonic acid-derived lipoxins and DHA-derived maresins and protectins are the most abundant specialized proresolving lipid mediators (SPMs) in brown fat of young control female mice. Obese aged mice exhibited significant reductions in the sum of lipoxins and of protectins. The most significantly reduced lipid mediators included Lipoxin B4, Maresin 2, 4S,14S-diHDHA, Protectin DX and Resolvin D6. In contrast, long-term feeding with a DHA-enriched diet increased the levels of most of the *n*-3 DPA, DHA, and EPA-derived SPMs, without restoring those derived from arachidonic acid.

4. The gene expression of *Prdm16* in brown adipose tissue positively correlated with the sum of lipoxins and with 4S,14S-diHDHA, a pathway marker of DHA-derived maresins. By contrast, the expression of *Ccl2* negatively correlated with the sum of SPMs and with the sums of the different resolvins and protectins classes. These data support a role for specific SPMs in regulating brown adipose tissue inflammation and function. However, chronic DHA supplementation was not able to reverse the impaired cold response of brown adipose tissue observed in obese aged mice. This suggests that obesity and aging impaired other mechanisms involved in cold-induced brown fat activation, that DHA supplementation could not restore.

5. Long-term, mild intensity exercise training (treadmill running) initiated in the late adulthood of obese mice induced significant metabolic adaptations in subcutaneous white adipose tissue. These included an increased expression of fatty acid oxidation genes (*Cpt1a, Acox1*) and the amelioration of inflammation, with a favorable modulation of pro/antiinflammatory genes (*Tnf* and *Il6* decrease, *Adipoq* and *Il4* increase) and lower total macrophages infiltration. Exercise also upregulated the mRNA levels of genes involved in mitochondrial biogenesis and thermogenesis (*Pgc1a, Tfam, Nrf1, Ucp1*), as well as of beige adipocytes selective genes (*Cd137, Tbx1*) in subcutaneous white fat. In contrast, brown adipose tissue of the obese aged mice was less responsive to exercise, revealing few changes on inflammation and fatty acid metabolism genes. However, the expression of functional brown adipocytes genes/proteins (*Pgc1a, Prdm16* and UCP1) was augmented in brown fat. At the systemic level, long-term treadmill running improved the HOMA-IR index and glucose tolerance. Altogether, these data suggest that the

long-term exercise-induced adaptations on subcutaneous white and brown adipose tissue of obese aged mice could contribute to its beneficial actions on glucose homeostasis.

6. In healthy postmenopausal women with overweight/obesity, a 16-week intervention with a DHA-rich fish oil-derived supplement or placebo (olive oil), alone or in combination with a progressive resistance training program caused similar moderate reductions in body weight and fat mass in the 4 intervention groups. However, the trained groups maintained the bone mineral content, increased upper limbs lean mass, and decreased lower limbs fat mass, together with an augmented muscle strength and quality compared to untrained groups. These effects occurred alongside an improved glucose tolerance in trained participants compared to the untrained ones. Regarding inflammatory parameters, the resistance training program also upregulated the expression of *ADIPONECTIN* in subcutaneous abdominal adipose tissue biopsies compared to the untrained groups. In placebo supplemented subjects, the resistance training program also downregulated the mRNA levels of *LEPTIN, CHEMERIN, IL6* and *CD11c* in subcutaneous fat biopsies, as well as decreased CRP circulating levels, in comparison to the untrained subjects.

7. In postmenopausal women with overweight/obesity, the DHA-rich supplement lowered markers of cardiovascular disease and metabolic inflammation, including diastolic blood pressure, circulating triglycerides levels, and the inflammatory platelet-to-lymphocyte ratio. The DHA-rich supplement also increased muscle quality in lower limbs. In untrained subjects, DHA supplementation also downregulated the expression of *LEPTIN, CHEMERIN, IL6* and *CD11c* in subcutaneous fat biopsies, as well as decreased CRP circulating levels, in comparison with the placebo supplemented subjects.

8. These data suggest the recommendation of resistance training programs and *n*-3 PUFA consumption to improve muscle strength and/or quality, as well as to reduce systemic and local adipose tissue inflammation, and cardiometabolic health in postmenopausal women with overweight/obesity. The combination of the DHA-rich supplement and the resistance training program does not seem to have any synergistic beneficial effects in this population.

General conclusion

In summary, this investigation demonstrates the relevance of partially replacing the saturated fat of a high-fat diet with *n*-3 PUFA, especially DHA. The increase in dietary *n*-3 PUFA, even in the context of a high-fat feeding maintained from youth (2 months old) to the old age (18 months old), induced healthier phenotypes in the subcutaneous white and interscapular brown fat depots of aged obese female mice. Since the induction of these phenotypes was accompanied by a dramatic increment in *n*-3 PUFA-derived specialized proresolving lipid mediators (SPMs) in the brown fat depot, it is likely that the results observed in the white fat depot could be also partially mediated by an augmented SPMs production.

On the other hand, our research in mice also reveals the beneficial effects of a continuous exercise training from the adulthood to the old age in aged obese female mice, by performing moderate aerobic exercise training, even in the context of a sustained high-fat feeding. However, exercise seems to have more impact on the subcutaneous white than the interscapular brown adipose depot, in terms of its ability to acquire a more antiinflammatory, fatty acid oxidative, and thermogenic gene and/or protein expression profile.

The local effects exerted by both interventions on the subcutaneous white and interscapular brown fat depots may contribute to the beneficial actions on lipid and glucose metabolism biomarkers observed at the systemic level. In general, our data suggest the significance of promoting dietary *n*-3 PUFA consumption and of performing moderate aerobic exercise training, to achieve a healthy aging.

Moreover, the results of the clinical trial conducted in postmenopausal women with overweight or obesity suggest the recommendation of performing resistance exercise training and to promote DHA-rich *n*-3 PUFA supplementation to this population. On the one hand, both interventions would entail effective strategies to reduce inflammatory markers at the adipose and systemic levels. On the other hand and specifically, DHA supplementation would induce an improvement in cardiometabolic biomarkers (diastolic blood pressure, triglycerides), while resistance exercise would induce local increments in muscle strength, quality and mass, and in fat mass losses, with an improved glucose tolerance. The results of the clinical trial allow us to conclude that the combination of both interventions does not lead to any relevant synergistic beneficial effect on the parameters analyzed in postmenopausal women with overweight or obesity

1. La suplementación crónica con DHA indujo un fenotipo más saludable en el tejido adiposo blanco subcutáneo de ratones hembra obesos envejecidos, caracterizado por: i) una reducción del tamaño de los adipocitos y una menor expresión de los principales genes lipogénicos (*Dgat1, Fasn, Scd1*), ii) la reducción del estado inflamatorio local, revelado por la expresión disminuida de genes proinflamatorios (*Tlr4, Ccl2, Lep*) y de marcadores de macrófagos M1 (*Cd11c*), junto con el aumento en los niveles de macrófagos M2 (CD206), y iii) el aumento en los niveles de ARNm de genes marcadores de adipocitos beige (*Cd137, Txb1, Tmem26*), así como la prevención en la caída de los niveles de la proteína termogénica UCP1 observada en ratones obesos envejecidos.

2. En el tejido adiposo pardo, la obesidad inducida por una alimentación crónica alta en grasa agravó la inflamación y la pérdida de función y actividad que ocurre durante el envejecimiento en ratones hembra. Esto se puso de manifiesto por la expresión reducida de reguladores clave en el desarrollo y función del tejido adiposo pardo (*Prdm16* y UCP1) y en el incremento de *Ccl2*, una quimiocina proinflamatoria. La suplementación crónica con DHA, en el contexto de una dieta rica en grasas, restauró parcialmente estas alteraciones en reguladores inflamatorios y termogénicos, incluso sin cambios morfológicos destacables. En conjunto, los efectos inducidos por la dieta enriquecida en DHA en el tejido adiposo blanco y pardo de ratones obesos envejecidos podrían contribuir a sus acciones beneficiosas sobre las mejoras observadas en los niveles de colesterol total, colesterol-LDL, e índices aterogénicos.

3. El análisis lipidómico del tejido adiposo pardo, reveló que las lipoxinas derivadas del ácido araquidónico, y las maresinas y protectinas derivadas del DHA, son los mediadores lipídicos especializados proresolutivos (SPMs) de inflamación más abundantes en la grasa parda de ratones hembra jóvenes. Los ratones envejecidos obesos mostraron reducciones significativas en la suma de lipoxinas y de protectinas. Los mediadores lipídicos más significativamente reducidos fueron la Lipoxina B_4, Maresina 2, 4S,14S-diHDHA, Protectina DX y Resolvina D6. Por el contrario, la alimentación a largo plazo con la dieta enriquecida en DHA aumentó los niveles de la mayoría de los SPMs derivados del omega-3 DPA, del DHA y del EPA, sin restaurar los SPMs derivados del AA.

4. La expresión del gen *Prdm16* se correlacionó positivamente con la suma de lipoxinas, así como con el 4S,14S-diHDHA, marcador de la vía de las maresinas derivadas de DHA. Por el contrario, la expresión de *Ccl2* se correlacionó negativamente con la suma de SPMs y con la suma de todas las clases de resolvinas y protectinas. Estos datos apoyan un papel de SPMs específicos en la regulación de la función e inflamación del tejido adiposo pardo. Sin embargo, la suplementación crónica con DHA no pudo revertir la respuesta deteriorada al frío del tejido adiposo pardo observada en ratones obesos envejecidos. Esto sugiere que la obesidad y el envejecimiento agravaron otros mecanismos involucrados en la activación de la grasa parda inducida por el frío, que la suplementación con DHA no puedo restaurar.

5. El entrenamiento de intensidad moderada a largo plazo (carrera en cinta rodante) iniciado en la edad adulta tardía de ratones obesos indujo adaptaciones metabólicas significativas en el tejido adiposo subcutáneo. Estas incluyeron el aumento en la expresión de genes de oxidación de ácidos grasos (*Cpt1a*, *Acox1*) y la mejora de la inflamación, con una modulación favorable de genes pro/antiinflamatorios (*Tnf* e *Il6* disminuidos*, Adipoq* e *Il4* aumentados) y una infiltración menor de macrófagos totales. El ejercicio también estimuló los niveles de mRNA de genes involucrados en la biogénesis mitocondrial y termogénesis (*Pgc1a, Tfam, Nrf1, Ucp1*) así como de genes selectivos de los

adipocitos beige (*Cd137, Tbx1*) en la grasa blanca subcutánea. Por el contrario, la grasa parda de los ratones obesos envejecidos respondió menos al ejercicio, revelando pocos cambios en los genes relacionados con la inflamación y el metabolismo de los ácidos grasos. Sin embargo, la expresión de genes/proteínas funcionales de adipocitos pardos (*Pgc1a, Prdm16* y UCP1) estaban aumentados en la grasa parda. A nivel sistémico, el entrenamiento a largo plazo mejoró el índice HOMA-IR y la tolerancia a la glucosa. En conjunto, estos datos sugieren que los efectos inducidos por el entrenamiento a largo plazo en el tejido adiposo blanco y pardo de ratones obesos envejecidos podrían contribuir a sus acciones beneficiosas sobre la homeostasis de la glucosa.

6. En mujeres postmenopáusicas sanas con sobrepeso/obesidad, una intervención de 16 semanas con un suplemento rico en DHA derivado del aceite de pescado o placebo (aceite de oliva), solo o en combinación con un programa de entrenamiento de fuerza progresivo, provocó una reducción moderada en el peso y la grasa corporal que fue similar en los 4 grupos de estudio. Sin embargo, los grupos entrenados mantuvieron el contenido mineral óseo, aumentaron la masa magra de las extremidades superiores, y disminuyeron la masa grasa de las extremidades inferiores, junto con un aumento de la fuerza y la calidad muscular, en comparación con los grupos no entrenados. Estos efectos ocurrieron junto con una mejora de la tolerancia a la glucosa en los grupos entrenados comparados con los no entrenados. Con respecto a los parámetros inflamatorios, el programa de entrenamiento de fuerza también incrementó la expresión de *ADIPONECTINA* en biopsias de tejido adiposo abdominal subcutáneo en comparación con los grupos no entrenados. En sujetos suplementados con placebo, el programa de entrenamiento de fuerza también redujo los niveles de ARNm de *LEPTINA*, *QUEMERINA*, *IL6* y *CD11c* en biopsias de grasa subcutánea, así como también redujo los niveles circulantes de proteína C reactiva, en comparación con los sujetos no entrenados.

7. En mujeres posmenopáusicas con sobrepeso/obesidad, el suplemento rico en DHA redujo los marcadores de enfermedad cardiovascular e inflamación metabólica, incluyendo la presión arterial diastólica, los niveles de triglicéridos circulantes y el ratio inflamatorio de plaquetas-linfocitos. El suplemento rico en DHA también aumentó la calidad muscular en las extremidades inferiores. En sujetos no entrenados, la suplementación con DHA también redujo la expresión de *LEPTINA*, *QUEMERINA*, *IL6* y *CD11c* en biopsias de grasa subcutánea, así como disminuyó los niveles circulantes de Proteína C-reactiva, en comparación con los sujetos suplementados con placebo.

8. Estos datos sugieren la recomendación de programas de entrenamiento de fuerza y el consumo de AGPI *n*-3 para mejorar la fuerza y/o la calidad muscular, así como para reducir la inflamación sistémica y local del tejido adiposo y la salud cardiometabólica en mujeres posmenopáusicas con sobrepeso/obesidad. La combinación del suplemento rico en DHA y el programa de fuerza parecen no tener ningún efecto beneficioso sinérgico en esta población.

Conclusión general

En resumen, esta investigación demuestra la relevancia de reemplazar parcialmente la grasa saturada de una dieta alta en grasa por AGPI *n*-3, especialmente por DHA. El aumento del contenido en AGPI *n*-3 de la dieta, incluso en el contexto de una alimentación alta en grasa mantenida desde la juventud (2 meses) hasta la vejez (18 meses), indujo fenotipos más saludables en los depósitos de grasa blanca subcutánea y parda

interescapular de ratones hembra obesos envejecidos. Dado que la inducción de estos fenotipos estuvo acompañada de un incremento dramático en los mediadores lipídicos especializados pro-resolutivos de inflamación (SPMs) derivados de AGPI *n*-3 en la grasa parda, es probable que los resultados observados en la grasa blanca también estén parcialmente mediados por una producción aumentada de SPMs.

Por otro lado, nuestra investigación también revela los efectos beneficiosos de un entrenamiento físico continuado desde la edad adulta hasta la vejez en ratones hembra obesos envejecidos, mediante la realización de un entrenamiento de ejercicio aeróbico moderado, incluso en el contexto de una alimentación alta en grasa mantenida a lo largo de la vida. Sin embargo, en este caso el ejercicio parece tener más impacto en el depósito blanco subcutáneo que en el pardo interescapular, en términos de su capacidad de adquirir un perfil de expresión génica y/o proteica más antiinflamatorio, oxidativo de ácidos grasos, y termogénico.

Los efectos locales ejercidos por ambas intervenciones en los depósitos de grasa blanca subcutánea y parda interescapular pueden contribuir a las acciones beneficiosas observadas a nivel sistémico en biomarcadores del metabolismo de lípidos y de glucosa. En general, nuestros datos sugieren la importancia de promover el consumo de AGPI *n*-3 y de realizar un ejercicio aeróbico moderado para lograr un envejecimiento saludable.

Además, los resultados del ensayo clínico realizado en mujeres posmenopáusicas con sobrepeso u obesidad sugieren la recomendación de realizar ejercicio de fuerza y promover la suplementación con AGPI *n*-3 ricos en DHA en esta población. Por un lado, ambas intervenciones supondrían estrategias efectivas para reducir los marcadores inflamatorios a nivel adiposo y sistémico. Por otra parte y de forma específica, la suplementación con DHA induciría una mejora en biomarcadores cardio-metabólicos (presión arterial diastólica, triglicéridos), mientras que el ejercicio de fuerza induciría aumentos locales en la fuerza, calidad y masa muscular, en la pérdida de masa grasa y una mejora en la tolerancia a la glucosa. Los resultados del ensayo clínico permiten concluir que la combinación de ambas intervenciones no conlleva ningún efecto beneficioso sinérgico relevante sobre los parámetros analizados en mujeres posmenopáusicas con sobrepeso u obesidad.

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References

The numbered references in this section pertain to the following sections: introduction, hypothesis, material and methods, and discussion.

1. World Health Organization. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. 2000;894:i-xii, 1-253.
2. World Health Organization. Obesity and overweight. 2020. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight
3. Kelly T, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. *Int J Obes*. 2008;32:1431-1437. doi:10.1038/ijo.2008.102
4. Finkelstein EA, Khavjou OA, Thompson H, et al. Obesity and severe obesity forecasts through 2030. *Am J Prev Med*. 2012;42:563-570. doi:10.1016/j.amepre.2011.10.026
5. World Health Organization. Global Health Estimates. Published 2019. https://www.who.int/data/global-health-estimates
6. United Nations Department of Economic and Social Affairs. World population prospects 2019: highlights.
7. Franceschi C, Garagnani P, Parini P, Giuliani C, Santoro A. Inflammaging: a new immune–metabolic viewpoint for age-related diseases. *Nat Rev Endocrinol*. 2018;14:576-590. doi:10.1038/s41574-018-0059-4
8. World Health Organization. Ageing and health. Published 2018. https://www.who.int/news-room/fact-sheets/detail/ageing-and-health
9. Eurostat. Causes of death statistics - people over 65 . Accessed February 22, 2021. https://ec.europa.eu/eurostat/statistics-explained/index.php/Causes_of_death_statistics_-_people_over_65
10. Underlying Cause of Death, 1999-2019 Results Form. Accessed February 22, 2021. https://wonder.cdc.gov/controller/datarequest/D76;jsessionid=A0695A1A277E692C18FD8BADE28C
11. Frasca D, Blomberg BB, Paganelli R. Aging, obesity, and inflammatory age-related diseases. *Front Immunol*. 2017;8:1745. doi:10.3389/fimmu.2017.01745
12. Fulop T, Larbi A, Dupuis G, et al. Immunosenescence and inflamm-aging as two sides of the same coin: friends or foes? *Front Immunol*. 2018;8:1960. doi:10.3389/fimmu.2017.01960
13. Franceschi C, Bonafè M, Valensin S, et al. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci*. 2000;908:244-254. doi:10.1111/j.1749-6632.2000.tb06651.x
14. JafariNasabian P, Inglis JE, Reilly W, Kelly OJ, Ilich JZ. Aging human body: changes in bone, muscle and body fat with consequent changes in nutrient intake. *J Endocrinol*. 2017;234:R37-R51. doi:10.1530/JOE-16-0603
15. Romero-Corral A, Somers VK, Sierra-Johnson J, et al. Accuracy of body mass index in diagnosing obesity in the adult general population. *Int J Obes*. 2008;32:959-966. doi:10.1038/ijo.2008.11
16. Ambikairajah A, Walsh E, Tabatabaei-Jafari H, Cherbuin N. Fat mass changes during menopause: a metaanalysis. *Am J Obstet Gynecol*. 2019;221:393-409.E50. doi:10.1016/j.ajog.2019.04.023
17. Monteiro R, Teixeira D, Calhau C. Estrogen signaling in metabolic inflammation. *Mediators Inflamm*. 2014;2014:615917. doi:10.1155/2014/615917
18. Sipilä S, Törmäkangas T, Sillanpää E, et al. Muscle and bone mass in middle-aged women: role of menopausal status and physical activity. *J Cachexia Sarcopenia Muscle*. 2020;11:698-709. doi:10.1002/jcsm.12547
19. Polotsky HN, Polotsky AJ. Metabolic implications of menopause. *Semin Reprod Med*. 2010;28:426-434. doi:10.1055/s-0030-1262902.
20. Kalinkovich A, Livshits G. Sarcopenic obesity or obese sarcopenia: a cross talk between age-associated adipose tissue and skeletal muscle inflammation as a main mechanism of the pathogenesis. *Ageing Res Rev*. 2017;35:200-221. doi:10.1016/j.arr.2016.09.008
21. Straub RH. The complex role of estrogens in inflammation. *Endocr Rev*. 2007;28:521-574. doi:10.1210/er.2007-0001
22. Mauvais-Jarvis F. Epidemiology of gender differences in diabetes and obesity. *Adv Exp Med Biol*. 2017;1043:3-8. doi:10.1007/978-3-319-70178-3_1
23. Ostan R, Monti D, Gueresi P, Bussolotto M, Franceschi C, Baggio G. Gender, aging and longevity

in humans: an update of an intriguing/neglected scenario paving the way to a gender-specific medicine. *Clin Sci*. 2016;130:1711-1725. doi:10.1042/CS20160004

24. Anand SS, Islam S, Rosengren A, et al. Risk factors for myocardial infarction in women and men: insights from the INTERHEART study. *Eur Heart J*. 2008;29:932-940. doi:10.1093/eurheartj/ehn018

25. Pucci G, Alcidi R, Tap L, Battista F, Mattace-Raso F, Schillaci G. Sex- and gender-related prevalence, cardiovascular risk and therapeutic approach in metabolic syndrome: a review of the literature. *Pharmacol Res*. 2017;120:34-42. doi:10.1016/j.phrs.2017.03.008

26. Newson L. Menopause and cardiovascular disease. *Post Reprod Heal*. 2018;24:44-49. doi:10.1177/2053369117749675

27. Zhang X, Liu L, Song F, Song Y, Dai H. Ages at menarche and menopause, and mortality among postmenopausal women. *Maturitas*. 2019;130:50-56. doi:10.1016/j.maturitas.2019.10.009

28. Gold EB. The timing of the age at which natural menopause occurs. *Obstet Gynecol Clin North Am*. 2011;38:425-440. doi:10.1016/j.ogc.2011.05.002

29. Al-Safi ZA, Polotsky AJ. Obesity and menopause. *Best Pract Res Clin Obstet Gynaecol*. 2015;29:548-553. doi:10.1016/j.bpobgyn.2014.12.002

30. Pérez LM, Pareja-Galeano H, Sanchis-Gomar F, Emanuele E, Lucia A, Gálvez BG. 'Adipaging': ageing and obesity share biological hallmarks related to a dysfunctional adipose tissue. *J Physiol*. 2016;594:3187-3207. doi:10.1113/JP271691

31. Villarroya F, Cereijo R, Gavaldà-Navarro A, Villarroya J, Giralt M. Inflammation of brown/beige adipose tissues in obesity and metabolic disease. *J Intern Med*. 2018;284:492-504. doi:10.1111/joim.12803

32. Kuryłowicz A, Kózniewski K. Anti-inflammatory strategies targeting metaflammation in type 2 diabetes. *Molecules*. 2020;25:2224. doi:10.3390/molecules25092224

33. Rafeh R, Viveiros A, Oudit GY, El-Yazbi AF. Targeting perivascular and epicardial adipose tissue inflammation: therapeutic opportunities for cardiovascular disease. *Clin Sci*. 2020;134:827-851. doi:10.1042/CS20190227

34. Ikeda K, Maretich P, Kajimura S. The common and distinct features of brown and beige adipocytes. *Trends Endocrinol Metab*. 2018;29:191-200. doi:10.1016/j.tem.2018.01.001

35. Kwok KHM, Lam KSL, Xu A. Heterogeneity of white adipose tissue: molecular basis and clinical implications. *Exp Mol Med*. 2016;48:e215. doi:10.1038/emm.2016.5

36. Giralt M, Villarroya F. White, brown, beige/brite: different adipose cells for different functions? *Endocrinology*. 2013;154:2992-3000. doi:10.1210/en.2013-1403

37. Peirce V, Carobbio S, Vidal-Puig A. The different shades of fat. *Nature*. 2014;510:76-83. doi:10.1038/nature13477

38. Wu J, Boström P, Sparks LM, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell*. 2012;150:366-376. doi:10.1016/j.cell.2012.05.016.Beige

39. Bonet ML, Oliver P, Palou A. Pharmacological and nutritional agents promoting browning of white adipose tissue. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2013;1831:969-985. doi:10.1016/j.bbalip.2012.12.002

40. Cypess AM, Lehman S, Williams G, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med*. 2009;360:1509-1517. doi:10.1056/NEJMoa0810780.

41. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol - Endocrinol Metab*. 2007;293:444-452. doi:10.1152/ajpendo.00691.2006

42. Lee P, Werner C, Kebebew E, Celi F. Functional thermogenic beige adipogenesis is inducible in human neck fat. *Int J Obes*. 2013;38:170-176. doi:10.1038/ijo.2013.82

43. Poher AL, Altirriba J, Veyrat-Durebex C, Rohner-Jeanrenaud F. Brown adipose tissue activity as a target for the treatment of obesity/insulin resistance. *Front Physiol*. 2015;6:1-9. doi:10.3389/fphys.2015.00004

44. Thyagarajan B, Foster MT. Beiging of white adipose tissue as a therapeutic strategy for weight loss in humans. *Horm Mol Biol Clin Investig*. 2017;31:j/hmbci.2017.31.issue-2/hmbci-2017-0016/hmbci-2017. doi:10.1515/hmbci-2017-0016.

45. Rabiee A. Beige fat maintenance; toward a sustained metabolic health. *Front Endocrinol (Lausanne)*. 2020;11:634. doi:10.3389/fendo.2020.00634

46. Darcy J, Tseng YH. ComBATing aging—does increased brown adipose tissue activity confer longevity? *GeroScience*. 2019;41:285-296. doi:10.1007/s11357-019-00076-0

47. Wang W, Seale P. Control of brown and beige fat development. *Physiol Behav*. 2016;17:691-702. doi:10.1038/nrm.2016.96.

48. Rodríguez A, Ezquerro S, Méndez-Giménez L, Becerril S, Frühbeck G. Revisiting the adipocyte: a model for integration of cytokine signaling in the regulation of energy metabolism. *Am J Physiol - Endocrinol Metab*. 2015;309:E691-E714. doi:10.1152/ajpendo.00297.2015

49. Pellegrinelli V, Carobbio S, Vidal-Puig A. Adipose tissue plasticity: how fat depots respond differently to pathophysiological cues. *Diabetologia*. 2016;59:1075-1088. doi:10.1007/s00125-016-3933-4

50. Reilly SM, Saltiel AR. Adapting to obesity with adipose tissue inflammation. *Nat Rev Endocrinol*. 2017;13:633-643. doi:10.1038/nrendo.2017.90

51. Pereira SS, Alvarez-Leite JI. Low-grade inflammation, obesity, and diabetes. *Curr Obes Rep*. 2014;3:422-431. doi:10.1007/s13679-014-0124-9

52. Choe SS, Huh JY, Hwang IJ, Kim JI, Kim JB. Adipose tissue remodeling: its role in energy metabolism and metabolic disorders. *Front Endocrinol (Lausanne)*. 2016;7:30. doi:10.3389/fendo.2016.00030

53. Cao H. Adipocytokines in obesity and metabolic disease. *J Endocrinol*. 2014;220:T47-T59. doi:10.1530/JOE-13-0339

54. Frank AP, De Souza Santos R, Palmer BF, Clegg DJ. Determinants of body fat distribution in humans may provide insight about obesity-related health risks. *J Lipid Res*. 2019;60:1710-1719. doi:10.1194/jlr.R086975

55. Goossens GH. The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. *Physiol Behav*. 2008;94:206-218. doi:10.1016/j.physbeh.2007.10.010

56. Veilleux A, Caron-Jobin M, Noël S, Laberge PY, Tchernof A. Visceral adipocyte hypertrophy is associated with dyslipidemia independent of body composition and fat distribution in women. *Diabetes*. 2011;60:1504-1511. doi:10.2337/db10-1039

57. Heinonen S, Saarinen L, Naukkarinen J, et al. Adipocyte morphology and implications for metabolic derangements in acquired obesity. *Int J Obes*. 2014;38:1423-1431. doi:10.1038/ijo.2014.31

58. Hwang YC, Hayashi T, Fujimoto WY, et al. Visceral abdominal fat accumulation predicts the conversion of metabolically healthy obese subjects to an unhealthy phenotype. *Int J Obes*. 2015;39:1365-1370. doi:10.1038/ijo.2015.75

59. Gustafson B, Hedjazifar S, Gogg S, Hammarstedt A, Smith U. Insulin resistance and impaired adipogenesis. *Trends Endocrinol Metab*. 2015;26:193-200. doi:10.1016/j.tem.2015.01.006

60. Virtue S, Vidal-Puig A. Adipose tissue expandability, lipotoxicity and the Metabolic Syndrome - An allostatic perspective. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2010;1801:338-349. doi:10.1016/j.bbalip.2009.12.006

61. Tchkonia T, Morbeck DE, Von Zglinicki T, et al. Fat tissue, aging, and cellular senescence. *Aging Cell*. 2010;9:667-684. doi:10.1111/j.1474-9726.2010.00608.x

62. Caso G, McNurlan MA, Mileva I, Zemlyak A, Mynarcik DC, Gelato MC. Peripheral fat loss and decline in adipogenesis in older humans. *Metabolism*. 2013;62:337-340. doi:10.1016/j.metabol.2012.08.007

63. Smith U, Li Q, Rydén M, Kirsty •, Spalding L. Cellular senescence and its role in white adipose tissue. *Int J Obes*. 2021;1:Epub ahead of print. doi:10.1038/s41366-021-00757-x

64. Xu M, Tchkonia T, Ding H, et al. JAK inhibition alleviates the cellular senescence-associated secretory phenotype and frailty in old age. *Proc Natl Acad Sci United Sattes Am*. 2015;112:E6301-E6310. doi:10.1073/pnas.1515386112

65. Longo M, Zatterale F, Naderi J, et al. Adipose tissue dysfunction as determinant of obesity-associated metabolic complications. *Int J Mol Sci*. 2019;20. doi:10.3390/ijms20092358

66. Guo S. Insulin signaling, resistance, and the metabolic syndrome: insights from mouse models to disease mechanisms. *J Endocrinol*. 2014;220:1-36. doi:10.1530/JOE-13-0327

67. McKie GL, Medak KD, Knuth CM, et al. Housing temperature affects the acute and chronic metabolic adaptations to exercise in mice. *J Physiol*. 2019;597:4581-4600. doi:10.1113/JP278221

68. Varghese M, Griffin C, Mckernan K, et al. Sex differences in inflammatory responses to adipose tissue lipolysis in diet-induced obesity. *Endocrinology*. 2019;160:293-312. doi:10.1210/en.2018-00797

69. Foley KP, Chen Y, Barra NG, et al. Inflammation promotes adipocyte lipolysis via IRE1 kinase. *J Biol Chem*. 2021;296:100440. doi:10.1016/J.JBC.2021.100440

70. Wang Y, Huang F. N-3 polyunsaturated fatty acids and inflammation in obesity: local effect and systemic benefit. *Biomed Res Int*. 2015;2015:581469. doi:10.1155/2015/581469

71. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol*. 2011;11:85-97. doi:nri2921 [pii]\r10.1038/nri2921

72. Grant RW, Dixit VD. Adipose tissue as an immunological organ. *Obesity*. 2015;23:512-518. doi:10.1002/oby.21003

73. Primeau V, Coderre L, Karelis AD, et al. Characterizing the profile of obese patients who are metabolically healthy. *Int J Obes (Lond)*. 2011;35:971-981. doi:10.1038/ijo.2010.216

74. Kloting N, Fasshauer M, Dietrich A, et al. Insulin-sensitive obesity. *Am J Physiol Endocrinol Metab*. 2010;299:E506-15. doi:10.1152/ajpendo.00586.2009

75. Jeremic N, Chatuverdi P, Tyagi SC. Browning of white fat: novel insight into factors, mechanisms and therapeutics. *J Cell Physiol*. 2016;232:61-68. doi:10.1002/jcp.25450

76. Bolsoni-Lopes A, Deshaies Y, Festuccia WT. Regulation of brown adipose tissue recruitment, metabolism and thermogenic function by peroxisome proliferator-activated receptor γ. *Temperature*. 2015;2:476-482. doi:10.1080/23328940.2015.1011564

77. Bartelt A, Bruns OT, Reimer R, et al. Brown adipose tissue activity controls triglyceride clearance. *Nat Med*. 2011;17:200-206. doi:10.1038/nm.2297

78. Stanford KI, Lynes MD, Takahashi H, et al. 12,13-diHOME: An exercise-induced lipokine that increases skeletal muscle fatty acid uptake. *Cell Metab*. 2018;27:1111-1120.e3. doi:10.1016/j.cmet.2018.03.020

79. Stanford KI, Middelbeek RJW, Townsend KL, et al. Brown adipose tissue regulates glucose homeostasis and insulin sensitivity. *J Clin Invest*. 2013;123:215-223. doi:10.1172/JCI62308

80. Hanssen MJW, Hoeks J, Brans B, et al. Short-term cold acclimation improves insulin sensitivity in patients with type 2 diabetes mellitus. *Nat Med*. 2015;21:6-10. doi:10.1038/nm.3891

81. Chondronikola M, Volpi E, Børsheim E, et al. Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. *Diabetes*. 2014;63:4089-4099. doi:10.2337/db14-0746

82. Villarroya J, Cereijo R, Villarroya F. An endocrine role for brown adipose tissue? *Am J Physiol Endocrinol Metab*. 2013;305:E567-72. doi:10.1152/ajpendo.00250.2013

83. Lynes MD, Leiria LO, Lundh M, et al. The cold-induced lipokine 12,13-diHOME promotes fatty acid transport into brown adipose tissue. *Nat Med*. 2017;23:631-637. doi:10.1038/nm.4297.

84. Leiria LO, Wang CH, Lynes MD, et al. 12-Lipoxygenase regulates cold adaptation and glucose metabolism by producing the omega-3 lipid 12-HEPE from brown fat. *Cell Metab*. 2019;30:768-783. doi:10.1016/j.cmet.2019.07.001

85. Hondares E, Iglesias R, Giralt A, et al. Thermogenic activation induces FGF21 expression and release in brown adipose tissue. *J Biol Chem*. 2011;286:12983-12990. doi:10.1074/jbc.M110.215889

86. Becker AS, Nagel HW, Wolfrum C, Burger IA. Anatomical grading for metabolic activity of brown adipose tissue. *PLoS One*. 2016;11:e0149458. doi:10.1371/journal.pone.0149458

87. Zhang Q, Ye H, Miao Q, et al. Differences in the metabolic status of healthy adults with and without active brown adipose tissue. *Wien Klin Wochenschr*. 2013;125:687-695. doi:10.1007/s00508-013-0431-2

88. Peterson CM, Lecoultre V, Frost EA, Simmons J, Redman LM, Ravussin E. The thermogenic responses to overfeeding and cold are differentially regulated. *Obes (Silver Spring)*. 2016;24:96-101. doi:10.1002/oby.21233

89. Yoneshiro T, Aita S, Matsushita M, et al. Recruited brown adipose tissue as an antiobesity agent in humans. *J Clin Invest*. 2013;123:3404-3408. doi:10.1172/JCI67803

90. Blondin DP, Labbé SM, Tingelstad HC, et al. Increased brown adipose tissue oxidative capacity in cold-acclimated humans. *J Clin Endocrinol Metab*. 2014;99:438-446. doi:10.1210/jc.2013-3901

91. Ouellet V, Routhier-Labadie A, Bellemare W, et al. Outdoor temperature, age, sex, body mass index, and diabetic status determine the prevalence, mass, and glucose-uptake activity of 18F-FDG-detected BAT in humans. *J Clin Endocrinol Metab*. 2011;96:192-199. doi:10.1210/jc.2010-0989

92. Saito M, Okamatsu-ogura Y, Matsushita M, et al. High Incidence of Metabolically Active Brown Adipose Effects of Cold Exposure and Adiposity. *Diabetes*. 2009;58:1526-1531. doi:10.2337/db09-0530.

93. Nedergaard J, Bengtsson T, Cannon B. New powers of brown fat: fighting the metabolic syndrome. *Cell Metab*. 2011;13:238-240. doi:10.1016/j.cmet.2011.02.009

94. Ouellet V, Labbé SM, Blondin DP, et al. Brown adipose tissue oxidative metabolism contributles to energy expenditure during cold exposure in humans. *J Clin Invest*. 2012;122:545. doi:10.1172/JCI60433DS1

95. Berry DC, Jiang Y, Arpke RW, et al. Cellular aging contributes to failure of cold-induced beige adipocyte formation in old mice and humans differential anti-adipogenic effects of eicosapentaenoic and docosahexaenoic acids in obesity. *Cell Metab*. 2017;25:166-181. doi:10.1016/j.cmet.2016.10.023

96. Bahler L, Verberne HJ, Admiraal WM, et al. Differences in sympathetic nervous stimulation of brown adipose tissue between the young and old, and the lean and obese. *J Nucl Med*. 2016;57:372-377. doi:10.2967/jnumed.115.165829

97. Cedikova M, Kripnerová M, Dvorakova J, et al. Mitochondria in White, Brown, and Beige Adipocytes. *Stem Cells Int*. 2016;2016:6067349. doi:10.1155/2016/6067349

98. Florez-Duquet M, Horwitz BA, McDonald RB. Cellular proliferation and UCP content in brown adipose tissue of cold- exposed aging Fischer 344 rats. *Am J Physiol - Regul Integr Comp Physiol*. 1998;274. doi:10.1152/ajpregu.1998.274.1.r196

99. Zoico E, Rubele S, De Caro A, et al. Brown and beige adipose tissue and aging. *Front Endocrinol (Lausanne)*. 2019;10:368. doi:10.3389/fendo.2019.00368

100. Yoneshiro T, Aita S, Matsushita M, et al. Age-related decrease in cold-activated brown adipose tissue and accumulation of body fat in healthy humans. *Obesity*. 2011;19:1755-1760. doi:10.1038/oby.2011.125

101. Orava J, Nuutila P, Noponen T, et al. Blunted metabolic responses to cold and insulin stimulation in brown adipose tissue of obese humans. *Obesity*. 2013;21:2279-2287. doi:10.1002/oby.20456

102. Xu F, Zheng X, Lin B, et al. Diet-induced obesity and insulin resistance are associated with brown fat degeneration in SIRT1-deficient mice. *Obesity*. 2016;24:634-642. doi:10.1002/oby.21393

103. Shimizu I, Aprahamian T, Kikuchi R, et al. Vascular rarefaction mediates whitening of brown fat in obesity. *J Clin Invest*. 2014;124:2099-2112. doi:10.1172/JCI71643

104. Graja A, Gohlke S, Schulz TJ. Aging of brown and beige/brite adipose tissue. *Handb Exp Pharmacol*. 2019;251:55-72. doi:10.1007/164_2018_151.

105. Tran K Van, Gealekman O, Frontini A, et al. The vascular endothelium of the adipose tissue gives rise to both white and brown fat cells. *Cell Metab*. 2012;15:222-229. doi:10.1016/j.cmet.2012.01.008

106. Wolf Y, Boura-Halfon S, Cortese N, et al. Brown-adipose-tissue macrophages control tissue innervation and homeostatic energy expenditure. *Nat Immunol*. 2017;18:665-674. doi:10.1038/ni.3746

107. Omran F, Christian M. Inflammatory Signaling and Brown Fat Activity. *Front Endocrinol (Lausanne)*. 2020;11:156. doi:10.3389/fendo.2020.00156

108. Alcalá M, Calderon-Dominguez M, Serra D, Herrero L, Viana M. Mechanisms of impaired brown adipose tissue recruitment in obesity. *Front Physiol*. 2019;10:94. doi:10.3389/fphys.2019.00094

109. Vijgen GHEJ, Bouvy ND, Teule GJJ, et al. Increase in brown adipose tissue activity after weight loss in morbidly obese subjects. *J Clin Endocrinol Metab*. 2012;97:E1229-33. doi:10.1210/jc.2012-1289

110. Hoeke G, Kooijman S, Boon MR, Rensen PCN, Berbeé JFP. Role of brown fat in lipoprotein

metabolism and atherosclerosis. *Circ Res*. 2016;118:173-182. doi:10.1161/CIRCRESAHA.115.306647

111. Valle A, Santandreu F, García-Palmer F, Roca P, Oliver J. The serum levels of 17β-estradiol, progesterone and triiodothyronine correlate with brown adipose tissue thermogenic parameters during aging. *Cell Physiol Biochem*. 2008;22:337-346. doi:10.1159/000149812

112. Fuller-Jackson JP, Dordevic AL, Clarke IJ, Henry BA. Effect of sex and sex steroids on brown adipose tissue heat production in humans. *Eur J Endocrinol*. 2020;183:343-355. doi:10.1530/EJE-20-0184

113. Barbatelli G, Murano I, Madsen L, et al. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *Am J Physiol Endocrinol Metab*. 2010;298:E1244-E1253. doi:10.1152/ajpendo.00600.2009

114. Smorlesi A, Frontini A, Giordano A, Cinti S. The adipose organ: white-brown adipocyte plasticity and metabolic inflammation. *Obes Rev*. 2012;13:83-96. doi:10.1111/j.1467-789X.2012.01039.x

115. Rosenwald M, Perdikari A, Rülicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. *Nat Cell Biol*. 2013;15:659-667. doi:10.1038/ncb2740

116. Sidossis LS, Porter C, Saraf MK, et al. Browning of subcutaneous white adipose tissue in humans after severe adrenergic stress. *Cell Metab*. 2015;22:219-227. doi:10.1016/j.cmet.2015.06.022

117. Cypess AM, White AP, Vernochet C, et al. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nat Med*. 2013;19:635-639. doi:10.1038/nm.3112.

118. Jespersen NZ, Larsen TJ, Peijs L, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. *Cell Metab*. 2013;17:798-805. doi:10.1016/j.cmet.2013.04.011

119. Shinoda K, Luijten IHN, Hasegawa Y, et al. Genetic and functional characterization of clonally derived adult human brown adipocytes Kosaku. *Nat Med*. 2015;21:389-394. doi:10.1038/nm.3819.Genetic

120. Tews D, Schwar V, Scheithauer M, et al. Comparative gene array analysis of progenitor cells from human paired deep neck and subcutaneous adipose tissue. *Mol Cell Endocrinol*. 2014;395:41-50. doi:10.1016/j.mce.2014.07.011

121. Nedergaard J, Cannon B. The browning of white adipose tissue: Some burning issues. *Cell Metab*. 2014;20:396-407. doi:10.1016/j.cmet.2014.07.005

122. Villarroya F, Vidal-Puig A. Beyond the sympathetic tone: The new brown fat activators. *Cell Metab*. 2013;17:638-643. doi:10.1016/j.cmet.2013.02.020

123. Moreno-Aliaga MJ, Pérez-Echarri N, Marcos-Gómez B, et al. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab*. 2011;14:242-253. doi:10.1016/j.cmet.2011.05.013

124. Qiu Y, Nguyen KD, Odegaard JI, et al. Eosinophils and type 2 cytokine signaling in macrophages orchestrate development of functional beige fat. *Cell*. 2014;157:1292-1308. doi:10.1016/j.cell.2014.03.066

125. Boon MR, van den Berg SAA, Wang Y, et al. BMP7 activates brown adipose tissue and reduces diet-induced obesity only at subthermoneutrality. *PLoS One*. 2013;8:e74083. doi:10.1371/journal.pone.0074083

126. Zhang HQ, Chen SY, Wang AS, et al. Sulforaphane induces adipocyte browning and promotes glucose and lipid utilization. *Mol Nutr Food Res*. 2016;60:2185-2197. doi:10.1002/mnfr.201500915

127. Kern PA, Finlin BS, Zhu B, et al. The effects of temperature and seasons on subcutaneous white adipose tissue in humans: Evidence for thermogenic gene induction. *J Clin Endocrinol Metab*. 2014;99:E2772-E2779. doi:10.1210/jc.2014-2440

128. Vidal P, Stanford KI. Exercise-induced adaptations to adipose tissue thermogenesis. *Front Endocrinol (Lausanne)*. 2020;11:270. doi:10.3389/fendo.2020.00270

129. Dewal RS, Stanford KI. Effects of exercise on brown and beige adipocytes. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2019;1864:71-78. doi:10.1016/j.bbalip.2018.04.013

130. Stanford KI, Middelbeek RJW, Townsend KL, et al. A novel role for subcutaneous adipose tissue in exercise-induced improvements in glucose homeostasis. *Diabetes*. 2015;64:2002-2014. doi:10.2337/db14-0704

131. Quesada-López T, Cereijo R, Turatsinze JV, et al. The lipid sensor GPR120 promotes brown fat activation and FGF21 release from adipocytes. *Nat Commun*. 2016;17:13479. doi:10.1038/ncomms13479

132. Zhuang P, Lu Y, Shou Q, et al. Differential anti-adipogenic effects of eicosapentaenoic and docosahexaenoic acids in obesity. *Mol Nutr Food Res*. 2019;63:e1801135. doi:10.1002/mnfr.201801135

133. Fernández-Galilea M, Félix-Soriano E, Colón-Mesa I, Escoté X, Moreno-Aliaga MJ. Omega-3 fatty acids as regulators of brown/beige adipose tissue: from mechanisms to therapeutic potential. *J Physiol Biochem*. 2019;76:251-257. doi:10.1007/s13105-019-00720-5

134. Mohamed-Ali V, Pinkney JH, Coppack SW. Adipose tissue as an endocrine and paracrine organ. *Int J Obes*. 1998;22:1145-1158. doi:10.1038/sj.ijo.0800770

135. Cinti S. The adipose organ at a glance. *Dis Model Mech*. 2012;5:588-594. doi:10.1242/dmm.009662

136. Zamboni M, Nori N, Brunelli A, Zoico E. How does adipose tissue contribute to inflammageing? *Exp Gerontol*. 2021;143:111162. doi:10.1016/j.exger.2020.111162

137. Mancuso P, Bouchard B. The impact of aging on adipose function and adipokine synthesis. *Front Endocrinol (Lausanne)*. 2019;10:137. doi:10.3389/fendo.2019.00137

138. Kumari R, Kumar S, Kant R. An update on metabolic syndrome: metabolic risk markers and adipokines in the development of metabolic syndrome. *Diabetes Metab Syndr Clin Res Rev*. 2019;13:2409-2417. doi:10.1016/j.dsx.2019.06.005

139. Esmaili S, Hemmati M, Karamian M. Physiological role of adiponectin in different tissues: a review. *Arch Physiol Biochem*. 2020;126:67-73. doi:10.1080/13813455.2018.1493606

140. Arai Y, Kamide K, Hirose N. Adipokines and Aging: Findings From Centenarians and the Very Old. *Front Endocrinol (Lausanne)*. 2019;10:142. doi:10.3389/fendo.2019.00142

141. Turer AT, Scherer PE. Adiponectin: mechanistic insights and clinical implications. *Diabetologia*. 2012;55:2319-2326. doi:10.1007/s00125-012-2598-x

142. Chandrasekar B, Boylston WH, Venkatachalam K, Webster NJG, Prabhu SD, Valente AJ. Adiponectin blocks interleukin-18-mediated endothelial cell death via APPL1-dependent AMP-activated protein kinase (AMPK) activation and IKK/NF-κB/PTEN suppression. *J Biol Chem*. 2008;283:24889-24898. doi:10.1074/jbc.M804236200

143. Yamaguchi N, Argueta JGM, Masuhiro Y, et al. Adiponectin inhibits Toll-like receptor family-induced signaling. *FEBS Lett*. 2005;579:6821-6826. doi:10.1016/j.febslet.2005.11.019

144. Kim JY, Van De Wall E, Laplante M, et al. Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *J Clin Invest*. 2007;117:2621-2637. doi:10.1172/JCI31021

145. Ohashi K, Parker JL, Ouchi N, et al. Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. *J Biol Chem*. 2010;285:6153-6160. doi:10.1074/jbc.M109.088708

146. Takemura Y, Ouchi N, Shibata R, et al. Adiponectin modulates inflammatory reactions via calreticulin receptor-dependent clearance of early apoptotic bodies. *J Clin Invest*. 2007;117:375-386. doi:10.1172/JCI29709

147. Gupta V, Mishra S, Mishra S, Kumar S, Gupta V. Association of Leptin: Adiponectin ratio and metabolic risk markers in postmenopausal women. *Immunol Lett*. 2018;196:63-67. doi:10.1016/j.imlet.2018.01.008

148. Yamauchi T, Kamon J, Waki H, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med*. 2001;7:941-946. doi:10.1038/90984

149. Baratta R, Amato S, Degano C, et al. Adiponectin relationship with lipid metabolism is independent of body fat mass: Evidence from both cross-sectional and intervention studies. In: *Journal of Clinical Endocrinology and Metabolism*. Vol 89. ; 2004:2665-2671. doi:10.1210/jc.2003-031777

150. Okamoto Y, Kihara S, Ouchi N, et al. Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 2002;106:2767-2770. doi:10.1161/01.CIR.0000042707.50032.19

151. Zhu W, Cheng KKY, Vanhoutte PM, Lam KSL, Xu A. Vascular effects of adiponectin: molecular mechanisms and potential therapeutic intervention. *Clin Sci*. 2008;114:361-374. doi:10.1042/CS20070347

152. Iwabu M, Okada-Iwabu M, Yamauchi T, Kadowaki T. Adiponectin/adiponectin receptor in disease and aging. Published online 2015. doi:10.1038/npjamd.2015.13

153. Parker-Duffen JL, Walsh K. Cardiometabolic effects of adiponectin. In: *Best Practice and Research: Clinical Endocrinology and Metabolism*. Vol 28. Bailliere Tindall Ltd; 2014:81-91. doi:10.1016/j.beem.2013.09.001

154. Cnop M, Havel PJ, Utzschneider KM, et al. Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: Evidence for independent roles of age and sex. *Diabetologia*. 2003;46:459-469. doi:10.1007/s00125-003-1074-z

155. Arita Y, Kihara S, Ouchi N, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun*. 1999;257:79-83. doi:10.1006/bbrc.1999.0255

156. Singh P, Sharma P, Sahakyan KR, et al. Differential effects of leptin on adiponectin expression with weight gain versus obesity. *Int J Obes*. 2016;40:266-274. doi:10.1038/ijo.2015.181

157. Aguilar-Salinas CA, García E, Robles L, et al. High adiponectin concentrations are associated with the metabolically healthy obese phenotype. *J Clin Endocrinol Metab*. 2008;93:4075-4079. doi:10.1210/jc.2007-2724

158. Li S, Shin HJ, Ding EL, Van Dam RM. Adiponectin levels and risk of type 2 diabetes: A systematic review and meta-analysis. *JAMA - J Am Med Assoc*. 2009;302:179-188. doi:10.1001/jama.2009.976

159. Derosa G, Maffioli P, Sahebkar A. Improvement of plasma adiponectin, leptin and C-reactive protein concentrations by orlistat: A systematic review and meta-analysis. *Br J Clin Pharmacol*. 2016;81:819-834. doi:10.1111/bcp.12874

160. Askarpour M, Alizadeh S, Hadi A, et al. Effect of bariatric surgery on the circulating level of adiponectin, chemerin, plasminogen activator inhibitor-1, leptin, resistin, and visfatin: a systematic review and meta-analysis. *Horm Metab Res*. 2020;52:207-215. doi:10.1055/a-1129-6785

161. Kizer JR, Arnold AM, Strotmeyer ES, et al. Change in circulating adiponectin in advanced old age: determinants and impact on physical function and mortality. the cardiovascular health study all stars study. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2010;65:1208-1214. doi:10.1093/gerona/glq122

162. Wang Y, Ma XL, Lau WB. Cardiovascular adiponectin resistance: the critical role of adiponectin receptor modification. *Trends Endocrinol Metab*. 2017;28:519-530. doi:10.1016/j.tem.2017.03.004

163. Gorgui J, Gasbarrino K, Georgakis MK, et al. Circulating adiponectin levels in relation to carotid atherosclerotic plaque presence, ischemic stroke risk, and mortality: A systematic review and meta-analyses. *Metabolism*. 2017;69:51-66. doi:10.1016/j.metabol.2017.01.002

164. Menzaghi C, Xu M, Salvemini L, et al. Circulating adiponectin and cardiovascular mortality in patients with type 2 diabetes mellitus: Evidence of sexual dimorphism. *Cardiovasc Diabetol*. 2014;13:130. doi:10.1186/s12933-014-0130-y

165. Jung DH, Kim JY, Kim JK, Koh SB, Park JK, Ahn SV. Relative contribution of obesity and serum adiponectin to the development of hypertension. *Diabetes Res Clin Pract*. 2014;103:51-56. doi:10.1016/j.diabres.2013.09.018

166. Henneman P, Janssens ACJW, Zillikens MC, et al. Menopause impacts the relation of plasma adiponectin levels with the metabolic syndrome. *J Intern Med*. 2010;267:402-409. doi:10.1111/j.1365-2796.2009.02162.x

167. Sodi R, Hazell MJ, Durham BH, Rees C, Ranganath LR, Fraser WD. The circulating concentration and ratio of total and high molecular weight adiponectin in post-menopausal women with and without osteoporosis and its association with body mass index and biochemical markers of bone metabolism. *Clin Biochem*. 2009;42:1375-1380. doi:10.1016/j.clinbiochem.2009.06.003

168. Chu MC, Cosper P, Orio F, Carmina E, Lobo RA. Insulin resistance in postmenopausal women with metabolic syndrome and the measurements of adiponectin, leptin, resistin, and ghrelin. *Am J Obstet Gynecol*. 2006;194:100-104. doi:10.1016/j.ajog.2005.06.073

169. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature*. 1994;372:425-432. doi:10.1038/372425a0

170. Villanueva EC, Myers MG. Leptin receptor signaling and the regulation of mammalian physiology. *Int J Obes*. 2008;32:S8-S12. doi:10.1038/ijo.2008.232

171. Montague CT, Prins JB, Sanders L, et al. Depot-related gene expression in human subcutaneous and omental adipocytes. *Diabetes*. 1998;47:1384-1391. doi:10.2337/diabetes.47.9.1384

172. Henneicke H, Kim S, Swarbrick MM, et al. Skeletal glucocorticoid signalling determines leptin resistance and obesity in aging mice. *Mol Metab*. 2020;42. doi:10.1016/j.molmet.2020.101098

173. Izquierdo AG, Crujeiras AB, Casanueva FF, Carreira MC. Leptin, obesity, and leptin resistance: where are we 25 years later? *Nutrients*. 2019;11. doi:10.3390/nu11112704

174. Sáinz N, Barrenetxe J, Moreno-Aliaga MJ, Martínez JA. Leptin resistance and diet-induced obesity: central and peripheral actions of leptin. *Metabolism*. 2015;64:35-46. doi:10.1016/j.metabol.2014.10.015

175. Gabriely I, Xiao Hui M, Yang XM, Rossetti L, Barzilai N. Leptin resistance during aging is independent of fat mass. *Diabetes*. 2002;51:1016-1021. doi:10.2337/diabetes.51.4.1016

176. Ma XH, Muzumdar R, Yang XM, Gabriely I, Berger R, Barzilai N. Aging is associated with resistance to effects of leptin on fat distribution and insulin action. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2002;57. doi:10.1093/gerona/57.6.B225

177. Carter S, Caron A, Richard D, Picard F. Role of leptin resistance in the development of obesity in older patients. *Clin Interv Aging*. 2013;8:829-844. doi:10.2147/CIA.S36367

178. Francisco V, Ruiz-Fernández C, Pino J, et al. Adipokines: Linking metabolic syndrome, the immune system, and arthritic diseases. *Biochem Pharmacol*. 2019;165:196-206. doi:10.1016/j.bcp.2019.03.030

179. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol*. 2006;6:772-783. doi:10.1038/nri1937

180. Hellström L, Wahrenberg H, Hruska K, Reynisdottir S, Arner P. Mechanisms behind gender differences in circulating leptin levels. *J Intern Med*. 2000;247:457-462. doi:10.1046/j.1365-2796.2000.00678.x

181. Elbers JMH, Asscheman H, Seidell JC, Frölich M, Meinders AE, Gooren LJG. Reversal of the sex difference in serum leptin levels upon cross-sex hormone administration in transsexuals. *J Clin Endocrinol Metab*. 1997;82:3267-3270. doi:10.1210/jcem.82.10.4284

182. Schautz B, Later W, Heller M, Peters A, Müller MJ, Bosy-Westphal A. Impact of age on leptin and adiponectin independent of adiposity. *Br J Nutr*. 2012;108:363-370. doi:10.1017/S0007114511005605

183. Bondue B, Wittamer V, Parmentier M. Chemerin and its receptors in leukocyte trafficking, inflammation and metabolism. *Cytokine Growth Factor Rev*. 2011;22:331-338. doi:10.1016/j.cytogfr.2011.11.004

184. Ernst MC, Sinal CJ. Chemerin: At the crossroads of inflammation and obesity. *Trends Endocrinol Metab*. 2010;21:660-667. doi:10.1016/j.tem.2010.08.001

185. Roman AA, Parlee SD, Sinal CJ. Chemerin: A potential endocrine link between obesity and type 2 diabetes. *Endocrine*. 2012;42:243-251. doi:10.1007/s12020-012-9698-8

186. Sell H, Laurencikiene J, Taube A, et al. Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells. *Diabetes*. 2009;58:2731-2740. doi:10.2337/db09-0277

187. Prieto-Hontoria PL, Pérez-Matute P, Fernández-Galilea M, et al. Effects of alpha-lipoic acid on chemerin secretion in 3T3-L1 and human adipocytes. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2016;1861:260-268. doi:10.1016/j.bbalip.2015.12.011

188. Malin S, Navaneethan S, Mulya A, Huang H, Kirwan J. Exercise-induced lowering of chemerin is associated with reduced cardiometabolic risk and glucose-stimulated insulin secretion in older adults. *J Nutr Heal Aging*. 2014;18:608-615. doi:10.1007/s12603-014-0459-7

189. Hu S, Shao Z, Zhang C, et al. Chemerin facilitates intervertebral disc degeneration via TLR4 and CMKLR1 and activation of NF-kB signaling pathway. *Aging (Albany NY)*. 2020;12:11732-11753. doi:10.18632/aging.103339

190. Goralski KB, McCarthy TC, Hanniman EA, et al. Chemerin, a novel adipokine that regulates adipogenesis and adipocyte metabolism. *J Biol Chem*. 2007;282:28175-28188. doi:10.1074/jbc.M700793200

191. Rouger L, Denis GR, Luangsay S, Parmentier M. ChemR23 knockout mice display mild obesity but no deficit in adipocyte differentiation. *J Endocrinol*. 2013;219:279-289. doi:10.1530/JOE-13-0106

192. Ernst MC, Haidl ID, Zuńīga LA, et al. Disruption of the chemokine-like receptor-1 (CMKLR1) gene is

associated with reduced adiposity and glucose intolerance. *Endocrinology*. 2012;153:672-682. doi:10.1210/en.2011-1490

193. Takahashi M, Inomata S, Okimura Y, et al. *Decreased Serum Chemerin Levels in Male Japanese Patients with Type 2 Diabetes: Sex Dimorphism*. Vol 60.; 2013.

194. Alfadda AA, Sallam RM, Chishti MA, et al. Differential patterns of serum concentration and adipose tissue expression of chemerin in obesity: Adipose depot specificity and gender dimorphism. *Mol Cells*. 2012;33:591-596. doi:10.1007/s10059-012-0012-7

195. Fried SK, Bunkin DA, Greenberg AS. Omental and Subcutaneous Adipose Tissues of Obese Subjects Release Interleukin-6: Depot Difference and Regulation by Glucocorticoid 1 . *J Clin Endocrinol Metab*. 1998;83:847-850. doi:10.1210/jcem.83.3.4660

196. Mohamed-Ali V, Goodrick S, Rawesh A, et al. Subcutaneous Adipose Tissue Releases Interleukin-6, But Not Tumor Necrosis Factor-α, *in Vivo* [1]. *J Clin Endocrinol Metab*. 1997;82:4196-4200. doi:10.1210/jcem.82.12.4450

197. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *J Am Med Assoc*. 2001;286:327-334. doi:10.1001/jama.286.3.327

198. Sabio G, Das M, Mora A, et al. A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. *Science (80-)*. 2008;322:1539-1543. doi:10.1126/science.1160794

199. Han MS, White A, Perry RJ, et al. Regulation of adipose tissue inflammation by interleukin 6. *Proc Natl Acad Sci U S A*. 2020;117:2751-2760. doi:10.1073/pnas.1920004117

200. Starr ME, Evers BM, Saito H. Age-Associated Increase in Cytokine Production During Systemic Inflammation: Adipose Tissue as a Major Source of IL-6. *Journals Gerontol Ser A Biol Sci Med Sci*. 2009;64A:723-730. doi:10.1093/gerona/glp046

201. Beavers KM, Ambrosius WT, Nicklas BJ, Rejeski WJ. Independent and combined effects of physical activity and weight loss on inflammatory biomarkers in overweight and obese older adults. *J Am Geriatr Soc*. 2013;61:1089-1094. doi:10.1111/jgs.12321

202. Grosicki GJ, Barrett BB, Englund DA, et al. Circulating Interleukin-6 Is Associated with Skeletal Muscle Strength, Quality, and Functional Adaptation with Exercise Training in Mobility-Limited Older Adults. *J frailty aging*. 2020;9:57-63. doi:10.14283/jfa.2019.30

203. Cron L, Allen T, Febbraio MA. The role of gp130 receptor cytokines in the regulation of metabolic homeostasis. *J Exp Biol*. 2016;219:259-265. doi:10.1242/jeb.129213

204. Pal M, Febbraio MA, Whitham M. From cytokine to myokine: The emerging role of interleukin-6 in metabolic regulation. *Immunol Cell Biol*. 2014;92:331-339. doi:10.1038/icb.2014.16

205. Wedell-Neergaard A-S, Lang Lehrskov L, Højgaard Christensen R, et al. Exercise-induced changes in visceral adipose tissue mass are regulated by IL-6 signaling: a randomized controlled trial. *Cell Metab*. 2019;29:844-855. doi:10.1016/j.cmet.2018.12.007

206. Balderas-Peña LMA, Macías-López GG, Zepeda-González A, González-Hernández I, Herrera-Rodríguez R, Fafutis-Morris M. Association between obesity, gender and preoperative inflammatory markers with postsurgical pain in live kidney donors. *Cir Cir*. 2011;79:526-533. doi:10.1007/s11999-013-3282-2

207. Young DG, Skibinski G, Mason JI, James K. The influence of age and gender on serum dehydroepiandrosterone sulphate (DHEA-S), IL-6, IL-6 soluble receptor (IL-6 sR) and transforming growth factor beta 1 (TGF-β1) levels in normal healthy blood donors. *Clin Exp Immunol*. 1999;117:476-481. doi:10.1046/j.1365-2249.1999.01003.x

208. Clément AA, Riesco E, Tessier S, et al. The relationship between adiposopathy and glucose-insulin homeostasis is not affected by moderate-intensity aerobic training in healthy women with obesity. *J Physiol Biochem*. 2018;74:591-601. doi:10.1007/s13105-018-0630-4

209. Sears B, Ricordi C. Anti-inflammatory nutrition as a pharmacological approach to treat obesity. *J Obes*. 2011;2011:431985. doi:10.1155/2011/431985

210. Miles EA, Childs CE, Calder PC. Long-chain polyunsaturated fatty acids (LCPUFAs) and the developing immune system: A narrative review. *Nutrients*. 2021;13:1-21. doi:10.3390/nu13010247

211. Calder PC. Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology? *Br J Clin Pharmacol*. 2013;75:645-662. doi:10.1111/j.1365-2125.2012.04374.x

212. American Diabetes Association. Nutrition recommendations and interventions for diabetes: a position statement of the American Diabetes Association. *Diabetes Care*. 2008;Suppl 1:S61-78. doi:10.2337/dc08-S061

213. U.S. Food and Drug Administration. Qualified Health Claims: Letters of Enforcement Discretion | FDA. Omega-3 fatty acids and reduced risk of coronary heart disease (Martek petition). Published 2004. https://www.fda.gov/food/food-labeling-nutrition/qualified-health-claims-letters-enforcement-discretion

214. FDA. FDA announces new qualified health claims for EPA and DHA omega-3 consumption and the risk of hypertension and coronary heart disease. Published June 2019. https://www.fda.gov/food/cfsan-constituent-updates/fda-announces-new-qualified-health-claims-epa-and-dha-omega-3-consumption-and-risk-hypertension-and

215. EFSA Panel on Dietetic Products Nutrition and Allergies. Scientific Opinion on Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. *EFSA J*. 2016;8:1461. doi:10.2903/j.efsa.2010.1461

216. Siscovick DS, Barringer TA, Fretts AM, et al. Omega-3 polyunsaturated fatty acid (fish oil) supplementation and the prevention of clinical cardiovascular disease: a science advisory from the american heart association. *Circulation*. 2017;135:e867-e884. doi:10.1161/CIR.0000000000000482

217. Flock MR, Skulas-Ray AC, Harris WS, Etherton TD, Fleming JA, Kris-Etherton PM. Determinants of erythrocyte omega-3 fatty acid content in response to fish oil supplementation: a dose-response randomized controlled trial. *J Am Heart Assoc*. 2013;2:20-23. doi:10.1161/JAHA.113.000513

218. Guo XF, Li X, Shi M, Li D. N-3 polyunsaturated fatty acids and metabolic syndrome risk: a meta-analysis. *Nutrients*. 2017;9:703. doi:10.3390/nu9070703

219. Mingay E, Veysey M, Lucock M, et al. Sex-dependent association between omega-3 index and body weight status in older Australians. *J Nutr Intermed Metab*. 2016;5:70-77. doi:10.1016/j.jnim.2016.04.001

220. Giltay EJ, Gooren LJG, Toorians AWFT, Katan MB, Zock PL. Docosahexaenoic acid concentrations are higher in women than in men because of estrogenic effects. *Am J Clin Nutr*. 2004;80:1167-1174. doi:10.1093/ajcn/80.5.1167

221. Rhee Y, Paik MJ, Kim KR, et al. Plasma free fatty acid level patterns according to cardiovascular risk status in postmenopausal women. *Clin Chim Acta*. 2008;392:11-16. doi:10.1016/j.cca.2008.02.012

222. Harris WS, Luo J, Pottala J V, et al. Red blood cell polyunsaturated fatty acids and mortality in the Women's Health Initiative Memory Study. *J Clin Lipidol*. 2017;11:250-259. doi:10.1016/j.jacl.2016.12.013.

223. Young IE, Parker HM, Cook RL, et al. Association between obesity and omega-3 status in healthy young women. *Nutrients*. 2020;12:1480. doi:10.3390/nu12051480

224. Martínez-Fernández L, Laiglesia LM, Huerta AE, Martínez JA, Moreno-Aliaga MJ. Omega-3 fatty acids and adipose tissue function in obesity and metabolic syndrome. *Prostaglandins Other Lipid Mediat*. 2015;121:24-41. doi:10.1016/j.prostaglandins.2015.07.003

225. Hiller B, Herdmann A, Nuernberg K. Dietary n-3 fatty acids significantly suppress lipogenesis in bovine muscle and adipose tissue: A functional genomics approach. *Lipids*. 2011;46:557-567. doi:10.1007/s11745-011-3571-z

226. Lorente-Cebrián S, Bustos M, Marti A, Fernández-Galilea M, Martinez JA, Moreno-Aliaga MJ. Eicosapentaenoic acid inhibits tumour necrosis factor-α-induced lipolysis in murine cultured adipocytes. *J Nutr Biochem*. 2012;23:218-227. doi:10.1016/j.jnutbio.2010.11.018

227. González-Périz A, Horrillo R, Ferré N, et al. Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB J*. 2009;23:1946-1957. Accessed May 15, 2020. https://www.ncbi.nlm.nih.gov/pubmed/19211925

228. Ruzickova J, Rossmeisl M, Prazak T, et al. Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. *Lipids*. 2004;39:1177-1185. doi:10.1007/s11745-004-1345-9

229. Buckley JD, Howe PRC. Long-chain omega-3 polyunsaturated fatty acids may be beneficial for reducing obesity-a review. *Nutrients*. 2010;2:1212-1230. doi:10.3390/nu2121212

230. Flachs P, Rossmeisl M, Kuda O, Kopecky J. Stimulation of mitochondrial oxidative capacity in white

fat independent of UCP1: A key to lean phenotype. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2013;1831:986-1003. doi:10.1016/j.bbalip.2013.02.003

231. Janovská P, Flachs P, Kazdová L, Kopecký J. Anti-obesity effect of n-3 polyunsaturated fatty acids in mice fed high-fat diet is independent of cold-induced thermogenesis. *Physiol Res*. 2013;62:153-161. doi:10.33549/physiolres.932464

232. Flachs P, Horakova O, Brauner P, et al. Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat. *Diabetologia*. 2005;48:2365-2375. doi:10.1007/s00125-005-1944-7

233. Oudart H, Groscolas R, Calgari C, et al. Brown fat thermogenesis in rats fed high-fat diets enriched with n-3 polyunsaturated fatty acids. *Int J Obes*. 1997;21:955-962. doi:10.1038/sj.ijo.0800500

234. Zhao M, Chen X. Eicosapentaenoic acid promotes thermogenic and fatty acid storage capacity in mouse subcutaneous adipocytes. *Biochem Biophys Res Commun*. 2014;450:1446-1451. doi:10.1016/j.bbrc.2014.07.010

235. Laiglesia LM, Lorente-Cebrián S, Prieto-Hontoria PL, et al. Eicosapentaenoic acid promotes mitochondrial biogenesis and beige-like features in subcutaneous adipocytes from overweight subjects. *J Nutr Biochem*. 2016;37:76-82. doi:10.1016/j.jnutbio.2016.07.019

236. Moreno-Aliaga MJ, Lorente-Cebrián S, Martínez JA. Regulation of adipokine secretion by n-3 fatty acids. *Proc Nutr Soc*. 2010;69:324-332. doi:10.1017/S0029665110001801

237. Figueras M, Olivan M, Busquets S, López-soriano FJ, Argilés JM. Effects of eicosapentaenoic acid (EPA) treatment on insulin sensitivity in an animal model of diabetes: improvement of the inflammatory status. *Obesity*. 2011;19:362-369. doi:10.1038/oby.2010.194

238. Flachs P, Mohamed-Ali V, Horakova O, et al. Polyunsaturated fatty acids of marine origin induce adiponectin in mice fed a high-fat diet. *Diabetologia*. 2006;49:394-397. doi:10.1007/s00125-005-0053-y

239. Ding WJ, Wang Y, Fan JG. Regulation of adipokines by polyunsaturated fatty acids in a rat model of non-alcoholic steatohepatitis. *Arch Iran Med*. 2014;17:563-568. doi:014178/AIM.008

240. Gammelmark A, Madsen T, Varming K, Lundbye-Christensen S, Schmidt EB. Low-dose fish oil supplementation increases serum adiponectin without affecting inflammatory markers in overweight subjects. *Nutr Res*. 2012;32:15-23. doi:10.1016/j.nutres.2011.12.007

241. Itoh M, Suganami T, Satoh N, et al. Increased adiponectin secretion by highly purified eicosapentaenoic acid in rodent models of obesity and human obese subjects. *Arterioscler Thromb Vasc Biol*. 2007;27:1918-1925. doi:10.1161/ATVBAHA.106.136853

242. Rossmeisl M, Jilkova ZM, Kuda O, et al. Metabolic effects of n-3 PUFA as phospholipids are superior to triglycerides in mice fed a high-fat diet : possible role of endocannabinoids. 2012;7:e38834. doi:10.1371/journal.pone.0038834

243. Kalupahana NS, Claycombe K, Newman SJ, et al. Eicosapentaenoic acid prevents and reverses insulin resistance in high-fat diet-induced obese mice via modulation of adipose tissue inflammation. *J Nutr*. 2010;140:1915-1922. doi:10.3945/jn.110.125732

244. Todoric J, Löffler M, Huber J, et al. Adipose tissue inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia*. 2006;49:2109-2119. doi:10.1007/s00125-006-0300-x

245. Titos E, Rius B, González-Périz A, et al. Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. *J Immunol*. 2011;187:5408-5418. doi:10.4049/jimmunol.1100225

246. Martínez-Fernández L, González-Muniesa P, Laiglesia LM, et al. Maresin 1 improves insulin sensitivity and attenuates adipose tissue inflammation in ob/ob and diet-induced obese mice. *FASEB J*. 2017;31:2135-2145. doi:10.1096/fj.201600859R

247. Oh DY, Talukdar S, Bae EJ, et al. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell*. 2010;142:687-698. doi:10.1016/j.cell.2010.07.041

248. González-Périz A, Clària J. Resolution of adipose tissue inflammation. *Sci World J*. 2010;10:832-856. doi:10.1100/tsw.2010.77

249. Luo X, Jia R, Yao Q, et al. Docosahexaenoic acid attenuates adipose tissue angiogenesis and insulin resistance in high fat diet-fed middle-aged mice via a SIRT1-dependent mechanism. *Mol Nutr*

Food Res. 2016;60:871-885. doi:10.1002/mnfr.201500714

250. Takahashi Y, Ide T. Dietary n-3 fatty acids affect mRNA level of brown adipose tissue uncoupling protein 1, and white adipose tissue leptin and glucose transporter 4 in the rat. *Br J Nutr*. 2000;84:175-184. doi:10.1017/S0007114500001409

251. Yang Z, Miyahara H, Mori T, Doisaki N, Hatanaka A. Beneficial effects of dietary fish-oil-derived monounsaturated fatty acids on metabolic syndrome risk factors and insulin resistance in mice. *J Agric Food Chem*. 2011;59:7482-7489. doi:10.1021/jf201496h.

252. Crescenzo R, Mazzoli A, Cancelliere R, et al. Polyunsaturated Fatty Acids Stimulate De novo Lipogenesis and Improve Glucose Homeostasis during Refeeding with High Fat Diet. *Front Physiol*. 2017;8:178. doi:10.3389/fphys.2017.00178

253. Worsch S, Heikenwalder M, Hauner H, Bader BL. Dietary n-3 long-chain polyunsaturated fatty acids upregulate energy dissipating metabolic pathways conveying anti-obesogenic effects in mice. doi:10.1186/s12986-018-0291-x

254. Bjursell M, Xu X, Admyre T, et al. The beneficial effects of n-3 polyunsaturated fatty acids on diet induced obesity and impaired glucose control do not require Gpr120. *PLoS One*. 2014;9:e114942. doi:10.1371/journal.pone.0114942

255. Bargut TCL, Silva-e-Silva ACAG, Souza-Mello V, Mandarim-de-Lacerda CA, Aguila MB. Mice fed fish oil diet and upregulation of brown adipose tissue thermogenic markers. *Eur J Nutr*. 2016;55:159-169. doi:10.1007/s00394-015-0834-0

256. Oliveira TE, Castro É, Belchior T, et al. Fish oil protects wild type and uncoupling protein 1-deficient mice from obesity and glucose intolerance by increasing energy expenditure. *Mol Nutr Food Res*. 2019;63:e1800813. doi:10.1002/mnfr.201800813

257. Kim M, Goto T, Yu R, et al. Fish oil intake induces UCP1 upregulation in brown and white adipose tissue via the sympathetic nervous system. *Sci Rep*. 2015;5:1-12. doi:10.1038/srep18013

258. Kim J, Okla M, Erickson A, Carr T, Natarajan SK, Chung S. Eicosapentaenoic acid potentiates brown thermogenesis through FFAR4-dependent up-regulation of miR-30b and miR-378. *J Biol Chem*. 2016;291:20551-20562. doi:10.1074/jbc.M116.721480

259. Ghandour RA, Colson C, Giroud M, et al. Impact of dietary ω3 polyunsaturated fatty acid supplementation on brown and brite adipocyte function. *J Lipid Res*. 2018;59:452-461. doi:10.1194/jlr.M081091

260. Pahlavani M, Ramalingam L, Miller EK, et al. Eicosapentaenoic Acid Reduces Adiposity, Glucose Intolerance and Increases Oxygen Consumption Independently of Uncoupling Protein 1. *Mol Nutr Food Res*. 2019;63:1-12. doi:10.1002/mnfr.201800821

261. Soni N, Ross AB, Scheers N, Nookaew I, Gabrielsson BG, Sandberg AS. The omega-3 fatty acids EPA and DHA, as a part of a murine high-fat diet, reduced lipid accumulation in brown and white adipose tissues. *Int J Mol Sci*. 2019;20:5895. doi:10.3390/ijms20235895

262. You M, Fan R, Kim J, Shin S-H, Chung S. Alpha-Linolenic Acid-Enriched Butter Promotes Fatty Brown Adipose Tissue. *Nutrients*. 2020;12:1-12.

263. Lopes PA, Bandarra NM, Martins S V., et al. Docosahexaenoic acid (DHA) at the sn-2 position of triacylglycerols increases DHA incorporation in brown, but not in white adipose tissue, of hamsters. *Int J Food Sci Nutr*. 2017;69:458-471. doi:10.1080/09637486.2017.1372390

264. Qin X, Park HG, Zhang JY, et al. Brown but not white adipose cells synthesize omega-3 docosahexaenoic acid in culture. *Prostaglandins Leukot Essent Fat Acids*. 2016;104:19-24. doi:10.1016/j.plefa.2015.11.001

265. Villarroya F, Cereijo R, Villarroya J, Giralt M. Brown adipose tissue as a secretory organ. *Nat Rev Endocrinol*. 2017;13:26-35. doi:10.1038/nrendo.2016.136

266. Xiang AS, Giles C, Loh RKC, et al. Plasma docosahexaenoic acid and eicosapentaenoic acid concentrations are positively associated with brown adipose tissue activity in humans. *Metabolites*. 2020;10:388. doi:10.3390/metabo10100388

267. Spite M, Clària J, Serhan CN. Resolvins, specialized proresolving lipid mediators, and their potential roles in metabolic diseases. *Cell Metab*. 2014;19:21-36. doi:10.1016/j.cmet.2013.10.006.

268. Serhan CN, Chiang N, Dalli J. The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution. *Semin Immunol*. 2015;27:200-215. doi:10.1016/j.smim.2015.03.004

269. Clària J, Nguyen BT, Madenci AL, Keith Ozaki C, Serhan CN. Diversity of lipid mediators in human adipose tissue depots. *Am J Physiol - Cell Physiol*. 2013;304:1141-1149. doi:10.1152/ajpcell.00351.2012

270. Crouch M, Al-Shaer A, Shaikh SR. Hormonal dysregulation and unbalanced specialized pro-resolving mediator biosynthesis cntribute toward impaired B cell outcomes in obesity. *Mol Nutr Food Res*. 2021;65:1-9. doi:10.1002/mnfr.201900924

271. Neuhofer A, Zeyda M, Mascher D, et al. Impaired local production of proresolving lipid mediators in obesity and 17-HDHA as a potential treatment for obesity-associated inflammation. *Diabetes*. 2013;62:1945-1956. doi:10.2337/db12-0828

272. Lopez EF, Kabarowski JH, Ingle KA, et al. Obesity superimposed on aging magnifies inflammation and delays the resolving response after myocardial infarction. *Am J Physiol - Hear Circ Physiol*. 2015;308:H269-H280. doi:10.1152/ajpheart.00604.2014

273. Cho YJ, Lim YH, Yun JM, Yoon HJ, Park M. Sex- and age-specific effects of energy intake and physical activity on sarcopenia. *Sci Rep*. 2020;10:9822. doi:10.1038/s41598-020-66249-6

274. López-Vicario C, Titos E, Walker ME, et al. Leukocytes from obese individuals exhibit an impaired SPM signature. *FASEB J*. 2019;33:7072-7083. doi:10.1096/fj.201802587R

275. Clària J, Dalli J, Yacoubian S, Gao F, Serhan CN. Resolvin D1 and Resolvin D2 govern local inflammatory tone in obese fat. *J Immunol*. 2012;189:2597-2605. doi:10.4049/jimmunol.1201272

276. Clària J, López-Vicario C, Rius B, Titos E. Pro-resolving actions of SPM in adipose tissue biology. *Mol Aspects Med*. 2017;58:83-92. doi:10.1016/j.mam.2017.03.004.

277. Crouch MJ, Kosaraju R, Guesdon W, et al. Frontline science: a reduction in DHA-derived mediators in male obesity contributes toward defects in select B cell subsets and circulating antibody. *J Leukoc Biol*. 2019;106:241-257. doi:10.1002/JLB.3HI1017-405RR

278. Martínez-Fernández L, González-Muniesa P, Sáinz N, et al. Maresin 1 regulates insulin signaling in human adipocytes as well as in adipose tissue and muscle of lean and obese mice. *J Physiol Biochem*. Published online 2020. doi:10.1007/s13105-020-00775-9

279. White PJ, Arita M, Taguchi R, Kang JX, Marette A. Transgenic restoration of long-chain n-3 fatty acids in insulin target tissues improves resolution capacity and alleviates obesity-linked inflammation and insulin resistance in high-fat-fed mice. *Diabetes*. 2010;59:3066-3073. doi:10.2337/db10-0054

280. Laiglesia LM, Lorente-Cebrián S, Martínez-Fernández L, et al. Maresin 1 mitigates liver steatosis in ob/ob and diet-induced obese mice. *Int J Obes*. 2018;42:572-579. doi:10.1038/ijo.2017.226

281. Jung TW, Kim HC, El-Aty AMA, Jeong JH. Maresin 1 attenuates NAFLD by suppression of endoplasmic reticulum stress via AMPK-SERCA2b pathway. *J Biol Chem*. 2018;293:3981-3988. doi:10.1074/jbc.RA117.000885

282. Han YH, Shin KO, Kim JY, et al. A maresin 1/RORα/12-lipoxygenase autoregulatory circuit prevents inflammation and progression of nonalcoholic steatohepatitis. *J Clin Invest*. 2019;129:1684-1698. doi:10.1172/JCI124219

283. White PJ, St-Pierre P, Charbonneau A, et al. Protectin DX alleviates insulin resistance by activating a myokine-liver glucoregulatory axis. *Nat Med*. 2014;20:664-669. doi:10.1038/nm.3549

284. González-Périz A, Horrillo R, Ferré N, et al. Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. *FASEB J*. 2009;23:1946-1957. doi:10.1096/fj.08-125674

285. Calder PC. Eicosapentaenoic and docosahexaenoic acid derived specialised pro-resolving mediators : Concentrations in humans and the effects of age , sex , disease and increased omega-3 fatty acid intake. *Biochimie*. 2020;178:105-123. doi:10.1016/j.biochi.2020.08.015

286. Itariu BK, Zeyda M, Hochbrugger EE, et al. Long-chain n-3 PUFAs reduce adipose tissue and systemic inflammation in severely obese nondiabetic patients: a randomized controlled trial. *Am J Clin Nutr*. 2012;96:1137-1149. doi:10.3945/ajcn.112.037432

287. Schuchardt JP, Ostermann AI, Stork L, et al. Effect of DHA supplementation on oxylipin levels in plasma and immune cell stimulated blood. *Prostaglandins Leukot Essent Fat Acids*. 2017;121:76-87. doi:10.1016/j.plefa.2017.06.007

288. Ostermann AI, West AL, Schoenfeld K, et al. Plasma oxylipins respond in a linear dose-response manner with increased intake of EPA and DHA: Results from a randomized controlled trial in healthy

humans. *Am J Clin Nutr*. 2019;109:1251-1263. doi:10.1093/ajcn/nqz016

289. Kulterer OC, Niederstaetter L, Herz CT, et al. The presence of active brown adipose tissue determines cold-induced energy expenditure and oxylipin profiles in humans. *J Clin Endocrinol Metab*. 2020;105:2203-2216. doi:10.1080/2331186X.2019.1662162

290. Dieckmann S, Maurer S, Fromme T, et al. Fatty Acid Metabolite Profiling Reveals Oxylipins as Markers of Brown but Not Brite Adipose Tissue. *Front Endocrinol (Lausanne)*. 2020;11:73. doi:10.3389/fendo.2020.00073

291. Titos E, Rius B, López-Vicario C, et al. Signaling and immunoresolving actions of Resolvin D1 in inflamed human visceral adipose tissue. *J Immunol*. 2016;197:3360-3370. doi:10.4049/jimmunol.1502522.

292. Spite M, Hellmann J, Tang Y, et al. Deficiency of the Leukotriene B 4 Receptor, BLT-1, Protects against Systemic Insulin Resistance in Diet-Induced Obesity . *J Immunol*. 2011;187:1942-1949. doi:10.4049/jimmunol.1100196

293. Horrillo R, González-Périz A, Martínez-Clemente M, et al. 5-Lipoxygenase Activating Protein Signals Adipose Tissue Inflammation and Lipid Dysfunction in Experimental Obesity. *J Immunol*. 2010;184:3978-3987. doi:10.4049/jimmunol.0901355

294. Madsen L, Pedersen LM, Lillefosse HH, et al. UCP1 induction during recruitment of brown adipocytes in white adipose tissue is dependent on cyclooxygenase activity. *PLoS One*. 2010;5:e11391. doi:10.1371/journal.pone.0011391

295. García-Alonso V, López-Vicario C, Titos E, et al. Coordinate functional regulation between microsomal prostaglandin e synthase-1 (mPGES-1) and peroxisome proliferator-activated receptor γ (PPARγ) in the conversion of white-to-brown adipocytes. *J Biol Chem*. 2013;288:28230-28242. doi:10.1074/jbc.M113.468603

296. García-Alonso V, Titos E, Alcaraz-Quiles J, et al. Prostaglandin E2 exerts multiple regulatory actions on human obese adipose tissue remodeling, inflammation, adaptive thermogenesis and lipolysis. *PLoS One*. 2016;11:e0153751. doi:10.1371/journal.pone.0153751

297. Elias I, Ferré T, Vilà L, et al. ALOX5AP overexpression in adipose tissue leads to LXA4 production and protection against diet-induced obesity and insulin resistance. *Diabetes*. 2016;65:2139-2150. doi:10.2337/db16-0040

298. Alsharari ZD, Risérus U, Leander K, et al. Serum fatty acids, desaturase activities and abdominal obesity - a population-based study of 60-year old men and women. *PLoS One*. 2017;12:e0170684. doi:10.1371/journal.pone.0170684

299. Bender N, Portmann M, Heg Z, Hofmann K, Zwahlen M, Egger M. Fish or n3-PUFA intake and body composition: a systematic review and meta-analysis. *Obes Rev*. 2014;15:657-665. doi:10.1111/obr.12189

300. Zhang YY, Liu W, Zhao TY, Tian HM. Efficacy of omega-3 polyunsaturated fatty acids supplementation in managing overweight and obesity: a meta-analysis of randomized clinical trials. *J Nutr Heal Aging*. 2017;21:187-192. doi:10.1007/s12603-016-0755-5

301. Mori TA, Bao DQ, Burke V, Puddey IB, Watts GF, Beilin LJ. Dietary fish as a major component of a weight-loss diet: effect on serum lipids, glucose, and insulin metabolism in overweight hypertensive subjects. *Am J Clin Nutr*. 1999;70:817-825. doi:10.1093/ajcn/70.5.817

302. Li N, Yue H, Jia M, et al. Effect of low-ratio n-6/n-3 PUFA on blood glucose: A meta-analysis. *Food Funct*. 2019;10:4557-4565. doi:10.1039/c9fo00323a

303. Akinkuolie AO, Ngwa JS, Meigs JB, Djoussé L. Omega-3 polyunsaturated fatty acid and insulin sensitivity: a meta-analysis of randomized controlled trials. *Clin Nutr*. 2011;30:702-707. doi:10.1016/j.clnu.2011.08.013.

304. Amigó N, Akinkuolie AO, Chiuve SE, Correig X, Cook NR, Mora S. Habitual fish consumption, n-3 fatty acids, and nuclear magnetic resonance lipoprotein subfractions in women. *J Am Heart Assoc*. 2020;9:e014963. doi:10.1161/JAHA.119.014963

305. AbuMweis S, Jew S, Tayyem R, Agraib L. Eicosapentaenoic acid and docosahexaenoic acid containing supplements modulate risk factors for cardiovascular disease: a meta-analysis of randomised placebo-control human clinical trials. *J Hum Nutr Diet*. 2018;31:67-84. doi:10.1111/jhn.12493

306. Lichtenstein AH, Appel LJ, Brands M, et al. Diet and lifestyle recommendations revision 2006: a

scientific statement from the American Heart Association nutrition committee. *Circulation*. 2006;114:82-96. doi:10.1161/CIRCULATIONAHA.106.176158

307. Li K, Huang T, Zheng J, Wu K, Li D. Effect of marine-derived n-3 polyunsaturated fatty acids on C-reactive protein, interleukin 6 and tumor necrosis factor α: A meta-analysis. *PLoS One*. 2014;9:1-28. doi:10.1371/journal.pone.0088103

308. O'Mahoney LL, Matu J, Price OJ, et al. Omega-3 polyunsaturated fatty acids favourably modulate cardiometabolic biomarkers in type 2 diabetes: A meta-analysis and meta-regression of randomized controlled trials. *Cardiovasc Diabetol*. 2018;17:98. doi:10.1186/s12933-018-0740-x

309. Farimani AR, Hariri M, Azimi-Nezhad M, Borji A, Zarei S, Hooshmand E. The effect of n-3 PUFAs on circulating adiponectin and leptin in patients with type 2 diabetes mellitus: a systematic review and meta-analysis of randomized controlled trials. *Acta Diabetol*. 2018;55:641-652. doi:10.1007/s00592-018-1110-6

310. Hariri M, Ghiasvand R, Shiranian A, Askari G, Iraj B, Salehi-Abargouei A. Does omega-3 fatty acids supplementation affect circulating leptin levels? A systematic review and meta-analysis on randomized controlled clinical trials. *Clin Endocrinol (Oxf)*. 2015;82:221-228. doi:10.1111/cen.12508

311. Allaire J, Couture P, Leclerc M, et al. A randomized, crossover, head-to-head comparison of eicosapentaenoic acid and docosahexaenoic acid supplementation to reduce inflammation markers in men and women: the Comparing EPA to DHA (ComparED) Study. *Am J Clin Nutr*. 2016;104:280-287. doi:10.3945/ajcn.116.131896

312. Allaire J, Vors C, Tremblay AJ, et al. High-dose DHA has more profound effects on LDL-related features than high-dose EPA: The ComparED study. *J Clin Endocrinol Metab*. 2018;103:2909-2917. doi:10.1210/jc.2017-02745

313. Allaire J, Harris WS, Vors C, et al. Supplementation with high-dose docosahexaenoic acid increases the Omega-3 Index more than high-dose eicosapentaenoic acid. *Prostaglandins Leukot Essent Fat Acids*. 2017;120:8-14. doi:10.1016/j.plefa.2017.03.008

314. Guo X fei, Li K lei, Li J mei, Li D. Effects of EPA and DHA on blood pressure and inflammatory factors: a meta-analysis of randomized controlled trials. *Crit Rev Food Sci Nutr*. 2019;59:3380-3393. doi:10.1080/10408398.2018.1492901

315. Abdelhamid AS, Brown TJ, Brainard JS, et al. Omega-3 fatty acids for the primary and secondary prevention of cardiovascular disease. *Cochrane Database Syst Rev*. 2018;7:CD003177. doi:10.1002/14651858.CD003177.pub3

316. Abbott KA, Burrows TL, Thota RN, Acharya S, Garg ML. Do v-3 PUFAs affect insulin resistance in a sex-specific manner? A systematic review and meta-analysis of randomized controlled trials. *Am J Clin Nutr*. 2016;104:1470-1484. doi:10.3945/ajcn.116.138172

317. Lohner S, Fekete K, Marosvölgyi T, Decsi T. Gender differences in the long-chain polyunsaturated fatty acid status: Systematic review of 51 publications. *Ann Nutr Metab*. 2013;62:98-112. doi:10.1159/000345599

318. Bischoff-Ferrari HA, Vellas B, Rizzoli R, et al. Effect of vitamin D supplementation, omega-3 fatty acid supplementation, or a strength-training exercise program on clinical outcomes in older adults: the DO-HEALTH randomized clinical trial. *JAMA - J Am Med Assoc*. 2020;324:1855-1868. doi:10.1001/jama.2020.16909

319. Zhang XW, Hou WS, Li M, Tang ZY. Omega-3 fatty acids and risk of cognitive decline in the elderly: a meta-analysis of randomized controlled trials. *Aging Clin Exp Res*. 2016;28:165-166. doi:10.1007/s40520-015-0381-9

320. Mazereeuw G, Lanctôt KL, Chau SA, Swardfager W, Herrmann N. Effects of omega-3 fatty acids on cognitive performance: A meta-analysis. *Neurobiol Aging*. 2012;33:1482.e17-1482.e29. doi:10.1016/j.neurobiolaging.2011.12.014

321. Custodero C, Mankowski RT, Lee SA, et al. Evidence-based nutritional and pharmacological interventions targeting chronic low-grade inflammation in middle-age and older adults: A systematic review and meta-analysis. *Ageing Res Rev*. 2018;46:42-59. doi:10.1016/j.arr.2018.05.004

322. Tan A, Sullenbarger B, Prakash R, McDaniel JC. Supplementation with eicosapentaenoic acid and docosahexaenoic acid reduces high levels of circulating proinflammatory cytokines in aging adults: A randomized, controlled study. *Prostaglandins Leukot Essent Fat Acids*. 2018;132:23-29. doi:10.1016/j.plefa.2018.03.010

323. Teixeira De Lemos E, Oliveira J, Páscoa Pinheiro J, Reis F. Regular physical exercise as a strategy

to improve antioxidant and anti-inflammatory status: Benefits in type 2 diabetes mellitus. *Oxid Med Cell Longev*. 2012;2012:741545. doi:10.1155/2012/741545

324. Benatti FB, Pedersen BK. Exercise as an anti-inflammatory therapy for rheumatic diseases - myokine regulation. *Nat Rev Rheumatol*. 2015;11:86-97. doi:10.1038/nrrheum.2014.193

325. Daou HN. Exercise as an anti-inflammatory therapy for cancer cachexia: a focus on interleukin-6 regulation. *Am J Physiol - Regul Integr Comp Physiol*. 2020;318:R296-R310. doi:10.1152/AJPREGU.00147.2019

326. Chodzko-Zajko WJ, Proctor DN, Fiatarone Singh MA, et al. American college of sports medicine position stand. Exercise and physical activity for older adults. *Med Sci Sports Exerc*. 2009;41:1510-1530. doi:10.1249/MSS.0b013e3181a0c95c

327. Jakicic JM, Powell KE, Campbell WW, et al. Physical activity and the prevention of weight gain in adults. *Med Sci Sport Exerc*. 2019;51:1262-1269. doi:10.1249/MSS.0000000000001938

328. Arnett DK, Blumenthal RS, Albert MA, et al. 2019 ACC/AHA Guideline on the primary prevention of cardiovascular disease: a report of the American College of Cardiology/American Heart Association task force on clinical practice guidelines. *Circulation*. 2019;140:e596-e646. doi:10.1161/CIR.0000000000000678

329. Colberg SR, Sigal RJ, Yardley JE, et al. Physical activity/exercise and diabetes: a position statement of the American Diabetes Association. *Diabetes Care*. 2016;39:2065-2079. doi:10.2337/dc16-1728

330. American College of Sports Medicine and the American Diabetes Association. Exercise and type 2 diabetes: American College of Sports Medicine and the American Diabetes Association Joint Position Statement. *Med Sci Sport Exerc*. 2010;42:2282-2303. doi:10.1249/MSS.0b013e3181eeb61c

331. Palacios G, Pedrero-Chamizo R, Palacios N, Maroto-Sánchez B, Aznar S, González-Gross M. Biomarkers of physical activity and exercise. *Nutr Hosp*. 2015;31:237-244. doi:10.3305/nh.2015.31.sup3.8771

332. Hassinen M, Lakka TA, Savonen K, et al. Cardiorespiratory fitness as a feature of metabolic syndrome in older men and women. *Diabetes Care*. 2008;31:1242-1247. doi:10.2337/dc07-2298

333. Sapp RM, Shill DD, Dash C, Hicks JC, Adams-Campbell LL, Hagberg JM. Circulating microRNAs and endothelial cell migration rate are associated with metabolic syndrome and fitness level in postmenopausal African American women. *Physiol Rep*. 2019;7:e14173. doi:10.14814/phy2.14173

334. Messier V, Malita FM, Rabasa-Lhoret R, Brochu M, Karelis AD. Association of cardiorespiratory fitness with insulin sensitivity in overweight and obese postmenopausal women: a Montreal Ottawa New Emerging Team study. *Metabolism*. 2008;57:1293-1298. doi:10.1016/j.metabol.2008.04.026

335. Ahmed T, French SD, Belanger E, Guerra RO, Zunzunegui MV, Auais M. Gender differences in trajectories of lower extremity function in older adults: findings from the International Mobility in Aging Study. *J Am Med Dir Assoc*. 2019;20:1199-1205.e4. doi:10.1016/j.jamda.2019.04.014

336. Soh Y, Won CW. Sex differences in association between body composition and frailty or physical performance in community-dwelling older adults. *Medicine (Baltimore)*. 2021;100:e24400. doi:10.1097/md.0000000000024400

337. Ebert H, Lacruz ME, Kluttig A, et al. Advanced glycation end products and their ratio to soluble receptor are associated with limitations in physical functioning only in women: results from the CARLA cohort. *BMC Geriatr*. 2019;19:299. doi:10.1186/s12877-019-1323-8

338. Sialino LD, Schaap LA, Van Oostrom SH, et al. Sex differences in physical performance by age, educational level, ethnic groups and birth cohort: the Longitudinal Aging Study Amsterdam. *PLoS One*. 2019;14:e0226342. doi:10.1371/journal.pone.0226342

339. Kirk B, Feehan J, Lombardi G, Duque G. Muscle, bone, and fat crosstalk: the biological role of myokines, osteokines, and adipokines. *Curr Osteoporos Rep*. 2020;18:388-400. doi:10.1007/s11914-020-00599-y

340. Heinonen I, Kalliokoski KK, Hannukainen JC, Duncker DJ, Nuutila P, Knuuti J. Organ-specific physiological responses to acute physical exercise and long-term training in humans. *Physiology*. 2014;29:421-436. doi:10.1152/physiol.00067.2013

341. Egan B, Zierath JR. Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell Metab*. 2013;17:162-184. doi:10.1016/j.cmet.2012.12.012

342. Hellsten Y, Nyberg M. Cardiovascular adaptations to exercise training. *Compr Physiol*. 2016;6:1-32. doi:10.1002/cphy.c140080

343. Lee S, Norheim F, Langleite TM, Gulseth HL, Birkeland KI, Drevon CA. Effects of long-term exercise on plasma adipokine levels and inflammation-related gene expression in subcutaneous adipose tissue in sedentary dysglycaemic, overweight men and sedentary normoglycaemic men of healthy weight. *Diabetologia*. 2019;62:1048-1064. doi:10.1007/s00125-019-4866-5

344. Blond MB, Rosenkilde M, Gram AS, et al. How does 6 months of active bike commuting or leisure-time exercise affect insulin sensitivity, cardiorespiratory fitness and intra-abdominal fat? A randomised controlled trial in individuals with overweight and obesity. *Br J Sports Med*. 2019;53:1183-1192. doi:10.1136/bjsports-2018-100036

345. Čížková T, Štěpán M, Daďová K, et al. Exercise training reduces inflammation of adipose tissue in the elderly: cross-sectional and randomized interventional trial. *J Clin Endocrinol Metab*. 2020;105:e4510–e4526. doi:10.1210/clinem/dgaa630

346. Liu Y, Dong G, Zhao X, Huang Z, Li P, Zhang H. Post-exercise effects and long-term training adaptations of hormone sensitive lipase lipolysis induced by high-intensity interval training in adipose tissue of mice. *Front Physiol*. 2020;11:535722. doi:10.3389/fphys.2020.535722

347. Ziegler AK, Damgaard A, Mackey AL, et al. An anti-inflammatory phenotype in visceral adipose tissue of old lean mice, augmented by exercise. *Sci Rep*. 2019;9:12069. doi:10.1038/s41598-019-48587-2

348. Martinez-Huenchullan SF, Ban LA, Olaya-Agudo LF, et al. Constant-moderate and high-intensity interval training have differential benefits on insulin sensitive tissues in high-fat fed mice. *Front Physiol*. 2019;10:459. doi:10.3389/fphys.2019.00459

349. Lehnig AC, Dewal RS, Baer LA, et al. Exercise training induces depot-specific adaptations to white and brown adipose tissue. *iScience*. 2019;11:425-439. doi:10.1016/j.isci.2018.12.033

350. Jones NL, Heigenhauser GJF, Kuksis A, Matsos CG, Sutton JR, Toews CJ. Fat metabolism in heavy exercise. *Clin Sci*. 1980;59:469-478. doi:10.1042/cs0590469

351. Wolfe RR. Fat metabolism in exercise. *Adv Exp Med Biol*. 1998;441:147-156. doi:10.1007/978-1-4899-1928-1_14

352. Wang N, Liu Y, Ma Y, Wen D. High-intensity interval versus moderate-intensity continuous training: Superior metabolic benefits in diet-induced obesity mice. *Life Sci*. 2017;191:122-131. doi:10.1016/j.lfs.2017.08.023

353. Bae JY. Aerobic exercise increases meteorin-like protein in muscle and adipose tissue of chronic high-fat diet-induced obese mice. *Biomed Res Int*. 2018;2018:6283932. doi:10.1155/2018/6283932

354. Bae JY, Woo J, Roh HT, et al. The effects of detraining and training on adipose tissue lipid droplet in obese mice after chronic high-fat diet. *Lipids Health Dis*. 2017;16:13. doi:10.1186/s12944-016-0398-x

355. Chen N, Lei T, Xin L, et al. Depot-specific effects of treadmill running and rutin on white adipose tissue function in diet-induced obese mice. *J Physiol Biochem*. 2016;72:453-467. doi:10.1007/s13105-016-0493-5

356. Rönn T, Volkov P, Tornberg A, et al. Extensive changes in the transcriptional profile of human adipose tissue including genes involved in oxidative phosphorylation after a 6-month exercise intervention. *Acta Physiol*. 2014;211:188-200. doi:10.1111/apha.12247

357. Stinkens R, Brouwers B, Jocken JW, et al. Exercise training-induced effects on the abdominal subcutaneous adipose tissue phenotype in humans with obesity. *J Appl Physiol*. 2018;125:1585-1593. doi:10.1152/japplphysiol.00496.2018

358. Pino MF, Parsons SA, Smith SR, Sparks LM. Active individuals have high mitochondrial content and oxidative markers in their abdominal subcutaneous adipose tissue. *Obesity*. 2016;24:2467-2470. doi:10.1002/oby.21669

359. Motiani P, Virtanen KA, Motiani KK, et al. Decreased insulin-stimulated brown adipose tissue glucose uptake after short-term exercise training in healthy middle-aged men. *Diabetes, Obes Metab*. 2017;19:1379-1388. doi:10.1111/dom.12947

360. Karpe F, Pinnick KE. Biology of upper-body and lower-body adipose tissue - link to whole-body phenotypes. *Nat Rev Endocrinol*. 2015;11:90-100. doi:10.1038/nrendo.2014.185

361. Rojas JXS, Frutos MGS, Horrillo D, et al. Differential development of inflammation and insulin resistance in different adipose tissue depots along aging in wistar rats: effects of caloric restriction. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2016;71:310-322. doi:10.1093/gerona/glv117

362. Gollisch KSC, Brandauer J, Jessen N, et al. Effects of exercise training on subcutaneous and visceral adipose tissue in normal- and high-fat diet-fed rats. *Am J Physiol - Endocrinol Metab*. 2009;297:495-504. doi:10.1152/ajpendo.90424.2008

363. Bradley RL, Jeon JY, Liu FF, Maratos-Flier E. Voluntary exercise improves insulin sensitivity and adipose tissue inflammation in diet-induced obese mice. *Am J Physiol - Endocrinol Metab*. 2008;295:586-594. doi:10.1152/ajpendo.00309.2007

364. Wasinski F, Bacurau RFP, Moraes MR, et al. Exercise and caloric restriction alter the immune system of mice submitted to a high-fat diet. *Mediators Inflamm*. 2013;2013:395672. doi:10.1155/2013/395672

365. Baek KW, Lee DI, Jeong MJ, et al. Effects of lifelong spontaneous exercise on the M1/M2 macrophage polarization ratio and gene expression in adipose tissue of super-aged mice. *Exp Gerontol*. 2020;141:111091. doi:10.1016/j.exger.2020.111091

366. Schafer MJ, White TA, Evans G, et al. Exercise prevents diet-induced cellular senescence in adipose tissue. *Diabetes*. 2016;65:1606-1615. doi:10.2337/db15-0291

367. Khalafi M, Mohebbi H, Symonds ME, et al. The impact of moderate-intensity continuous or high-intensity interval training on adipogenesis and browning of subcutaneous adipose tissue in obese male rats. *Nutrients*. 2020;12:925. doi:10.3390/nu12040925

368. Kolahdouzi S, Talebi-Garakani E, Hamidian G, Safarzade A. Exercise training prevents high-fat diet-induced adipose tissue remodeling by promoting capillary density and macrophage polarization. *Life Sci*. 2019;220:32-43. doi:10.1016/j.lfs.2019.01.037

369. Giori IG, Magliano DC, Alexandre-Santos B, et al. Enalapril and treadmill running reduce adiposity, but only the latter causes adipose tissue browning in mice. *J Cell Physiol*. 2021;236:900-910. doi:10.1002/jcp.29900

370. Aldiss P, Lewis JE, Lupini I, et al. Exercise training in obese rats does not induce browning at thermoneutrality and induces a muscle-like signature in brown adipose tissue. *Front Endocrinol (Lausanne)*. 2020;11:97. doi:10.3389/fendo.2020.00097

371. Mendham AE, Larsen S, George C, et al. Exercise training results in depot-specific adaptations to adipose tissue mitochondrial function. *Sci Rep*. 2020;10:3785. doi:10.1038/s41598-020-60286-x

372. Raun SH, Henriquez-Olguín C, Karavaeva I, et al. Housing temperature influences exercise training adaptations in mice. *Nat Commun*. 2020;11:1-16. doi:10.1038/s41467-020-15311-y

373. Xu X, Ying Z, Cai M, et al. Exercise ameliorates high-fat diet-induced metabolic and vascular dysfunction, and increases adipocyte progenitor cell population in brown adipose tissue. *Am J Physiol - Regul Integr Comp Physiol*. 2011;300:1115-1125. doi:10.1152/ajpregu.00806.2010

374. Barbosa MA, Guerra-Sá R, De Castro UGM, et al. Physical training improves thermogenesis and insulin pathway, and induces remodeling in white and brown adipose tissues. *J Physiol Biochem*. 2018;74:441-454. doi:10.1007/s13105-018-0637-x

375. De Matteis R, Lucertini F, Guescini M, et al. Exercise as a new physiological stimulus for brown adipose tissue activity. *Nutr Metab Cardiovasc Dis*. 2013;23:582-590. doi:10.1016/j.numecd.2012.01.013

376. Ohyama K, Nogusa Y, Suzuki K, Shinoda K, Kajimura S, Bannai M. A combination of exercise and capsinoid supplementation additively suppresses diet-induced obesity by increasing energy expenditure in mice. *Am J Physiol - Endocrinol Metab*. 2015;308:E315-E323. doi:10.1152/ajpendo.00354.2014

377. Wu M V., Bikopoulos G, Hung S, Ceddia RB. Thermogenic capacity is antagonistically regulated in classical brown and white subcutaneous fat depots by high fat diet and endurance training in rats: Impact on whole-body energy expenditure. *J Biol Chem*. 2014;289:34129-34140. doi:10.1074/jbc.M114.591008

378. Tanaka R, Fuse S, Kuroiwa M, et al. Vigorous-intensity physical activities are associated with high brown adipose tissue density in humans. *Int J Environ Res Public Health*. 2020;17:18-25. doi:10.3390/ijerph17082796

379. Sanchez-Delgado G, Martinez-Tellez B, Garcia-Rivero Y, et al. Association between brown adipose

tissue and bone mineral density in humans. *Int J Obes*. 2019;43:1516-1525. doi:10.1038/s41366-018-0261-4

380. Vosselman MJ, Hoeks J, Brans B, et al. Low brown adipose tissue activity in endurance-trained compared with lean sedentary men. *Int J Obes*. 2015;39:1696-1702. doi:10.1038/ijo.2015.130

381. Motiani P, Teuho J, Saari T, et al. Exercise training alters lipoprotein particles independent of brown adipose tissue metabolic activity. *Obes Sci Pract*. 2019;5:258-272. doi:10.1002/osp4.330

382. Colpani V, Oppermann K, Spritzer PM. Association between habitual physical activity and lower cardiovascular risk in premenopausal, perimenopausal, and postmenopausal women: a population-based study. *Menopause*. 2013;20:525-531. doi:10.1097/gme.0b013e318271b388

383. Ward E, Gold EB, Johnson WO, et al. Patterns of cardiometabolic health as midlife women transition to menopause: a prospective multiethnic study. *J Clin Endocrinol Metab*. 2019;104:1404-1412. doi:10.1210/jc.2018-00941

384. Pillon NJ, Gabriel BM, Dollet L, et al. Transcriptomic profiling of skeletal muscle adaptations to exercise and inactivity. *Nat Commun*. 2020;11:470. doi:10.1038/s41467-019-13869-w.

385. Hsu KJ, Liao C De, Tsai MW, Chen CN. Effects of exercise and nutritional intervention on body composition, metabolic health, and physical performance in adults with sarcopenic obesity: A meta-analysis. *Nutrients*. 2019;11. doi:10.3390/nu11092163

386. MacKenzie-Shalders K, Kelly JT, So D, Coffey VG, Byrne NM. The effect of exercise interventions on resting metabolic rate: a systematic review and meta-analysis. *J Sports Sci*. 2020;38:1635-1649. doi:10.1080/02640414.2020.1754716

387. Bouchonville M, Armamento-Villareal R, Shah K, et al. Weight loss, exercise or both and cardiometabolic risk factors in obese older adults: Results of a randomized controlled trial. *Int J Obes*. 2014;38:423-431. doi:10.1038/ijo.2013.122

388. De Farias JM, Bom KF, Tromm CB, et al. Effect of physical training on the adipose tissue of diet-induced obesity mice: interaction between reactive oxygen species and lipolysis. *Horm Metab Res*. 2013;45:190-196. doi:10.1055/s-0032-1323740

389. Sardeli A V., Komatsu TR, Mori MA, Gáspari AF, Chacon-Mikahil MPT. Resistance training prevents muscle loss induced by caloric restriction in obese elderly individuals: a systematic review and meta-analysis. *Nutrients*. 2018;10:423. doi:10.3390/nu10040423

390. Peterson MD, Sen A, Gordon PM. Influence of resistance exercise on lean body mass in aging adults: a meta-analysis. *Med Sci Sports Exerc*. 2011;43:249-258. doi:10.1249/MSS.0b013e3181eb6265.

391. Giné-Garriga M, Roqué-Fíguls M, Coll-Planas L, Sitjà-Rabert M, Salvà A. Physical exercise interventions for improving performance-based measures of physical function in community-dwelling, frail older adults: a systematic review and meta-analysis. *Arch Phys Med Rehabil*. 2014;95. doi:10.1016/j.apmr.2013.11.007

392. Kammire DE, Walkup MP, Ambrosius WT, et al. Effect of weight change following intentional weight loss on bone health in older adults with obesity. *Obesity*. 2019;27:1839-1845. doi:10.1002/oby.22604

393. Dolan E, Swinton PA, Sale C, Healy A, O'Reilly J. Influence of adipose tissue mass on bone mass in an overweight or obese population: Systematic review and meta-analysis. *Nutr Rev*. 2017;75:858-870. doi:10.1093/nutrit/nux046

394. Sardeli AV, Tomeleri CM, Cyrino ES, Fernhall B, Cavaglieri CR, Chacon-Mikahil MPT. Effect of resistance training on inflammatory markers of older adults: a meta-analysis. *Exp Gerontol*. 2018;111:188-196. doi:10.1016/j.exger.2018.07.021

395. Abd El-Kader SM, Gari AM, Salah El-Den AEM. Impact of moderate versus mild aerobic exercise training on inflammatory cytokines in obese type 2 diabetic patients: A randomized clinical trial. *Afr Health Sci*. 2013;13:857-863. doi:10.4314/ahs.v13i4.1

396. Yu N, Ruan Y, Gao X, Sun J. Systematic review and meta-analysis of randomized, controlled trials on the effect of exercise on serum leptin and adiponectin in overweight and obese individuals. *Horm Metab Res*. 2017;49:164-173. doi:10.1055/s-0042-121605

397. Rostás I, Pótó L, Mátrai P, et al. In middle-aged and old obese patients, training intervention reduces leptin level: A meta-analysis. *PLoS One*. 2017;12:e0182801. doi:10.1371/journal.pone.0182801

398. Kistler-Fischbacher M, Weeks BK, Beck BR. The effect of exercise intensity on bone in postmenopausal women (part 1): a systematic review. *Bone*. 2021;143:115696. doi:10.1016/j.bone.2020.115696

399. Kistler-Fischbacher M, Weeks BK, Beck BR. The effect of exercise intensity on bone in postmenopausal women (part 2): a meta-analysis. *Bone*. 2021;143:115696. doi:10.1016/j.bone.2020.115697

400. Zhao R, Zhao M, Xu Z. The effects of differing resistance training modes on the preservation of bone mineral density in postmenopausal women: a meta-analysis. *Osteoporos Int*. 2015;26:1605-1618. doi:10.1007/s00198-015-3034-0

401. Campbell PT, Campbell KL, Wener MH, et al. A yearlong exercise intervention decreases CRP among obese postmenopausal women. *Med Sci Sports Exerc*. 2009;41:1533-1539. doi:10.1249/MSS.0b013e31819c7feb

402. Tomeleri CM, Ribeiro AS, Souza MF, et al. Resistance training improves inflammatory level, lipid and glycemic profiles in obese older women: a randomized controlled trial. *Exp Gerontol*. 2016;84:80-87. doi:10.1016/j.exger.2016.09.005

403. Saeidi A, Jabbour G, Ahmadian M, et al. Independent and combined effects of antioxidant supplementation and circuit resistance training on selected adipokines in postmenopausal women. *Front Physiol*. 2019;10:484. doi:10.3389/fphys.2019.00484

404. Kolahdouzi S, Baghadam M, Kani-Golzar FA, et al. Progressive circuit resistance training improves inflammatory biomarkers and insulin resistance in obese men. *Physiol Behav*. 2019;205:15-21. doi:10.1016/j.physbeh.2018.11.033

405. Zeng J, Peng L. Comparison of the effect of resistance training on the body compositions of different women groups: a systematic review and meta-analysis of randomized controlled trials. *J Sports Med Phys Fitness*. 2020;60:1118-1127. doi:10.23736/S0022-4707.20.10533-4

406. Nunes PRP, Barcelos LC, Oliveira AA, et al. Effect of resistance training on muscular strength and indicators of abdominal adiposity, metabolic risk, and inflammation in postmenopausal women: controlled and randomized clinical trial of efficacy of training volume. *Age (Omaha)*. 2016;38. doi:10.1007/s11357-016-9901-6

407. Gadelha AB, Paiva FML, Gauche R, de Oliveira RJ, Lima RM. Effects of resistance training on sarcopenic obesity index in older women: A randomized controlled trial. *Arch Gerontol Geriatr*. 2016;65:168-173. doi:10.1016/j.archger.2016.03.017

408. Nunes PRP, Barcelos LC, Oliveira AA, et al. Effect of resistance training on muscular strength and indicators of abdominal adiposity, metabolic risk, and inflammation in postmenopausal women: controlled and randomized clinical trial of efficacy of training volume. *Age (Omaha)*. 2016;38. doi:10.1007/s11357-016-9901-6

409. ten Hoor GA, Kok G, Peters GJY, Frissen T, Schols AMWJ, Plasqui G. The psychological effects of strength exercises in people who are overweight or obese: a systematic review. *Sport Med*. 2017;47:2069-2081. doi:10.1007/s40279-017-0748-5

410. Thein LA, Thein JM, Landry GL. Ergogenic aids. *Phys Ther*. 1995;75:426-439. doi:10.1093/ptj/75.5.426

411. Philpott JD, Witard OC, Galloway SDR. Applications of omega-3 polyunsaturated fatty acid supplementation for sport performance. *Res Sport Med*. 2019;27:219-237. doi:10.1080/15438627.2018.1550401

412. Smith GI, Julliand S, Reeds DN, Sinacore DR, Klein S, Mittendorfer B. Fish oil-derived n-3 PUFA therapy increases muscle mass and function in healthy older adults. *Am J Clin Nutr*. 2015;102:115-122. doi:10.3945/ajcn.114.105833

413. Lalia AZ, Dasari S, Robinson MM, et al. Influence of omega-3 fatty acids on skeletal muscle protein metabolism and mitochondrial bioenergetics in older adults. *Aging (Albany NY)*. 2017;9:1096-1129. doi:10.18632/aging.101210

414. Strandberg E, Edholm P, Ponsot E, et al. Influence of combined resistance training and healthy diet on muscle mass in healthy elderly women: a randomized controlled trial. *J Appl Physiol*. 2015;119:918-925. doi:10.1152/japplphysiol.00066.2015

415. Strandberg E, Ponsot E, Piehl-Aulin K, Falk G, Kadi F. Resistance training alone or combined with n-3 PUFA-rich diet in older women: effects on muscle fiber hypertrophy. *Journals Gerontol - Ser A Biol Sci Med Sci*. 2019;74:489-493. doi:10.1093/gerona/gly130

416. Rodacki CLN, Rodacki ALF, Pereira G, et al. Fish-oil supplementation enhances the effects of strength training in elderly women. *Am J Clin Nutr*. 2012;95:428-436. doi:10.3945/ajcn.111.021915

417. Da Boit M, Sibson R, Sivasubramaniam S, et al. Sex differences in the effect of fish-oil supplementation on the adaptive response to resistance exercise training in older people: a randomized controlled trial. *Am J Clin Nutr*. 2017;105:151-158. doi:10.3945/ajcn.116.140780

418. Pastor R, Tur JA. Response to exercise in older adults who take supplements of antioxidants and/or omega-3 polyunsaturated fatty acids: A systematic review. *Biochem Pharmacol*. 2020;173:113649. doi:10.1016/j.bcp.2019.113649

419. Cornish SM, Myrie SB, Bugera EM, Chase JE, Turczyn D, Pinder M. Omega-3 supplementation with resistance training does not improve body composition or lower biomarkers of inflammation more so than resistance training alone in older men. *Nutr Res*. 2018;60:87-95. doi:10.1016/j.nutres.2018.09.005

420. Logan SL, Spriet LL. Omega-3 fatty acid supplementation for 12 weeks increases resting and exercise metabolic rate in healthy community- dwelling older females. *PLoS One*. 2015;10:e0144828. doi:10.1371/journal.pone.0144828

421. Lv ZT, Zhang JM, Zhu WT. Omega-3 polyunsaturated fatty acid supplementation for reducing muscle soreness after eccentric exercise: a systematic review and meta-analysis of randomized controlled trials. *Biomed Res Int*. 2020;2020:8062017. doi:10.1155/2020/8062017

422. Anthony R, Macartney MJ, Peoples GE. The influence of long-chain omega-3 fatty acids on eccentric exercise-induced delayed muscle soreness: reported outcomes are compromised by study design issues. *Int J Sport Nutr Exerc Metab*. 2021;Jan 20:1-11. doi:10.1123/ijsnem.2020-0238

423. Dad'ová K, Petr M, Šteffl M, et al. Effect of calanus oil supplementation and 16 week exercise program on selected fitness parameters in older women. *Nutrients*. 2020;12:481. doi:10.3390/nu12020481

424. Brezinova M, Cajka T, Oseeva M, et al. Exercise training induces insulin-sensitizing PAHSAs in adipose tissue of elderly women. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2020;1865:158576. doi:10.1016/j.bbalip.2019.158576

425. Lee SR, Khamoui A V., Jo E, et al. Effect of conjugated linoleic acids and omega-3 fatty acids with or without resistance training on muscle mass in high-fat diet-fed middle-aged mice. *Exp Physiol*. 2017;102:1500-1512. doi:10.1113/EP086317

426. Barbeau PA, Holloway TM, Whitfield J, et al. α-Linolenic acid and exercise training independently, and additively, decrease blood pressure and prevent diastolic dysfunction in obese Zucker rats. *J Physiol*. 2017;595:4351-4364. doi:10.1113/JP274036

427. Livshits G, Kalinkovich A. Inflammaging as a common ground for the development and maintenance of sarcopenia, obesity, cardiomyopathy and dysbiosis. *Ageing Res Rev*. 2019;56:100980. doi:10.1016/j.arr.2019.100980

428. Kim K-B, Kim K, Kim C, et al. Effects of exercise on the body composition and lipid profile of individuals with obesity: a systematic review and meta-analysis. *J Obes Metab Syndr*. 2019;28:278-294. doi:10.7570/jomes.2019.28.4.278

429. Nilsson MI, Bourgeois JM, Nederveen JP, et al. Lifelong aerobic exercise protects against inflammaging and cancer. *PLoS One*. 2019;14:e0210863. doi:10.1371/journal.pone.0210863

430. Levine ME, Lu AT, Chen BH, et al. Menopause accelerates biological aging. *Proc Natl Acad Sci U S A*. 2016;113:9327-9332. doi:10.1073/pnas.1604558113

431. Hill AM, Buckley JD, Murphy KJ, Howe PRC. Combining fish-oil supplements with regular aerobic exercise improves body composition and cardiovascular disease risk factors. *Am J Clin Nutr*. 2007;85:1267-1274. doi:10.1093/ajcn/85.5.1267

432. DeFina LF, Marcoux LG, Devers SM, Cleaver JP, Willis BL. Effects of omega-3 supplementation in combination with diet and exercise on weight loss and body composition. *Am J Clin Nutr*. 2011;93:455-462. doi:10.3945/ajcn.110.002741

433. World Health Organization. *Men, Ageing and Health: Achieving Health across the Life Span*.; 2001.

434. Stewart A, Marfell-Jones M, Olds T, de Ridder H. *International Standards for Anthropometric Assessment*. New Zealand: ISAK: LowerHutt; 2011.

435. Aranceta Bartrina J, Arija Val V V., Maíz Aldalur E, et al. Dietary Guidelines for the Spanish

population (SENC, diciembre 2016); the new graphic icon of healthy food. *Nutr Hosp*. 2016;33:e95581. doi:10.20960/nh.827

436. Schröder H, Fitó M, Estruch R, et al. A short screener is valid for assessing mediterranean diet adherence among older spanish men and women. *J Nutr*. 2011;141:1140-1145. doi:10.3945/jn.110.135566

437. Martínez-González MA, López-Fontana C, Varo JJ, Sánchez-Villegas A, Martinez JA. Validation of the Spanish version of the physical activity questionnaire used in the Nurses' Health Study and the Health Professionals' Follow-up Study. *Public Health Nutr*. 2005;8:920-927. doi:10.1079/phn2005745

438. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *J Am Coll Dent*. 2014;81:14-18. doi:10.1093/acprof:oso/9780199241323.003.0025

439. Munro IA, Garg ML. Prior supplementation with long chain omega-3 polyunsaturated fatty acids promotes weight loss in obese adults: a double-blinded randomised controlled trial. *Food Funct*. 2013;4:650-658. doi:10.1039/c3fo60038f

440. Munro IA, Garg ML. Dietary supplementation with long chain omega-3 polyunsaturated fatty acids and weight loss in obese adults. *Obes Res Clin Pract*. 2013;7:e173-e181. doi:10.1016/j.orcp.2011.11.001

441. Munro IA, Garg ML. Dietary supplementation with n-3 PUFA does not promote weight loss when combined with a very-low-energy diet. *Br J Nutr*. 2012;108:1466-1474. doi:10.1017/S0007114511006817

442. Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). *EFSA J*. 2012;10:2815. doi:10.2903/j.efsa.2012.2815

443. Mataix-Verdú J, Mañas Almendros M, Llopis González J, Martínez de Victoria E. *Tablas de Composición de Alimentos*. Universidad de Granada; 1993.

444. Cuff DJ, Meneilly GS, Martin A, Ignaszewski A, Tildesley HD, Frohlich JJ. Effective exercise modality to reduce insulin resistance in women with type 2 diabetes. *Diabetes Care*. 2003;26:2977-2982. doi:10.2337/diacare.26.11.2977

445. American College of Sports Medicine. Progression models in resistance training for healthy adults. *Med Sci Sports Exerc*. 2009;41:687-708. doi:10.1249/MSS.0b013e3181915670

446. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*. 1972;18:499-502. doi:10.1093/clinchem/18.6.499

447. Simental-Mendía LE, Rodríguez-Morán M, Guerrero-Romero F. The product of fasting glucose and triglycerides as surrogate for identifying insulin resistance in apparently healthy subjects. *Metab Syndr Relat Disord*. 2008;6:299-304. doi:10.1089/met.2008.0034

448. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28:412-419. doi:10.1007/BF00280883

449. Frühbeck G, Catalán V, Rodríguez A, Gómez-Ambrosi J. Adiponectin-leptin ratio: a promising index to estimate adipose tissue dysfunction. Relation with obesity-associated cardiometabolic risk. *Adipocyte*. 2018;7:57-62. doi:10.1080/21623945.2017.1402151

450. Fest J, Ruiter R, Ikram MA, Voortman T, Van Eijck CHJ, Stricker BH. Reference values for white blood-cell-based inflammatory markers in the Rotterdam Study: a population-based prospective cohort study. *Sci Rep*. 2018;8:10566. doi:10.1038/s41598-018-28646-w

451. Graja A, Schulz TJ. Mechanisms of aging-related impairment of brown adipocyte development and function. *Gerontology*. 2015;61:211-217. doi:10.1159/000366557

452. Rogers NH, Landa A, Park S, Smith RG. Aging leads to a programmed loss of brown adipocytes in murine subcutaneous white adipose tissue. *Aging Cell*. 2012;11:1074-1083. doi:10.1111/acel.12010

453. Itariu BK, Zeyda M, Hochbrugger EE, et al. Long-chain n-3 PUFAs reduce adipose tissue and systemic inflammation in severely obese nondiabetic patients: A randomized controlled trial. *Am J Clin Nutr*. 2012;96:1137-1149. doi:10.3945/ajcn.112.037432

454. Bentham J, Di Cesare M, Bilano V, et al. Worldwide trends in body-mass index, underweight,

overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. *Lancet*. 2017;390:2627-2642. doi:10.1016/S0140-6736(17)32129-3

455. van Greevenbroek MMJ, Schalkwijk CG, Stehouwer CDA. Dysfunctional adipose tissue and low-grade inflammation in the management of the metabolic syndrome: current practices and future advances. *F1000Research*. 2016;5:F1000 Faculty Rev-2515. doi:10.12688/f1000research.8971.1

456. Palmer AK, Kirkland JL. Aging and adipose tissue: potential interventions for diabetes and regenerative medicine. *Exp Gerontol*. 2016;86:97-105. doi:10.1016/j.exger.2016.02.013

457. Campbell WW, Kraus WE, Powell KE, et al. High-intensity interval training for cardiometabolic disease prevention. *Med Sci Sport Exerc*. 2019;51:1220-1226. doi:10.1249/MSS.0000000000001934

458. Skulas-Ray AC, Wilson PWF, Harris WS, et al. Omega-3 fatty acids for the management of hypertriglyceridemia: a science advisory from the American Heart Association. *Circulation*. 2019;140:E673-E691. doi:10.1161/CIR.0000000000000709

459. Moon HJ, Kim TH, Byun DW, Park Y. Positive correlation between erythrocyte levels of n-3 polyunsaturated fatty acids and bone mass in postmenopausal Korean women with osteoporosis. *Ann Nutr Metab*. 2012;60:146-153. doi:10.1159/000337302

460. Dipietro L, Campbell WW, Buchner DM, et al. Physical activity, injurious falls, and physical function in aging. *Med Sci Sport Exerc*. 2019;51:1303-1313. doi:10.1249/MSS.0000000000001942

461. Kumanyika SK, Obarzanek E, Stettler N, et al. Population-based prevention of obesity: the need for comprehensive promotion of healthful eating, physical activity, and energy balance: A scientific statement from American Heart Association Council on Epidemiology and Prevention, Interdisciplinary Commi. *Circulation*. 2008;118:428-464. doi:10.1161/CIRCULATIONAHA.108.189702

462. Zamboni M, Mazzali G. Obesity in the elderly: an emerging health issue. *Int J Obes*. 2012;36:1151-1152. doi:10.1038/ijo.2012.120

463. Valencak TG, Osterrieder A, Schulz TJ. Sex matters: the effects of biological sex on adipose tissue biology and energy metabolism. *Redox Biol*. 2017;12:806-813. doi:10.1016/j.redox.2017.04.012

464. Laurson KR, McCann DA, Senchina DS. Age, sex, and ethnicity may modify the influence of obesity on inflammation. *J Investig Med*. 2011;59:27-31. doi:10.2310/JIM.0b013e318200151a

465. Liu KA, Dipietro Mager NA. Women's involvement in clinical trials: historical perspective and future implications. *Pharm Pract (Granada)*. 2016;14:708. doi:10.18549/PharmPract.2016.01.708

466. Zucker I, Beery AK. Males still dominate animal studies. *Nature*. 2010;465:690. doi:10.1038/465690a

467. Gallego MPO, López PB, Armero MAT, Alemán JA, Albero JS, López PJT. Prevalencia del síndrome metabólico y sus componentes en mujeres menopáusicas españolas. *Nutr Hosp*. 2015;32:656-666. doi:10.3305/nh.2015.32.2.9211

468. Flurkey K, Currer J, Harrison D. *The Mouse in Aging Research*. (Fox JG et al, ed.). American College Laboratory Animal Medicine (Elsevier); 2007.

469. Xu X, Liu C, Xu Z, et al. Altered adipocyte progenitor population and adipose-related gene profile in adipose tissue by long-term high-fat diet in mice. *Life Sci*. 2012;90:1001-1009. doi:10.1016/j.lfs.2012.05.016

470. Bargut TCL, Souza-Mello V, Mandarim-De-Lacerda CA, Aguila MB. Fish oil diet modulates epididymal and inguinal adipocyte metabolism in mice. *Food Funct*. 2016;7:1468-1476. doi:10.1039/c5fo00909j

471. Burhans MS, Hagman DK, Kuzma JN, Schmidt KA, Kratz M. Contribution of adipose tissue inflammation to the development of type 2 diabetes mellitus. *Compr Physiol*. 2018;9:1-58. doi:10.1002/cphy.c170040

472. Raclot T, Groscolas R, Langin D, Ferré P. Site-specific regulation of gene expression by n-3 polyunsaturated fatty acids in rat white adipose tissues. *J Lipid Res*. 1997;38:1963-1972. doi:10.1016/S0022-2275(20)37127-3

473. Hensler M, Bardova K, Jilkova Z, et al. The inhibition of fat cell proliferation by n-3 fatty acids in dietary obese mice. *Lipids Health Dis*. 2011;10:128. doi:10.1186/1476-511X-10-128

474. Rokling-Andersen MH, Rustan AC, Wensaas AJ, et al. Marine n-3 fatty acids promote size reduction of visceral adipose depots, without altering body weight and composition, in male Wistar rats fed a high-fat diet. *Br J Nutr*. 2009;102:995-1006. doi:10.1017/S0007114509353210

475. Engin A. Adiponectin-resistance in obesity. *Adv Exp Med Biol*. 2017;960:415-441. doi:10.1007/978-3-319-48382-5_18

476. Nguyen KD, Qiu Y, Cui X, et al. Alternatively activated macrophages produce catecholamines to sustain adaptive thermogenesis. *Nature*. 2011;480:104-108. doi:10.1038/nature10653

477. Kim M, Goto T, Yu R, et al. Fish oil intake induces UCP1 upregulation in brown and white adipose tissue via the sympathetic nervous system. *Sci Rep*. 2015;5:18013. doi:10.1038/srep18013

478. Bargut TCL, Martins FF, Santos LP, Aguila MB, Mandarim-de-Lacerda CA. Administration of eicosapentaenoic and docosahexaenoic acids may improve the remodeling and browning in subcutaneous white adipose tissue and thermogenic markers in brown adipose tissue in mice. *Mol Cell Endocrinol*. 2019;482:18-27. doi:10.1016/j.mce.2018.12.003

479. Serhan CN, Yang R, Martinod K, et al. Maresins: Novel macrophage mediators with potent antiinflammatory and proresolving actions. *J Exp Med*. 2009;206:15-23. doi:10.1084/jem.20081880

480. Hellmann J, Tang Y, Kosuri M, Bhatnagar A, Spite M. Resolvin D1 decreases adipose tissue macrophage accumulation and improves insulin sensitivity in obese-diabetic mice. *FASEB J*. 2011;25:2399-2407. doi:10.1096/fj.10-178657

481. Villarroya J, Gallego-Escuredo JM, Delgado-Anglés A, et al. Aging is associated with increased FGF21 levels but unaltered FGF21 responsiveness in adipose tissue. *Aging Cell*. 2018;17:e12822. doi:10.1111/acel.12822

482. Milet C, Bléher M, Allbright K, et al. Egr1 deficiency induces browning of inguinal subcutaneous white adipose tissue in mice. *Sci Rep*. 2017;7:16153. doi:10.1038/s41598-017-16543-7

483. Bléher M, Meshko B, Cacciapuoti I, et al. Egr1 loss-of-function promotes beige adipocyte differentiation and activation specifically in inguinal subcutaneous white adipose tissue. *Sci Rep*. 2020;10:15842. doi:10.1038/s41598-020-72698-w

484. Ichimura A, Hirasawa A, Poulain-Godefroy O, et al. Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. *Nature*. 2012;483:350-354. doi:10.1038/nature10798

485. Chen Y, Zhang D, Ho KW, et al. GPR120 is an important inflammatory regulator in the development of osteoarthritis. *Arthritis Res Ther*. 2018;20:163. doi:10.1186/s13075-018-1660-6

486. Houtkooper RH, Argmann C, Houten SM, et al. The metabolic footprint of aging in mice. *Sci Rep*. 2011;1:134. doi:10.1038/srep00134

487. Villarroya J, Flachs P, Redondo-Angulo I, et al. Fibroblast growth factor-21 and the beneficial effects of long-chain n-3 polyunsaturated fatty acids. *Lipids*. 2014;49:1081-1089. doi:10.1007/s11745-014-3948-x

488. Muzumdar R, Allison DB, Huffman DM, et al. Visceral adipose tissue modulates mammalian longevity. *Aging Cell*. 2008;7:438-440. doi:10.1111/j.1474-9726.2008.00391.x

489. Kuk JL, Katzmarzyk PT, Nichaman MZ, Church TS, Blair SN, Ross R. Visceral fat is an independent predictor of all-cause mortality in men. *Obesity*. 2006;14:336-341. doi:10.1038/oby.2006.43

490. Huffman DM, Barzilai N. Role of visceral adipose tissue in aging. *Biochim Biophys Acta - Gen Subj*. 2009;1790:1117-1123. doi:10.1016/j.bbagen.2009.01.008

491. Jelenik T, Rossmeisl M, Kuda O, et al. AMP-activated protein kinase α2 subunit is required polyunsaturated fatty acids. *Diabetes*. 2010;59:2737-2746. doi:10.2337/db09-1716.

492. Yang J, Sáinz N, Félix-soriano E, et al. Effects of long-term DHA supplementation and physical exercise on non-alcoholic fatty liver development in obese aged female mice. *Nutrients*. 2021;13:501. doi:10.3390/nu13020501

493. Srikakulapu P, Mcnamara CA. B lymphocytes and adipose tissue inflammation. *Arterioscler Thromb Vasc Biol*. 2020;40:1110-1122. doi:10.1161/ATVBAHA.119.312467

494. Seale P, Kajimura S, Yang W, et al. Transcriptional control of brown fat determination by PRDM16. *Cell Metab*. 2007;6:38-54. doi:10.1016/j.cmet.2007.06.001

495. Harms MJ, Ishibashi J, Wang W, et al. Identity and function in adult mice. *Cell Metab*. 2014;19:593-604. doi:10.1016/j.cmet.2014.03.007.

496. Félix-Soriano E, Sáinz N, Gil-Iturbe E, et al. Changes in brown adipose tissue lipid mediator signatures with aging, obesity, and DHA supplementation in female mice. *FASEB J*. 2021;00:e21592. doi:10.1096/fj.202002531R

497. Geng L, Liao B, Jin L, et al. Exercise alleviates obesity-induced metabolic dysfunction via enhancing FGF21 sensitivity in adipose tissues. *Cell Rep*. 2019;26:2738-2752.e4. doi:10.1016/j.celrep.2019.02.014

498. Frederiksen L, Nielsen TL, Wraae K, et al. Subcutaneous rather than visceral adipose tissue is associated with adiponectin levels and insulin resistance in young men. *J Clin Endocrinol Metab*. 2009;94:4010-4015. doi:10.1210/jc.2009-0980

499. Ahtiainen JP, Sallinen J, Häkkinen K, Sillanpää E. Inter-individual variation in response to resistance training in cardiometabolic health indicators. *Scand J Med Sci Sport*. 2020;30:1040-1053. doi:10.1111/sms.13650

500. Ralston GW, Kilgore L, Wyatt FB, Buchan D, Baker JS. Weekly training frequency effects on strength gain: a meta-analysis. *Sport Med - Open*. 2018;4:36. doi:10.1186/s40798-018-0149-9

501. Ihalainen JK, Inglis A, Mäkinen T, et al. Strength training improves metabolic health markers in older individual regardless of training frequency. *Front Physiol*. 2019;10:32. doi:10.3389/fphys.2019.00032

502. Cunha PM, Tomeleri CM, Nascimento MA, et al. Comparision of low and high volume of resistance training on body fat and blood biomarkers in untrained older women: a randomized clinical trial. *J Strength Cond Res*. 2019;35:1-8. doi:10.1519/jsc.0000000000003245

503. Boutcher SH, Dunn SL. Factors that may impede the weight loss response to exercise-based interventions. *Obes Rev*. 2009;10:671-680. doi:10.1111/j.1467-789X.2009.00621.x

504. Churchward-Venne TA, Tieland M, Verdijk LB, et al. There are no nonresponders to resistance-type exercise training in older men and women. *J Am Med Dir Assoc*. 2015;16:400-411. doi:10.1016/j.jamda.2015.01.071

505. Klonoff EA, Annechild A, Landrine H. Predicting exercise adherence in women: the role of psychological and physiological factors. *Prev Med (Baltim)*. 1994;23:257-262. doi:10.1006/pmed.1994.1036

506. Schedlowski M, Enck P, Rief W, Bingel U. Neuro-bio-behavioral mechanisms of placebo and nocebo responses: implications for clinical trials and clinical practice. *Pharmacol Rev*. 2015;67:697-730. doi:10.1124/pr.114.009423

507. Kozłowski S, Chwalbińska-Moneta J, Vigš M, Kaciuba-UŚciłko H, Nazar K. Greater serum GH response to arm than to leg exercise performed at equivalent oxygen uptake. *Eur J Appl Physiol Occup Physiol*. 1983;52:131-135. doi:10.1007/BF00429040

508. Aminoff T, Smolander J, Korhonen O, Louhevaara V. Prediction of acceptable physical work loads based on responses to prolonged arm and leg exercise. *Ergonomics*. 1998;41:109-120. doi:10.1080/001401398187350

509. Bergman BC, Perreault L, Strauss A, et al. Intramuscular triglyceride synthesis: Importance in muscle lipid partitioning in humans. *Am J Physiol - Endocrinol Metab*. 2018;314:E152-E164. doi:10.1152/ajpendo.00142.2017

510. Hurley BF, Nemeth PM, Martin WH, Hagberg JM, Dalsky GP, Holloszy JO. Muscle triglyceride utilization during exercise: Effect of training. *J Appl Physiol*. 1985;60:562-567. doi:10.1152/jappl.1986.60.2.562

511. Mosca L, Banka CL, Benjamin EJ, et al. Evidence-based guidelines for cardiovascular disease prevention in women: 2007 Update. *Circulation*. 2007;115:1481-1501. doi:10.1161/CIRCULATIONAHA.107.181546

512. Curtis EM, Harvey NC, D'Angelo S, et al. Bone mineral content and areal density, but not bone area, predict an incident fracture risk: a comparative study in a UK prospective cohort. *Arch Osteoporos*. 2016;11:39. doi:10.1007/s11657-016-0293-0

513. Watson SL, Weeks BK, Weis LJ, Harding AT, Horan SA, Beck BR. High-intensity resistance and impact training improves bone mineral density and physical function in postmenopausal women with osteopenia and osteoporosis: the LIFTMOR randomized controlled trial. *J Bone Miner Res*. 2018;33:211-220. doi:10.1002/jbmr.3284

514. Holzer G, Von Skrbensky G, Holzer LA, Pichl W. Hip fractures and the contribution of cortical versus

trabecular bone to femoral neck strength. *J Bone Miner Res*. 2009;24:468-474. doi:10.1359/jbmr.081108

515. Liu TT, Li XD, Wang WZ, Zhang JG, Yang DZ. Efficacy of weight adjusted bone mineral content in osteoporosis diagnosis in Chinese female population. *Chin Med J (Engl)*. 2019;132:772-781. doi:10.1097/CM9.0000000000000143

516. Diaz Brinton R. Minireview: translational animal models of human menopause: challenges and emerging opportunities. *Endocrinology*. 2012;153:3571-3578. doi:10.1210/en.2012-1340

517. Cholewa J, Guimarães-Ferreira L, da Silva Teixeira T, et al. Basic models modeling resistance training: an update for basic scientists interested in study skeletal muscle hypertrophy. *J Cell Physiol*. 2014;229:1148-1156. doi:10.1002/jcp.24542

518. Manzanares G, Brito-Da-Silva G, Gandra PG. Voluntary wheel running: patterns and physiological effects in mice. *Brazilian J Med Biol Res*. 2019;52:e7830. doi:10.1590/1414-431X20187830

519. Colson C, Ghandour RA, Dufies O, et al. Diet supplementation in ω3 polyunsaturated fatty acid favors an anti-inflammatory basal environment in mouse adipose tissue. *Nutrients*. 2019;11:438. doi:10.3390/nu11020438

520. Mas E, Croft KD, Zahra P, Barden A, Mori TA. Resolvins D1, D2, and other mediators of self-limited resolution of inflammation in human blood following n-3 fatty acid supplementation. *Clin Chem*. 2012;58:1476-1484. doi:10.1373/clinchem.2012.190199

521. Polus A, Zapala B, Razny U, et al. Omega-3 fatty acid supplementation influences the whole blood transcriptome in women with obesity, associated with pro-resolving lipid mediator production. *Biochim Biophys Acta - Mol Cell Biol Lipids*. 2016;1861:1746-1755. doi:10.1016/j.bbalip.2016.08.005

522. Barden AE, Mas E, Croft KD, Phillips M, Mori TA. Specialized proresolving lipid mediators in humans with the metabolic syndrome after n-3 fatty acids and aspirin. *Am J Clin Nutr*. 2015;102:1357-1364. doi:10.3945/ajcn.115.116384

523. Walker CG, Browning LM, Mander AP, et al. Age and sex differences in the incorporation of EPA and DHA into plasma fractions, cells and adipose tissue in humans. *Br J Nutr*. 2014;111:679-689. doi:10.1017/S0007114513002985

524. Mengel LA, Seidl H, Brandl B, et al. Gender differences in the response to short-term cold exposure in young adults. *J Clin Endocrinol Metab*. 2020;105:dgaa110. doi:10.1210/clinem/dgaa110

525. Fletcher LA, Kim K, Leitner BP, et al. Sexual dimorphisms in adult human brown adipose tissue. *Obesity*. 2020;28:241-246. doi:10.1002/oby.22698

526. Varghese M, Griffin C, McKernan K, Eter L, Abrishami S, Kanakadurga S. Female adipose tissue has improved adaptability and metabolic health compared to males in aged obesity. *Aging (Albany NY)*. 2020;12:1725-1746. doi:10.18632/aging.102709