EFFECTS OF FISH OIL AND ITS COMBINATION WITH GRAPE SEED POLYPHENOLS OR BUCKWHEAT D-FAGOMINE ON CARDIOMETABOLIC RISK FACTORS AND OXIDATIVE STRESS IN RATS

Bernat Miralles Pérez

UNIVERSITAT ROVIRA i VIRGILI

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DOCTORAL THESIS
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Supervised by Dr. Marta Romeu Ferran

Department of Basic Medical Sciences

Functional Nutrition, Oxidation and Cardiovascular Diseases Research Group

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I STATE that the present study, entitled "Effects of Fish Oil and its Combination with Grape Seed Polyphenols or Buckwheat D-Fagomine on Cardiometabolic Risk Factors and Oxidative Stress and in Rats", presented by Bernat Miralles Pérez for the award of the degree of Doctor, has been carried out under my supervision at the Department of Basic Medical Sciences of this university.

Reus, May 24, 2021

Doctoral Thesis Supervisor

Marta Romeu Ferran

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Acknowledgements

The present doctoral thesis was supported by the Spanish Ministry of Economy and Competitiveness (grant numbers AGL2009-12374-C03-02, AGL2013-49079-C2-1,2-R, AGL2017-83599-R and RTI2018-095659-B-I00). Furthermore, this doctoral thesis has been possible with the support of the Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitat de Catalunya, the European Union (EU) and the European Social Fund (ESF) (2018 FI_B 00919, 2019 FI_B1 00160, 2020 FI_B2 00119). The Universitat Rovira i Virgili is also gratefully acknowledged for the early doctoral fellowship (2017PMF-PIPF-38), mobility grant (AEE2019–Biomedicine) and Open Access publishing support (2020OPEN-15).

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Abbreviations

$^{\bullet}OH$	Hydroxyl radical
$^{1}O_2$	Singlet oxygen
4-HHE	4-hydroxyhexanal
4-HNE	4-hydroxynonenal
ALA	α-linolenic acid
ALT	Alanine aminotransferase
AP-1	Activator protein 1
ARA	Arachidonic acid
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
ATP III	National Cholesterol Education Program Adult Treatment Panel III
CAT	Catalases
COX	Cyclooxygenases
CRP	C-reactive protein
CVD	Cardiovascular diseases
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
EFSA	European Food Safety Agency
EPA	Eicosapentaenoic acid
FG	D-fagomine
FRAP	Ferric reducing ability of plasma
GLUT	Glucose transporters
GPR120	G-protein-coupled receptor 120
GPx	Glutathione peroxidases
GR	Glutathione reductases
GSE	Grape seed extract
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferases
H_2O_2	Hydrogen peroxide
HbA1c	Glycated hemoglobin
HDL-C	High-density lipoprotein cholesterol
HF	High-fat
IDF	International Diabetes Federation
IKK	IκB kinase
IL-6	Interleukin-6
INSR	Insulin receptor tyrosine kinase
IRS1	Insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
Keap1	Kelch like ECH associated protein 1

$L^{\bullet}$	Lipid radical
LDL	Low-density lipoprotein particles
LDL-C	Low-density lipoprotein cholesterol
LOH	Lipid alcohol
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid hydroperoxide
LOX	Lipoxygenases
MDA	Malondialdehyde
NAFLD	Non-alcoholic fatty liver disease
NEAC	Non-enzymatic antioxidant capacity
NEFA	Non-esterified fatty acids
NF-κB	Nuclear factor kappa B
NOX	NADPH oxidases
Nrf2	Nuclear factor-erythroid 2-related factor 2
$O_2^{\bullet-}$	Superoxide anion radical
OH^{-}	Hydroxyl anion
ORAC	Oxygen radical absorbance capacity
PI3K	Phosphatidylinositol 3 kinase
PLA_2	Phospholipases A_2
PPAR	Peroxisome proliferator-activated receptor
PTP1B	Protein tyrosine phosphatase 1B
PUFA	Polyunsaturated fatty acids
$RO^{\bullet}$	Alkoxyl radical
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
SCD	Stearoyl CoA desaturase
SFA	Saturated fatty acids
SOD	Superoxide dismutases
TBARS	Thiobarbituric Acid-Reactive Substances
TLR	Toll-like receptors
TNFα	Tumor necrosis factor α
VLDL	Very low-density lipoprotein particles
WHO	World Health Organization
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

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Preface

PREFACE

PREFACE

Worldwide prevalence of cardiometabolic diseases such as obesity and diabetes have alarmingly increased in the past decades. The Word Health Organization have reported that there are about 650 million adults with obesity (2016), and about 422 million adults with diabetes (2014), which in turn increase the risk for developing cardiovascular events. In parallel, significant changes in lifestyle and eating habits have been seen in human populations. Traditional dietary patterns, which are based on local availability of foods and highly related to culture and traditions, have shifted to a Westernized dietary pattern. The western diet provides highly processed and palatable foods with low nutritional quality, containing high amounts of saturated fatty acids (SFA), omega-6 polyunsaturated fatty acids (ω-6 PUFA), refined sugars, and overall energy.

Exceeding energy demands of the body together with low energy expenditure plays a major role in the onset, development, and progression of cardiometabolic diseases in mammals by affecting metabolic and inflammatory pathways. Overload of dietary fat promotes production of reactive species including oxygen species, nitrogen species and carbonyl species, which are oxidants that lead to oxidative stress. Oxidative stress is by definition “an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage” [1]. There are two classes of oxidative stress based on its intensity: oxidative eustress and oxidative distress [2]. On one hand, oxidative eustress is a well-controlled, low-intensity and reversible increase in oxidative stress—e.g., postprandial oxidative stress [2]. Oxidative eustress is essential for normal functioning of numerous cell processes, including metabolic signaling, reversible oxidative modification of proteins, immune response, proliferation, differentiation and apoptosis [3,4]. On the other hand, oxidative

distress is a high-intensity oxidative stress that induces irreversible oxidative damage to biomolecules such as lipids, proteins and nucleic acids, resulting in disturbance in redox signaling pathways that can evolve into a pathological condition [2]. In the present thesis, the term oxidative stress is used to refer to oxidative distress. Oxidative stress results from excessive quantity of oxidants or a down-regulation or depletion of antioxidants. The maintenance of appropriate redox homeostasis is a critical event to prevent disease (Figure 1).

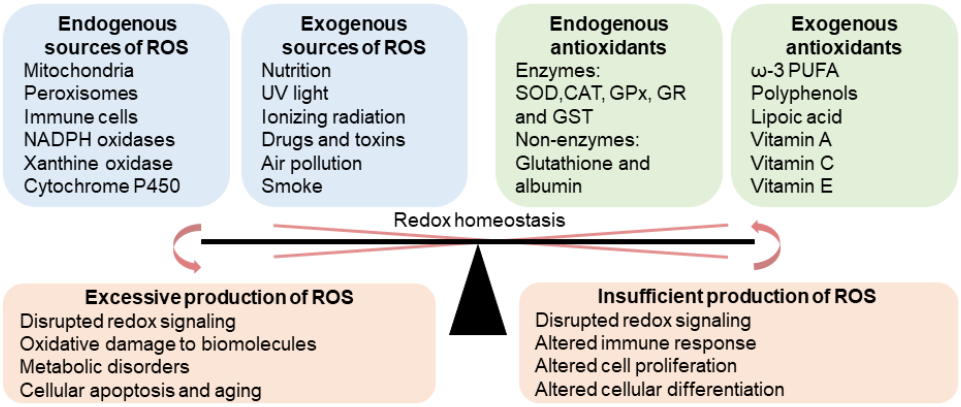

Figure 1. Key players in oxidative stress and related health outcomes. Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; ω-3 PUFA, omega-3 polyunsaturated fatty acids.

Oxidative stress is associated with normal aging and with numerous metabolic disorders, including obesity, insulin resistance and metabolic syndrome [5,6]. A major challenge in the field of oxidative stress research is to find reliable biomarkers for the early prediction of disease and the onset and monitoring of disease progression [7]. Numerous biomarkers of oxidative stress have been described in disease [2,8]. As shown in Table 1, there are five groups of

biomarkers [9]. Biomarkers in the Group Type 0 are direct measurements of ROS, whereas the other groups (Type 1–4) are indirect measurements of ROS. The indirect methods include: (1) measurements of oxidatively damaged biomolecules, (2) stable product of oxidative stress, (3) ROS-producing and antioxidant enzymes as well as non-enzymatic antioxidants, and finally (4) genetic factors and mutations in ROS-producing or antioxidant enzymes.

Table 1. Classification of oxidative stress biomarkers *.

Type 0	Specific ROS (e.g., $O_2^{\bullet-}$ and H_2O_2)
Type 1	Oxidatively damaged biomolecules (e.g., protein carbonyls, GSSG/GSH, oxidized LDL, conjugated diene hydroperoxides, TBARS, MDA, 4-HNE and 4-HHE)
Type 2	Stable product of ROS-producing enzymes (e.g., acid uric)
Type 3	ROS-producing and antioxidant enzyme activities as well as non-enzymatic antioxidants (e.g., XO, SOD, CAT, GPx, GR, GSSG/GSH, ORAC and FRAP)
Type 4	Genetic factors and mutations in ROS-producer or antioxidant enzymes

* Adapted from: Ghezzi, 2020 [9].
Abbreviations: ROS, reactive oxygen species; GSSG, oxidized glutathione; GSH, reduced glutathione; LDL, low-density lipoprotein; TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; 4-HHE, 4-hydroxyhexenal; XO, xanthine oxidase; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; ORAC, oxygen radical absorbance capacity; FRAP, ferric reducing ability of plasma.

Because ROS have high reactivity and are highly removed by antioxidants, direct measurements of ROS in biological samples require specialized techniques including Electro Spin [10], which are not usually present in conventional laboratories. Numerous advances have been made in the last decades for measuring of ROS, but some problems related to artifact formation, lack of sensibility, and lack of specificity must be kept in mind [10].

The assessment of more stable parameters of oxidative stress is often preferred, including amount of oxidatively damaged biomolecules, amount of stable

products of ROS-producing enzymes, activities of ROS-producing enzymes, activities of antioxidant enzymes, amounts of non-enzymatic antioxidants, and non-enzymatic antioxidant capacity (NEAC). The measurement of enzymatic activity remains critical for assessing oxidative stress because of high expression of antioxidant enzyme may not result in the corresponding high activity probably due to post-translational modifications or feedback inhibition [11]. The concept of NEAC encompasses antioxidant power of both endogenous and exogenous non-enzymatic antioxidants in a biological sample, typically blood fraction [12]. There are two types of approaches for estimating of NEAC: direct or indirect measurements [12]. First, direct measurements of NEAC are based on inhibitory effect of antioxidants on oxidation produced by specific oxidizing agent. For example, the oxygen radical absorbance capacity (ORAC) assay is a direct measurement of NEAC, which assesses the inhibitory effect of antioxidants of sample on oxidation produced by 2,2′-azobis(2-amidinopropane) dihydrochloride as a source of peroxyl radical ($ROO^{\bullet}$) to fluorescein [13]. On the other hand, indirect measurements of NEAC are based on the reductive effects of antioxidants on oxidants and their derivates. For example, the ferric reducing ability of plasma (FRAP) assay is an indirect measurement of NEAC, which assesses the reductive effects of antioxidants of a sample on ferric ion producing a colored ferrous-tripyridyltriazine complex [14].

Measuring just a single biomarker of oxidative stress is not a useful strategy for assessing oxidative stress and its clinical relevance [15]. This approach may give misleading conclusions because of the complexity of redox metabolism and the fact that changes in specific biomarkers of oxidative stress may depend on the condition studied [8,15]. The measurement of numerous biomarkers of oxidative

stress in healthy and in pathological conditions may provide a highly comprehensible picture of oxidative stress, allowing us to evaluate the clinical relevance of preventive or therapeutic interventions appropriately [8].

Improving quality of dietary fat profile may be an important target to prevent the onset of cardiometabolic diseases. In this respect, rodents are well-known experimental models of cardiometabolic disorders induced by calorically-dense diets [16], and, concretely, rats seem to be an appropriate model to test the effects of functional food on early stages of insulin resistance under overload of dietary fat conditions [17]. The present doctoral thesis will explore the influence of dietary supplementation with ω-3 PUFA from fish oil, individually or in combination with other bioactive compounds such as polyphenols and iminocyclitols, compared to supplementation with soybean oil or coconut oil, as sources of ω-6 PUFA and SFA, respectively. To achieve this purpose and keeping in mind distinct conditions, we used either healthy rats or rats of both genders under calorically-dense diet conditions. This thesis is intended to find useful nutritional strategies to protect against the mechanisms underlying the onset of cardiometabolic disorders.

Summary

SUMMARY

SUMMARY

The past few decades have seen an alarming increase in the worldwide prevalence of obesity, atherogenic dyslipidemia, and metabolic syndrome. The increase in the prevalence of these conditions is highly related to unhealthy dietary habits and sedentary lifestyles. Overload of dietary fat promotes disturbances in several cardiometabolic processes, among which we find the onset and progression of insulin resistance. The pathogenesis of insulin resistance is clearly related to oxidative stress, inflammation as well as accumulation of bioactive lipids such as diacylglycerols and ceramides.

The replacement of saturated fatty acids to unsaturated fatty acids exerts beneficial influence on cardiometabolic health in healthy individuals and in individuals at high risk of disease. Concretely, ω-3 polyunsaturated fatty acids (ω-3 PUFA) from fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are associated with lower risk for developing cardiometabolic disorders. There is accumulating evidence that, when provided individually, DHA may exert more potent effects than EPA. Despite that, DHA is especially susceptible to peroxidation. Then, when provided in excess, DHA may lead to deleterious effects on health. Antioxidant compounds such as polyphenols from grape seeds can prevent oxidation of PUFA during digestion as well as in cell membranes, suggesting that the combination of ω-3 PUFA and polyphenols could exert great benefits on cardiometabolic health. On the other hand, buckwheat D-fagomine, which is an iminocyclitol capable of acting on gut microbiota, can reduce postprandial glucose and can prevent the activation of pro-inflammatory pathways induced by lipopolysaccharides. Thus, the combination of D-fagomine and ω-3 PUFA could also potentially enhance cardiometabolic health through early acting on gut microbiota.

SUMMARY

The aim of the present thesis is to explore the influence of fish oil and its combination with grape seed polyphenols or buckwheat D-fagomine on cardiometabolic risk factors and oxidative stress in healthy rats and in rats fed a high fat diet.

The first part of this thesis evaluates the effects of a fish oil rich in DHA on cardiometabolic risk factors and oxidative stress in healthy rats compared to fish oil containing EPA/DHA in a balanced 1:1 ratio, soybean oil, and coconut oil. Fish oil rich in DHA provides greater benefits on plasma lipid profile and fat accumulation in muscle than the obtained with fish oil containing EPA/DHA 1:1, but markedly promotes oxidative damage to lipids and proteins, with a concomitant increase in antioxidant response.

The second part of this thesis evaluates the effects of fish oil containing EPA/DHA 1:1 and its combination with grape seed polyphenols on oxidative stress and bioactive lipid accumulation in the liver of rats fed a high-fat high-sucrose diet. The supplementation with fish oil containing EPA/DHA 1:1 clearly increases abundances of PUFA-containing diacylglycerols, whereas individual supplementation with grape seed polyphenols markedly decreases abundances of hepatic lipids, and potentiates the endogenous antioxidant response. Unlike individual supplementations, the combination of fish oil containing EPA/DHA 1:1 and polyphenols induces similar accumulation of monounsaturated fatty acid-containing diacylglycerols and ceramides in the liver than that induced by the high-fat diet without supplementation. Nevertheless, individual supplementations are not as effective as combined supplementation to prevent the onset of insulin resistance under fat overload conditions, suggesting that the combined supplementation promotes accumulation of fat in lipid droplets.

SUMMARY

The final part of this thesis evaluates the effects of fish oil containing EPA/DHA 1:1 and its combination with buckwheat D-fagomine on oxidative stress and related cardiometabolic risk factor in rats fed a high-fat diet. The supplementation with fish oil containing EPA/DHA 1:1 is the main responsible for the beneficial effects on oxidative stress observed under fat overload conditions. Nevertheless, individual supplementations are not as effective as combined supplementation to prevent the onset of insulin resistance. The combination of fish oil containing EPA/DHA 1:1 and its combination with buckwheat D-fagomine also enhances glutathione status and decreases antioxidant enzyme activities, suggesting lower oxidative stress.

The increase in dietary intake of ω-3 PUFA from fish oil supplements containing EPA/DHA 1:1, alone or combined with polyphenols or D-fagomine, may be a useful nutritional strategy to protect against the mechanisms underlying the onset of cardiometabolic disorders in healthy individuals and in individuals at high risk of disease.

1. Introduction

INTRODUCTION

1.1. OXIDATIVE STRESS

1.1.1. REACTIVE OXYGEN SPECIES

Normal functioning of aerobic organism such as mammals requires nutrients and oxygen for production of energy through cellular respiration. Mitochondria are the main producers of energy by means of the electron transport chain in the inner membrane. The electron transport chain consists of a series of electron carriers that ends in production of adenosine triphosphate (ATP), using oxygen as the last electron acceptor. During the first step in mitochondrial oxidative phosphorylation, a proportion of electrons leaks especially from complex I, II and III. It is estimated that electron leakage through normal mitochondrial functioning is 0.2–2% of total consumed oxygen [18]. These electrons react with molecular oxygen leading to production of reactive oxygen species (ROS). In fact, mitochondria are primary sources of endogenous ROS [19], which have a very short half-life due to high reactivity and high clearance by antioxidants (Figure 2).

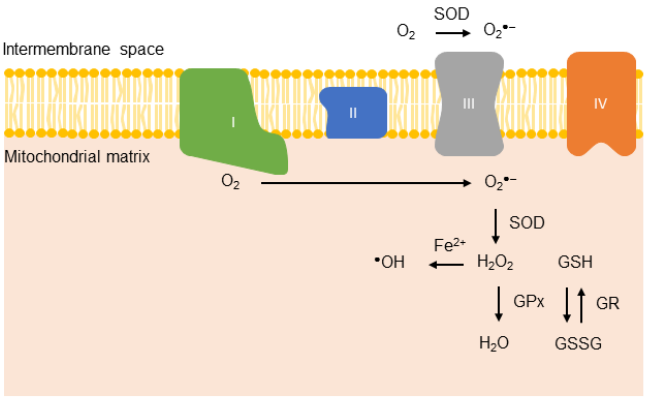

Figure 2. Production of reactive oxygen species by mitochondria, and antioxidant defense systems. Abbreviations: SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione.

ROS are oxidants, a type of electrophilic molecules that are capable to take electrons from another molecule (nucleophile) without forming an adduct [20]. There are two types of ROS based on its reactivity: oxygen free radical and oxygen non-radical species [2].

On one hand, oxygen radical species are highly reactive and short half-life molecules due to possession of an unpaired electron—the dot in formulae symbolize the unpaired electron. These radical species include superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), alkoxyl radical ($RO^{\bullet}$) and peroxyl radical ($ROO^{\bullet}$), among others. On the other hand, oxygen non-radical species are less reactive and more stable than radicals are. These non-radical species include hydrogen peroxide (H_2O_2), lipid hydroperoxide (LOOH) and singlet oxygen ($^1O_2$), among others. In particular, the H_2O_2 is the main metabolite of redox metabolism, and a key player in redox signaling pathways in the cell [3,4].

Importantly, ROS can be interconverted among them. Non-radical H_2O_2 in presence of transition metals such us ferrous ion produces radical $^{\bullet}OH$ and hydroxyl anion (OH^-) via Fenton reaction. Then, the $^{\bullet}OH$, a highly reactive radical, may initiate oxidative damage to biomolecules such as lipids, proteins and nucleic acids.

Besides the electron transport chain, other important sources of ROS in mammals include peroxisomes—due to oxidation of fatty acids—and oxidase enzymes such as NADPH oxidases (NOX) and xanthine oxidase (XO). NOX (EC 1.6.3.1) are transmembrane glycoproteins expressed in numerous types of cells that transport electrons across membranes. Concretely, NOX catalyzes the reduction of molecular oxygen to $O_2^{\bullet-}$ and H_2O_2. The NOX family comprises NOX

1–5 and DUOX 1–2, and plays a key role in numerous biological functions [21], including in immunity defense and inflammation as well as in cellular signaling. Next, XO (EC 1.17.3.2) is a molybdo-flavoenzyme mainly localized in cytosol but also in peroxisomes and microsomes. XO catalyzes the oxidation of hypoxanthine and xanthine to uric acid and $O_2^{\bullet-}$ or H_2O_2. The enzyme predominantly exists in the liver and intestines as xanthine dehydrogenase (XDH, EC 1.17.1.4), which not produce ROS. Nevertheless, XDH can be converted to XO either reversibly by oxidation of cysteine residues or irreversibly by proteolysis [22]. Thus, XO is a secondary producer of ROS and a marker of tissue injury.

1.1.2. ANTIOXIDANTS

A broad spectrum of antioxidant definitions has been proposed. In general, an antioxidant is by definition “any substance that delays, prevents, or remove oxidative damage to a target molecule” [18].

Mammals present powerful endogenous antioxidant defense systems to protect against excessive production of ROS and other electrophiles. These antioxidant systems attempt to minimize oxidative stress and subsequent oxidative damage to biomolecules, being the first line of defense. It is important to note that endogenous antioxidants may differ among tissues, type of cells and subcellular compartments. There are two types of endogenous antioxidant defenses: enzymatic or non-enzymatic antioxidants.

1.1.2.1. Endogenous Antioxidant Enzymes

Major antioxidant enzyme systems in mammals include superoxide dismutases (SOD), catalases (CAT), glutathione peroxidases (GPx), glutathione reductases (GR) and glutathione S-transferases (GST) [18].

SOD (EC 1.15.1.1) are metalloproteins that catalyze dismutation of $O_2^{\bullet-}$ to H_2O_2. There are three isoforms of SOD in distinct cell compartments: SOD1 contains Copper/Zinc and is mainly localized in cytosol and mitochondrial intermembrane space, SOD2 contains manganese and is localized in mitochondrial matrix, and SOD3 contains Copper/Zinc and is localized in extracellular space.

CAT (EC 1.11.1.6) are hemoproteins with manganese that catalyze degradation of H_2O_2 into water and oxygen. CAT are mainly located in peroxisomes of cells, except for erythrocytes.

GPx (EC 1.11.1.9) are selenoproteins that catalyze degradation of H_2O_2 or LOOH into water or corresponding alcohol and oxygen, using reduced glutathione (GSH) as cofactor. GPx have higher affinity for H_2O_2 than CAT. Four isoforms of GPx exist in distinct cell compartments (e.g., cytosol, membrane, mitochondria, peroxisomes, and nucleus) and in extracellular space. Importantly, GPx4 is capable of acting within membranes and lipoproteins, reducing peroxidized fatty acids to corresponding alcohols. The GPx work together with GR (EC 1.8.1.7), which are flavoproteins that catalyze the conversion of oxidized glutathione (GSSG) to GSH, using NADPH as cofactor. Thus, the recycling system composed of GPx and GR supplies GSH again.

GST (EC 2.5.1.18) are a superfamily of enzymes that catalyze the conjugation of endogenous electrophiles, such as reactive carbonyl species, and xenobiotic compounds with GSH. The GST are mainly located in cytosol, but also in membrane of organelles (e.g., mitochondria, endoplasmic reticulum and microsomes). In particular, the cytosolic GST include several classes based on

amino acid sequence similarities: alpha, kappa, mu, omega, pi, sigma, theta and zeta.

1.1.2.2. Endogenous Non-Enzymatic Antioxidants

Non-enzymatic endogenous antioxidants in mammals include GSH, albumin, uric acid, melatonin and bilirubin.

GSH is a low-molecular weight thiol containing glutamic acid, cysteine, and glycine, ubiquitously present in mammalian tissues but especially in the liver [23]. It is the primary reducing agent both in mitochondria and in the endoplasmic reticulum [24], maintaining a reduced state by either directly or indirectly scavenging of ROS [20]. Gamma-glutamylcysteine synthetase is the limiting enzyme in de novo synthesis of GSH, which also include glutathione synthetase [23]. As commented earlier, GSH is also a cofactor of GPx, and is recycled by GR activity. The measurement of the ratio of GSH to its oxidized counterpart GSSG estimates redox status of the cell.

Albumin is the most abundant protein in circulation. It exerts numerous important roles, including maintaining of osmotic pressure and transport of numerous molecules—e.g., fatty acids and hormones. Furthermore, albumin is a key player in antioxidant defense of blood by means of binding of metal ions (e.g., iron and copper), scavenging of ROS, and enzymatic activity [25].

1.1.2.3. Exogenous Antioxidants

Diet can provide numerous bioactive compounds to the organism, especially those dietary patterns rich in fruits and vegetables. Several of dietary compounds possess antioxidant capabilities by scavenging of ROS or by inducing

endogenous antioxidant response, and then exhibiting potential benefits to health. These exogenous antioxidants include vitamin C, α-tocopherol (vitamin E), vitamin A and related carotenoids (e.g., lycopene and carotene), lipoic acid ω-3 polyunsaturated fatty acids (PUFA) and polyphenols. Furthermore, amino acids, minerals and other vitamins are indirect players in antioxidant defense, being cofactors or precursors of enzymes or GSH. Exogenous and endogenous antioxidants cooperate to minimize oxidative stress and subsequent oxidative damage to biomolecules.

1.1.3. OXIDATIVE DAMAGE TO BIOMOLECULES

High-intensity oxidative stress results in damage to biomolecules such as lipids, proteins and nucleic acids. Under normal conditions, lipolytic enzymes and proteasomes remove damaged lipids and proteins, respectively, for the maintenance of the cell function. Nevertheless, accumulation of oxidatively damaged molecules in body fluids and within cell or tissue is a hallmark of numerous cardiometabolic disorders such as obesity, insulin resistance and metabolic syndrome [5,6].

1.1.3.1. Oxidative damage to lipids

Oxidative damage to lipids mainly affects PUFA, with two or more double bonds in their hydrocarbon chains, due to weakness of hydrogen at the site nearby to unsaturation. The oxidation of PUFA can occur either via non-enzymatic or enzymatic pathways [26].

The non-enzymatic pathway comprises three steps: initiation, propagation and termination or inhibition (Figure 3). First, a free radical such as •OH abstracts a hydrogen atom from a PUFA, resulting in a lipid carbon-centered radical (L•). Importantly, the carbon-centered radical tends to rearrange to form a conjugated diene, which is more stable than carbon-centered radical is. The L• reacts with molecular oxygen to yield a lipid peroxyl radical (LOO•), which in turn react with another PUFA to produce a LOOH and a new L•. After that, the new L• can further react with molecular oxygen propagating the process. The process ends when an antioxidant such as albumin, GSH or α-tocopherol inhibits the propagation step by means of scavenging of LOO• [26–28].

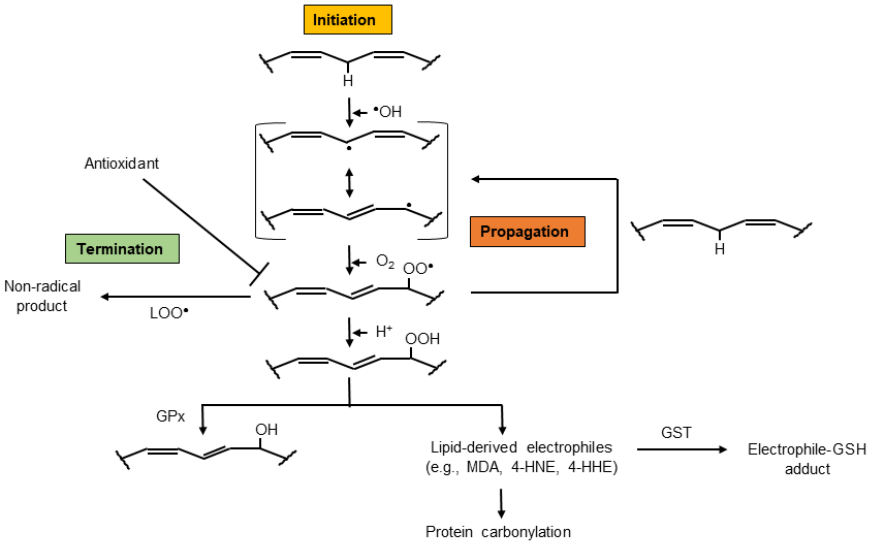

Figure 3. Lipid peroxidation of polyunsaturated fatty acids. Abbreviations: LOO•, lipid peroxyl radical; GPx, glutathione peroxidase; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; 4-HHE, 4-hydroxyhexanal; GST, glutathione S-transferase; GSH, glutathione.

The non-enzymatic pathway can affect either non-esterified PUFA or esterified PUFA. Because PUFA are abundant in glycerophospholipids of cell membranes, their oxidation may negatively modulate membrane properties such us fluidity, increasing the risk for developing disease [29].

On the other hand, the enzymatic pathway usually requires previous release of PUFA from glycerophospholipids of cell membranes. In this respect, phospholipases A_2 (PLA_2) are enzymes that catalyze the hydrolysis of the ester bond at *sn*-2 position of complex lipids yielding a non-esterified PUFA. Once released into the cytosol, PUFA can undergo oxidation through the action of cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 monooxygenases, producing a large number and classes of LOOH.

GPx can reduce the LOOH derived from either enzymatic or non-enzymatic pathways to corresponding stable alcohol (LOH). Alternatively, the LOOH can be further metabolized to reactive carbonyl species such as α,β-unsaturated aldehydes, including malondialdehyde (MDA), 4-hydroxynonenal (4-HNE) and 4-hydroxyhexanal (4-HHE), among others. These species are end products of lipid peroxidation and can be non-specifically estimated in a sample by means of the thiobarbituric acid-reactive substances (TBARS) assay [30].

Unlike ROS, end products of lipid peroxidation (e.g., MDA, 4-HNE and 4-HHE) are electrophilic molecules that are capable to take electrons from nucleophiles forming an adduct [20]. Once generated, end products of lipid peroxidation are rapidly conjugated with GSH by GST for detoxification purposes or, conversely, they react with other proteins causing oxidative damage (Figure 3).

1.1.3.2. Oxidative damage to proteins

Numerous diseases have been associated with oxidation of proteins [31]. One of the most studied process of protein oxidation is protein carbonylation. Importantly, protein carbonyls are early biomarkers of oxidative stress due to their relative stability compared to GSSG and MDA [32]. Oxidative damage to albumin is a well-known biomarker of oxidative stress [7].

Protein carbonylation is an irreversible post-translational modification of a protein resulting in a protein that contains a reactive carbonyl moiety [33]. ROS can directly oxidize protein in proline, arginine, lysine and threonine residues, whereas end products of lipid peroxidation act mainly via Michael adduction, yielded in cysteine, histidine or lysine residues [34]. Cysteine residues of protein are especially susceptible to oxidation [34].

Modification of protein can modulate activity of protein leading to disturbance in cellular processes. Generally, protein carbonylation decreases activity of protein, which can result in deleterious effects, such as inflammation, and evolve into disease. Nevertheless, protein carbonylation also plays a key role in activation of signaling pathways. In this respect, the carbonylation of reactive cysteine residues of Kelch like ECH associated protein 1 (Keap1) allow the release of nuclear factor-erythroid 2-related factor 2 (Nrf2) and subsequent translocation of Nrf2 to the nucleus [35]. Nrf2 is a transcription factor that regulates expression of cytoprotective genes, including those related to oxidative stress and detoxification of other electrophilic compounds [36], maintaining tightly regulated the redox homeostasis. On one hand, under unstressed conditions, Nrf2 is linked to Keap1 in the cytoplasm, and is constantly degraded. On the other hand, under high exposure to oxidants, reactive cysteine residues of Keap1 are oxidized,

resulting in a translocation of Nrf2 to the nucleus. Once into the nucleus, Nrf2 activates target genes involved in antioxidant response and electrophilic detoxification for preventing molecular damage (Figure 4) [37].

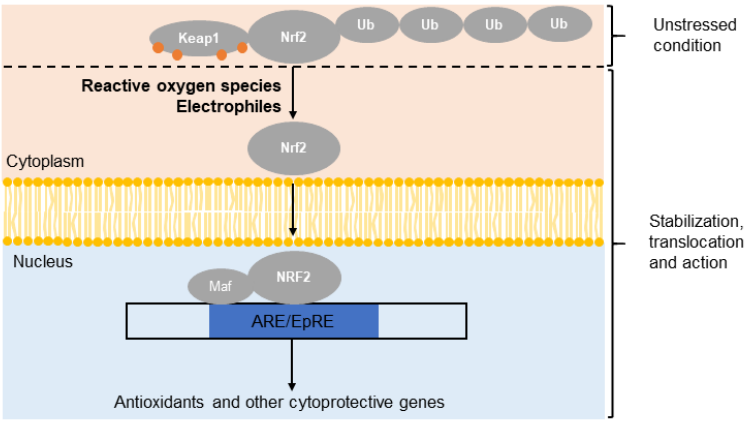

Figure 4. Mechanism of Nrf2 to induce expression of cytoprotective enzymes. Abbreviations: Keap1, kelch like ECH associated protein 1; Nrf2, nuclear factor-erythroid 2-related factor 2; Ub, ubiquitin; ARE, antioxidant response element; EpRE, electrophile responsive element.

1.1.3.3. Oxidative damage to nucleic acids

Excessive production of ROS and end products of lipid peroxidation can also induce oxidative damage to nucleic acid bases—purines and pyrimidines—and 2-deoxyribose/ribose of DNA and RNA [38–40]. Early oxidative damage to DNA leads to low DNA replication and low cell division. Mitochondrial DNA may be more susceptible to oxidative damage than nuclear DNA, probably due to the proximity to the electron transport chain [41]. Damage to RNA leads to errors in translation and impairment of protein synthesis. Under normal conditions, oxidative modifications to DNA/RNA are tightly repaired by a variety of mechanisms but, if the damage rate is over than the repair rate, accumulation of damaged nucleic acids can lead to mutations, carcinogenesis and cell death,

evolving into disease. Among nucleic acid bases, guanine is especially vulnerable to oxidation by the •OH, which can add to position 8 in the purine ring yielding to C8-OH-adduct radical. During the repair process, this is further oxidized to several species including 8-oxo-7,8-dihydroguanine, which are often used to estimate oxidative stress.

1.2. METABOLIC SYNDROME

The past few decades have seen an alarming increase in the worldwide prevalence of non-communicable diseases, including obesity, diabetes and cardiovascular diseases (CVD) [42,43]. CVD such as heart disease and stroke are major causes of morbidity and mortality not only in economically developed countries but also in developing countries, especially in males [42,44].

Unhealthy dietary habits, lack of physical activity in addition to other biological and genetic factors significantly increases the risk of cardiometabolic alterations. Concretely, metabolic syndrome is a cluster of metabolic alterations such as central obesity, atherogenic dyslipidemia, high blood pressure and high fasting blood glucose that often occur simultaneously. The metabolic syndrome is highly prevalent worldwide [45] and considerably increases the risk for developing type 2 diabetes and CVD. Many scientific organizations and experts, including the International Diabetes Federation (IDF), the National Cholesterol Education Program Adult Treatment Panel III (ATP III) and the World Health Organization (WHO), have established specific criteria to define the metabolic syndrome in humans (Table 2) [46].

Table 2. Criteria for clinical diagnosis of metabolic syndrome [45,47,48].

Measure	IDF *	NCEP-ATP III †	WHO ‡
Central obesity (waist circumference) §	>94 cm for men >80 cm for women	>102 cm for men >88 cm for women	>90 cm for men >85 cm for women
Increased TG ‡	>1.7 mmol/L	>1.7 mmol/L	>1.7 mmol/L
Decreased HDL-C &	<1.03 mmol/L for men <1.29 mmol/L for women	<1.03 mmol/L for men <1.29 mmol/L for women	<0.9 mmol/L for men <1.0 mmol/L) for women
Increased BP &	Systolic ≥130 and/or diastolic ≥85 mm Hg	Systolic ≥130 and/or diastolic ≥85 mm Hg	Systolic ≥140 and/or diastolic ≥90 mm Hg
Increased FG &	≥100 mg/dL (5.6 mmol/L) or diabetes	≥110 mg/dL (6.1 mmol/L) or diabetes	≥110 mg/dL (6.1 mmol/L) or diabetes

* Presence of central obesity plus two or more of the criteria.
† Presence of three or more of the criteria.
‡ Presence of diabetes or impaired fasting glucose plus two or more of the other criteria.
§ Specific cutoff for each ethnicity; cutoff indicated here is for European origin.
& Cutoff or drug treatment for this alteration.
Abbreviations: IDF, International Diabetes Federation; NCEP-ATP III, National Cholesterol Education Program Adult Treatment Panel III; WHO, Word Health Organization, TG, triacylglycerol; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; FG, fasting glucose.

1.2.1. OBESITY, DYSLIPIDEMIA AND ECTOPIC FAT

Obesity is a highly prevalent condition worldwide [43] that is characterized by the expansion of white adipose tissue due to an increase in both the size of adipocytes (hypertrophy) and the number of adipocytes (hyperplasia) [49]. It is generally due to high energy intake along with low energy expenditure but also genetic factors play an important role. Central obesity is a critical component of the metabolic syndrome, being also a major risk factor for developing atherogenic dyslipidemia, ectopic fat accumulation and insulin resistance, especially when accumulation of fat occurs in visceral depots [50].

Dyslipidemia is an aberrant concentration of circulating lipids that can be caused by genetic factors and/or secondarily developed because of unhealthy lifestyle and pathological conditions such as obesity, insulin resistance or diabetes. Concretely, atherogenic dyslipidemia include increased concentrations of

triacylglycerol, total cholesterol, and low-density lipoprotein cholesterol (LDL-C) along with decreased concentration of high-density lipoprotein cholesterol (HDL-C). This type of aberrant lipid profile is a risk factor for developing cardiovascular diseases [51].

The liver plays a critical role in lipid metabolism and subsequently in lipid plasma profile. The liver is responsible for synthesis, assembly and exporting of lipids—mainly of triacylglycerol and secondary of esterified cholesterol—via very LDL (VLDL) particles. The VLDL particles undergo triacylglycerol abstraction by extrahepatic tissues including adipose tissue and muscle for storage and oxidation, respectively, via recognition of ApoC-II and activation of lipoprotein lipase. This event results in LDL particles rich in cholesterol, which in turn is delivered from LDL particles to extrahepatic tissues via recognition of Apo-B100 by LDL receptors. Finally, remaining LDL particles return to the liver. On the other hand, HDL particles are important players in reverse transport of cholesterol. The HDL particles carry cholesterol from extrahepatic tissues and macrophages to the liver, where it is recognized by scavenger receptor class B-1 for excretion or conversion to bile acids. The reverse transport of cholesterol is critical for prevention of atherosclerosis and CVD.

Obesity is also a risk factor for aberrant accumulation of fat in the liver and subsequent development of non-alcoholic fatty liver disease (NAFLD). When the expanded adipose tissue fails to store lipids, non-esterified fatty acids (NEFA) are released into circulation. The liver, as well as other organs such muscles, incorporates these NEFA. This process is commonly referred to as ectopic fat deposition. Obesity is not the only way for ectopic fat accumulation but also overload of sucrose is a critical player through the induction of *de novo*

lipogenesis. The histological analysis of the liver is the gold standard method for diagnosis of NAFLD [52]. The aberrant accumulation of lipids in the liver is associated with dysfunction of mitochondria, inflammation and oxidative stress. These features contribute to the progression from simple liver steatosis to steatohepatitis, and consequent increase in the risk of fibrosis, cirrhosis and hepatocellular carcinoma [53]. Impaired hepatic function may be estimated by means of enzyme activities in plasma including those of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are released into circulation by damaged hepatocytes.

1.2.2. INFLAMMATION

Inflammation is an acute physiological response against foreign, dangerous insults. In fact, it is a critical process for repair and remodeling of damaged tissue [54]. There are two stages of inflammation: initiation, which comprises productive and transition phases, and resolution.

The inflammatory response is generally resolved once the dangerous insult is removed. Nevertheless, when the resolution of inflammation does not occur, more immune cells such as monocytes infiltrate the damaged tissue. Once into the tissue, monocytes undergo differentiation to macrophages; followed by polarization from M2 to M1, which is the classically active form. Macrophages recognize dangerous signals by means of pattern-recognition receptors such as toll-like receptors (TLR). High number of activated immune cells produces large amounts of inflammatory mediators including cytokines such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6), chemokines and metabolites of

arachidonic acid (ARA, 20:4 ω-6). Furthermore, activated immune cells produce ROS by NOX for killing purposes.

The unresolved inflammatory response can progressively evolve into a chronic inflammation with a concomitant loss of its regulation and its tolerance. Then, inflammation becomes a pathological condition [55]. Chronic low-grade inflammation is a main feature of obesity [56,57]. The inflammatory state in obesity differs from classic inflammation because, in this particular case, the insult for the onset and maintenance of inflammatory response is the excess of nutrients provided to metabolic cells such as adipocytes [58].

Saturated fatty acids (SFA) can induce activation of inflammatory pathways [54]. NEFA interact with TLR and fatty acid transport protein in the plasma membrane of cell. Once into the cell, NEFA activate several serine kinases such as IκB kinase (IKK) and c-Jun N-terminal kinase (JNK). In turn, these kinases induce pro-inflammatory signaling pathways IKK/nuclear factor kappa B (NF-κB) and JNK/activator protein 1 (AP-1), increasing expression of pro-inflammatory genes.

During progression to obesity, the expansion and decreasing of vascularization of white adipose tissue leads to a decrease in flux of oxygen into the tissue [59]. Hypoxia promotes infiltration of immune cells into the tissue, which may occur in response to high release of chemokines such as monocyte chemotactic protein-1 [60] by activation of the NF-κB signaling pathway in adipocytes [61]. In fact, macrophages are the predominant immune cell in adipose tissue in obesity [56]. Furthermore, obesity promotes polarization of macrophages from M2 to M1 by pro-inflammatory mediators.

Gut microbiota dysbiosis in obesity also participates in activation of macrophages through increasing circulating lipopolysaccharides derived from gram-negative bacteria, which are recognized by TLR at the surface of macrophages [62]. Activated macrophages and adipocytes significantly produce cytokines (e.g., TNFα, IL-6), chemokines, NEFA, metabolites of ARA and ROS, exacerbating a vicious circle.

Early local inflammation in expanded adipose tissue may contribute to inflammation in other tissues via releasing of NEFA and pro-inflammatory mediators into circulation. In this respect, TNFα and IL-6 mainly derived from adipose tissue induce the secretion of C-reactive protein (CRP) by the liver, which is a well-known biomarker of inflammation [63].

1.2.3. GLUCOSE HOMEOSTASIS

Glucose is the main energy source of mammals. Glucose homeostasis is tightly maintained by action of glucagon and insulin, which are secreted from the Islets of Langerhans of the pancreas.

Insulin is an endocrine peptide hormone produced by the β-pancreatic cells in response to high availability of nutrients in the bloodstream (i.e., after a meal). Insulin signaling cascade includes several coordinated events. Once in circulation, insulin acts by binding to insulin receptor tyrosine kinase (INSR) on the plasma membrane of the cell. The binding of insulin with INSR induces auto-phosphorylation on tyrosine residues of INSR and subsequent recruitment of its substrates, such as insulin receptor substrate 1 (IRS1). After that, the IRS1 undergoes phosphorylation on tyrosine residues, which in turn leads to activation

of signaling pathways, including the mitogenic pathways and the metabolic pathways [64].

As far as metabolic signaling is concerned, insulin modulates metabolism of target tissues, especially of skeletal muscle, liver and adipose tissue, via the phosphatidylinositol 3 kinase (PI3K). The recruitment of PI3K by IRS leads to activation of protein kinase B (also referred to as AKT) and subsequently translocation of glucose transporters (GLUT) from cytosolic vesicles to the plasma membrane. The GLUT internalize glucose into the cells, resulting in high glycogenesis along with low gluconeogenesis in skeletal muscle and in the liver. Furthermore, insulin stimulates lipogenesis in adipose tissue and in the liver, promoting storage of lipids in the form of triacylglycerol rather than oxidation [64]. Low-grade, local and temporal production of ROS derived from NOX in the plasma membrane beneficially participate in the insulin signaling cascade by inhibition of phosphatases, such as protein tyrosine phosphatase 1B (PTP1B)—which inhibit the INSR, amplifying the insulin signaling [64,65].

1.2.4. INSULIN RESISTANCE

Insulin resistance is a deficient response of the target tissues to insulin. Impaired insulin signaling is a common characteristic in such cardiometabolic disorders as obesity, metabolic syndrome, type 2 diabetes and NAFLD [66]. The main effects of insulin resistance include low glucose uptake by muscles, high glucose production by the liver and high lipolysis by adipose tissue. All together lead to an overall increase in glucose and NEFA concentrations in circulation [64]. Early stages of insulin resistance, which can progressively evolve into type 2 diabetes,

are characterized by a compensatory increase in secretion of insulin from enlarged β-pancreatic cells [67].

High-fat (HF) diet promotes insulin resistance by inhibition of insulin signaling cascade. There are three mechanisms that may be involved in the onset of insulin resistance under HF diet conditions: (1) oxidative stress (2), inflammation, and (3) ectopic fat accumulation [64].

First, high-intensity oxidative stress may be a mechanistic event for the onset of insulin resistance. HF diet induces an excessive production of ROS through β-oxidation of fatty acids especially in mitochondria, leading to an oxidized environment into the cell [68]. This oxidized environment promotes the activation of stress-sensitive kinases (e.g., IKK and JNK) [65]. In turn, the activation of stress-sensitive kinases contributes to the onset of insulin resistance by inhibition of both the insulin receptor through phosphorylation on serine residues—instead of tyrosine—of IRS1 and the protein kinase B activity. In contrast, there is accumulating evidence that ROS may negatively affect trafficking of cytosolic GLUT-containing vesicles independently of IRS1 and protein kinase B [69,70].

Second, chronic low-grade inflammation may be a mechanistic event for the onset of insulin resistance in obesity [71]. In this respect, pro-inflammatory TNFα may impair insulin signaling by both the phosphorylation on serine residues of IRS1 and the activation of IKK and JNK activities (Figure 5) [54]. Nevertheless, other studies have suggested that inflammation is not a cause for insulin resistance, at least, in expanded visceral adipose tissue [72], suggesting that accumulation of lipid intermediates such as diacylglycerol (DAG) and ceramide species is an earlier event in the onset of insulin resistance, whereas systemic

inflammation evolve into an important factor in more advanced stages of insulin resistance once obesity is fully established [73].

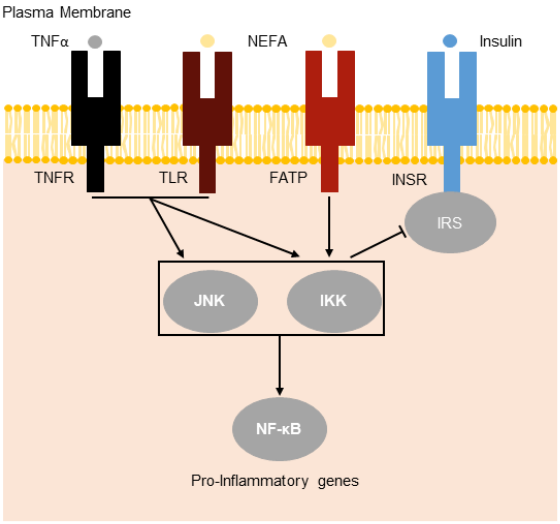

Figure 5. Relationship between inflammation and insulin resistance. Abbreviations: TNFα, tumor necrosis factor α; TNFR, tumor necrosis factor receptor; NEFA, non-esterified fatty acids; TLR, toll-like receptor; FATP, fatty acid transport protein; INSR, insulin receptor; JNK, c-Jun N-terminal kinase; IKK, IκB kinase; NF-κB, nuclear factor kappa B.

Finally, excessive accumulation of lipid intermediates such as DAG and ceramide species in tissues may be a mechanistic event for the onset of insulin resistance. In fact, both the DAGs and the ceramides have been proposed as early predictors of diabetes [74,75]. These two types of bioactive lipids can activate certain isoforms of protein kinase C that results in an impairment of the insulin signaling (Figure 6) [75]. On one hand, DAGs are glycerolipids that are comprised of a glycerol backbone in which two of the hydroxyl groups are esterified with a long-chain fatty acid [76]. They are lipid intermediates in synthesis/hydrolysis of triacylglycerols. DAG acts as a lipid second messenger in the cell. Concretely, *sn*-1,2-DAG can activate protein kinase C, which in turn inhibits the INSR in the plasma membrane by phosphorylation. On the other hand, ceramides are

sphingolipids that are comprised of a sphingoid backbone linked to a long-chain fatty acid through an amine bond [77]. They are lipid intermediates in synthesis/hydrolysis of complex sphingolipids, including sphingomyelin. Thus, ceramide is a precursor of molecules involved in the integrity of cell membrane and a signaling lipid. Concretely, ceramides can activate protein kinase C, which in turn inhibits translocation of protein kinase B from cytosol to the plasma membrane.

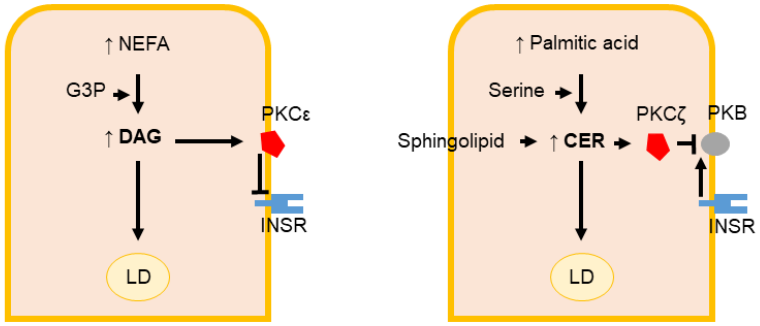

Figure 6. Relationship between accumulation of bioactive lipids and insulin resistance. Abbreviations: NEFA, non-esterified fatty acids; G3P, glycerol-3-phosphate; DAG, diacylglycerol; PKC, protein kinase C; INSR, insulin receptor; LD, lipid droplet; CER, ceramide; PKB, protein kinase B.

1.3. FOODS AND BIOACTIVE COMPOUNDS

Certain groups of food as central components characterize dietary patterns (e.g., Mediterranean diet, Nordic diet and Vegetarian diet). In particular, the current concept of Mediterranean diet—traditionally followed by population of Mediterranean region—include: (1) high quantity of fruits, vegetables, whole grains, dairy products, nuts, seeds and legumes, (2) moderate quantity of fish and seafood, white meat, eggs, olive oil, and (3) low quantity of red meat, processed meat, solid fats and sweets [78].

The prevalence of cardiometabolic disorders has increased in parallel to significant changes in lifestyle of human populations. These changes are especially relevant in dietary patterns [79,80]. Diet of Western societies have evolved into a dietary pattern rich in highly processed, highly palatable and high-energy-dense food, meat, soft drinks, as well as solid fats and refined oils. Together, the diet provides high amount of total fat—mainly SFA and ω-6 PUFA, sucrose and sodium. Meanwhile, the Westernized dietary pattern is poor in vegetables, fruits, fish and whole grains, which contain greater quantity of vitamins, minerals, fiber, ω-3 PUFA and other biologically active compounds.

The Western diet is associated with deleterious outcomes on metabolic health [81,82]. Because the pathogenesis of insulin resistance is clearly related to oxidative stress, inflammation and bioactive lipid accumulation, targeting them by food and its related bioactive compounds may potentially have preventive effects on the onset of disease.

1.3.1. FISH OIL

Fish oil is rich in ω-3 PUFA, especially eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3). EPA and DHA are mainly provided by dietary intake (food and supplements) or, in very low proportion, by conversion from essential α-linolenic acid (ALA, 18:3 ω-3)—which cannot be synthetized in mammal tissues and must be provided by diet.

Oily fish (e.g., anchovy, salmon, sardine and tuna) and other marine products such as algae are sources of EPA and DHA, whereas vegetable food such as linseed, flaxseed, walnuts and rapeseed are sources of ALA [83]. Currently, the European Food Safety Agency (EFSA) [84] recommends an adequate intake of 250 mg of EPA + DHA (1–2 servings of oily fish a week) based on cardiovascular health considerations in the general population. Furthermore, the EFSA [85] suggests safe dietary doses of combined EPA + DHA (up to 5 g a day), individual EPA (up to 1.8 g a day) and individual DHA (up to 1 g a day). The amount of oily fish consumed has slightly increased during past decades in Mediterranean region [79], however, the intake remains low. Then, the use of fish oil supplements—providing individually EPA and DHA or combined in variable proportions—can be a useful strategy to achieve the recommendations of EPA and DHA. In fact, fish supplements are commonly used in some Western societies as an inexpensive, quick and safe way to increase ω-3 PUFA intake [86].

The biosynthetic pathway of EPA and DHA from ALA comprises a series of desaturation and elongation steps performed by desaturase and elongase enzymes (Figure 7) [87,88]. Desaturases add a double bond in the hydrocarbon

chain of the fatty acid, whereas elongases add carbons. Importantly, ω-3 PUFA compete against ω-6 PUFA for desaturases and elongases.

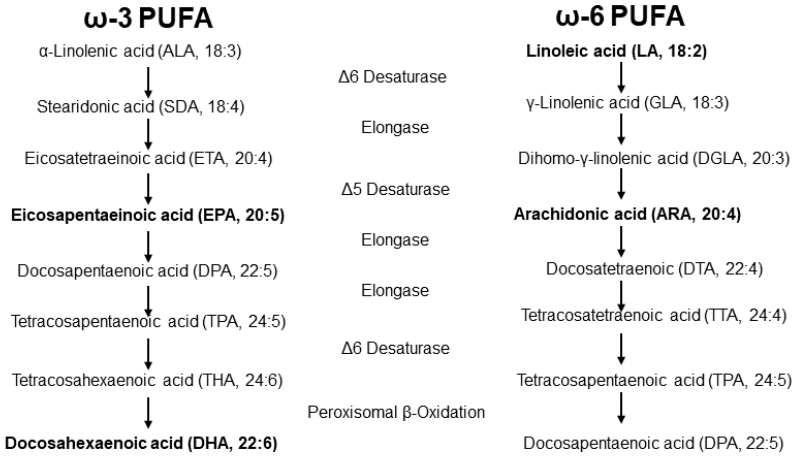

Figure 7. Metabolism of omega-3 and omega-6 polyunsaturated fatty acids.

Type of dietary fat consumed clearly affects fatty acid composition in blood and in tissues in a dose-dependent manner [88]. In this respect, the supplementation with EPA and DHA promotes their incorporation in cell membranes and in tissues [88,89]. High amounts of EPA and DHA in cell membranes and in tissues exhibits beneficial effects on cardiometabolic health by modulating lipid metabolism, cell and organelle membrane properties as well as anti-inflammatory and antioxidant responses, among others [90,91].

Various mechanisms can mediate in promoting the beneficial effects of oily fish and fish oil supplements on cardiometabolic health. First, ω-3 PUFA exert hypolipidemic effect through the activation of the peroxisome proliferator-activated receptor (PPAR)α, which in turn induces fatty acid β-oxidation and cholesterol uptake from plasma, along with inhibition of lipogenesis [92–94].

Second, ω-3 PUFA in cell and organelle membranes may beneficially modulate its properties, including fluidity, ion permeability and protein function [90,95]. Third, ω-3 PUFA exert anti-inflammatory effect by binding to G-protein-coupled receptor 120 (GPR120), inhibiting IKK and JNK activities and then preventing activation of the NF-κB signaling pathway [96–99]. During hydrolysis from phospholipids and oxidation steps, the ω-3 PUFA and the ω-6 PUFA may also compete for enzymes such as PLA_2, COX and LOX for producing oxidized metabolites [100]. Oxidized lipids derived from ω-3 PUFA by COX and LOX (e.g., resolvins, protectins and maresins) exhibit anti-inflammatory and pro-resolving properties [100]. Furthermore, eicosanoids derived from EPA induce lower inflammatory responses than those from ω-6 PUFA (e.g., series 2 of prostaglandins and thromboxanes as well as series 4 of leukotrienes) [55]. Fourth, ω-3 PUFA from fish oil exert antioxidant effect likely by activation of the Nrf2 signaling pathway [101,102], resulting in an increase in the endogenous antioxidants [101–103]. Finally, ω-3 PUFA exert beneficial effect on adipose tissue by increasing adipogenesis via activation of ciliary GPR120/cAMP pathway, activating nuclear transcription factors including PPARγ and, consequently, increasing expression of adipogenic genes such as that of adiponectin [104].

Despite their beneficial effects, the ω-3 PUFA contains multiple double bonds in the hydrocarbon chain that make them prone to oxidation by ROS. Lipid peroxidation is highly dependent on the degree of unsaturation in a fatty acid. Thus, DHA is particularly vulnerable to oxidation. High intake of DHA increases peroxidability index in cell membranes and tissues, and then increases end products of lipid peroxidation [105–107]. Importantly, oxidized lipids provided by

diet induce cellular and tissue damage, which is particularly evident in intestines [108] and even in subcutaneous white adipose tissue [109]. Dietary oxidized fat increases the risk of CVD in a dose dependent manner [110,111]. For this reason, PUFA-rich products require appropriate storage conditions and even addition of antioxidants [112].

1.3.2. GRAPE SEED EXTRACT

Grape seeds contain polyphenols, which are compounds that contain multiple phenolic rings with one or more hydroxyl groups. Polyphenols are secondary metabolites of plants that contribute to defense against microorganisms and predators, attractant for pollinators and seed-dispersing animals, among many other critical functions.

One of the most studied families of polyphenols are flavonoids, which in turn includes flavonols, flavones, isoflavones, flavavones, anthocyanidins, and flavon-3-ols subclasses. In particular, flavan-3-ols range from monomers such as (epi)catechin and (epi)gallocatechin to oligomeric and polymeric proanthocyanidins [113].

The most abundant proanthocyanidin is exclusively made up of (epi)catechin; this type of proanthocyanidin is also referred to as procyanidin. Grape seeds are important sources of proanthocyanidins. Furthermore, they are notably present in other fruits (e.g., apples, pears, some berries), cocoa and nuts. Thus, dietary patterns rich in vegetables and fruits provide considerable amounts of proanthocyanidins, among several other types of polyphenols. Furthermore, it can be provided by supplements. The estimated intake of proanthocyanidins in a Mediterranean population may range from 67 to 286 mg a day [114].

Although proanthocyanidins—like the other polyphenols—are non-essential dietary compounds, numerous studies have shown their beneficial effects against cardiometabolic disorders [113,115] by modulating glucose metabolism, lipid metabolism, inflammation and oxidative stress. In particular, proanthocyanidins exhibit antioxidant properties via scavenging of ROS [116], inhibiting of ROS-producing enzymes [117] and increasing endogenous antioxidant response mediated by Nrf2 [118].

1.3.3. BUCKWHEAT D-FAGOMINE

Buckwheat contains D-fagomine (FG), which is a polyhydroxylated piperidine alkaloid, classified as an iminocyclitol—analogue of saccharides that contains nitrogen instead of oxygen [119]. Like polyphenols, FG is a secondary metabolite of plants, which protects them against microorganisms and insects [120].

FG is not only present in buckwheat but also in leaves of mulberry [121]. The estimated dietary intake of FG from buckwheat flour-based products (e.g., bread, cookies and beer) in Mediterranean population range from 3 to 17 mg a day [122].

Because of its similarities with saccharides, FG is considered a gut glycosidase inhibitor [123]. Glycosidases catalyze the hydrolysis of glycosidic bonds in saccharides. In this respect, supplementation with FG attenuates the increase in postprandial glucose after receiving either sucrose or starch [124]. Furthermore, FG beneficially modulates gut microbiota by limiting the adhesion of detrimental bacteria to gut mucosa [124], and increases fecal excretion of Enterobacteriales (e.g., *Escherichia coli*) in rats after receiving HF diet [125]. These beneficial changes in gut microbiota may prevent, at least in part, the onset of several cardiometabolic alterations including body weight gain, dyslipidemia, insulin

resistance and chronic low-grade inflammation under HF diet conditions [126,127].

1.3.4. RATIONALE FOR COMBINING BIOACTIVE COMPOUNDS

The Mediterranean diet is considered a healthy dietary pattern that is associated with numerous protective effects against cardiometabolic disorders [128]. Unlike the Western diet, the Mediterranean dietary pattern provides an appropriate fat profile particularly rich in monounsaturated FA and optimal levels of ω-6 PUFA to ω-3 PUFA—high ratio of ω-6 PUFA to ω-3 PUFA is associated with high risk for developing inflammation, obesity and insulin resistance [129,130].

Besides ω-3 PUFA, the Mediterranean diet is a source of numerous non-nutrient bioactive compounds such as polyphenols that can beneficially contribute to the effects resulting from the diet. Overall, the interaction of bioactive compounds can exert great benefits on cardiometabolic health by modulation of antioxidant and anti-inflammatory pathways as well as abundances of bioactive lipids [131,132].

Improving the quality of dietary fat may be a key factor to prevent the onset of metabolic diseases [133]. Furthermore, in recent years, nutritional studies have shifted from examination of the effects of supplementation with a single bioactive compound on health to combining them. Combining bioactive compounds may exert complementary or synergic effects resulting in greater benefits than providing them individually. For example, the combined supplementation of ω-3 PUFA from fish oil with proanthocyanidins from a grape seed extract (GSE) prevents the oxidation of PUFA during digestion as well as in cell membranes [134], suggesting that the combination could exert great benefits on cardiometabolic health. The addition of FG to feeding rich in ω-3 PUFA could also

enhance cardiometabolic health through early acting on gut microbiota, reducing postprandial glucose and preventing activation of pro-inflammatory pathways induced by lipopolysaccharides.

Therefore, targeting oxidative stress, inflammation and bioactive lipid accumulation by combining bioactive compounds may be a powerful preventive strategy to protect against the onset of obesity, insulin resistance and metabolic syndrome.

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF FISH OIL AND ITS COMBINATION WITH GRAPE SEED POLYPHENOLS OR BUCKWHEAT D-FAGOMINE ON CARDIOMETABOLIC RISK FACTORS AND OXIDATIVE STRESS IN RATS
Bernat Miralles Pérez

2. Hypothesis

HYPOTHESIS

The present doctoral thesis comprises three experiments that are highly connected among them by way of the use of fish oil rich in ω-3 PUFA. This thesis is based on previous works of our research group, which showed that supplementation with fish oil containing EPA/DHA in a balanced 1:1 ratio exerts greater benefits on cardiometabolic risk factors and oxidative stress in healthy rats than those obtained with unbalanced ratios, when compared to soybean oil and linseed oil. Despite that, there is accumulating evidence that, when provided individually, DHA may exert more potent effects than EPA. DHA is especially susceptible to peroxidation, which in turn may lead to deleterious effects on health. For these reasons, Experiment 1 evaluates the effects of increasing concentrations of DHA in fish oil on cardiometabolic risk factors and on numerous biomarkers of oxidative stress in healthy rats. Then, following experiments (Experiments 2 and 3) evaluate the effects of fish oil containing EPA/DHA 1:1 and its combination with GSE or FG in rats fed a high-fat diet. Therefore, the hypothesis of the research experiments derived from the present doctoral thesis are as follows:

2.1. EXPERIMENT 1. Fish Oil Rich in Docosahexaenoic Acid

Increasing concentrations of DHA in fish oil beneficially affects cardiovascular risk factors and oxidative stress in healthy male Sprague–Dawley rats.

2.2. EXPERIMENT 2. Fish Oil and Grape Seed Extract

The combined supplementation with fish oil containing EPA/DHA 1:1 and GSE exerts beneficial influence on oxidative stress and bioactive lipid species in the liver of female Wistar–Kyoto rats fed a high-fat diet.

2.3. EXPERIMENT 3. Fish Oil and Buckwheat D-Fagomine

The combined supplementation with fish oil containing EPA/DHA 1:1 and FG exerts beneficial influence on oxidative stress and related cardiometabolic risk factors in male Sprague–Dawley rats fed a high-fat diet.

3. Objectives

OBJECTIVES

The aim of this thesis was, first, to evaluate the effects of increasing concentrations of DHA in fish oil on cardiometabolic risk factors and oxidative stress in healthy rats, second, to evaluate those of the combination of fish oil containing EPA/DHA 1:1 and GSE or FG in rats on a high-fat diet. This main objective can be divided into secondary objectives as follows:

3.1. EXPERIMENT 1. Fish Oil Rich in Docosahexaenoic Acid

- To examine the effects of increasing concentrations of DHA on feed intake and biometric data in healthy male Sprague–Dawley rats compared to coconut oil, soybean oil, and fish oil containing EPA/DHA 1:1.
- To examine the effect of increasing concentrations of DHA on glucose metabolism in healthy male Sprague–Dawley rats compared to coconut oil, soybean oil, and fish oil containing EPA/DHA 1:1.
- To examine the effects of increasing concentrations of DHA on circulating and tissue lipid profiles in healthy male Sprague–Dawley rats compared to coconut oil, soybean oil, and fish oil containing EPA/DHA 1:1.
- To examine the effect of increasing concentrations of DHA on inflammation in healthy male Sprague–Dawley rats compared to coconut oil, soybean oil, and fish oil containing EPA/DHA 1:1.
- To examine the effects of increasing concentrations of DHA on biomarkers of oxidative stress in blood and in tissues of healthy male Sprague–Dawley rats compared to coconut oil, soybean oil, and fish oil containing EPA/DHA 1:1.

3.2. EXPERIMENT 2. Fish Oil and Grape Seed Extract

- To examine the effects of combined supplementation with fish oil containing EPA/DHA 1:1 and GSE on biometric data and glucose metabolism in female Wistar–Kyoto rats fed a high-fat high-sucrose diet.
- To examine the effects of combined supplementation with fish oil containing EPA/DHA 1:1 and GSE on biomarkers of oxidative stress in the liver of female Wistar–Kyoto rats fed a high-fat high-sucrose diet.
- To explore the effects of combined supplementation with fish oil containing EPA/DHA 1:1 and GSE on abundances of diacylglycerol and ceramide species in the liver of female Wistar–Kyoto rats fed a high-fat high-sucrose diet.

3.3. EXPERIMENT 3. Fish Oil and Buckwheat D-Fagomine

- To examine the effects of combined supplementation with fish oil containing EPA/DHA 1:1 and FG on biometric data and glucose metabolism in male Sprague–Dawley rats fed a high-fat diet.
- To examine the effect of combined supplementation with fish oil containing EPA/DHA 1:1 and FG on plasma lipid profile in male Sprague–Dawley rats fed a high-fat diet.
- To examine the effects of combined supplementation with fish oil containing EPA/DHA 1:1 and FG on hepatic fat and hepatic function in male Sprague–Dawley rats fed a high-fat diet.
- To examine the effect of combined supplementation with fish oil containing EPA/DHA 1:1 and FG on inflammation in male Sprague–Dawley rats fed a high-fat diet.
- To examine the effects of combined supplementation with fish oil containing EPA/DHA 1:1 and FG on biomarkers of oxidative stress in blood and tissues of male Sprague–Dawley rats fed a high-fat diet.

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF FISH OIL AND ITS COMBINATION WITH GRAPE SEED POLYPHENOLS OR BUCKWHEAT D-FAGOMINE ON CARDIOMETABOLIC RISK FACTORS AND OXIDATIVE STRESS IN RATS
Bernat Miralles Pérez

4. Results

RESULTS

RESULTS

The results of the present doctoral thesis have been published or submitted for publication by means of the following scientific papers:

Paper 1: Effects of a Fish Oil Rich in Docosahexaenoic Acid on Cardiometabolic Risk Factors and Oxidative Stress in Healthy Rats

Paper 2: Effects of Fish Oil and Grape Seed Extract Combination on Hepatic Endogenous Antioxidants and Bioactive Lipids in Diet-Induced Early Stages of Insulin Resistance in Rats

Paper 3: The Effects of the Combination of Buckwheat D-Fagomine and Fish Omega-3 Fatty Acids on Oxidative Stress and Related Risk Factors in Pre-Obese Rats

RESULTS

4.1. PAPER 1. Effects of a Fish Oil Rich in Docosahexaenoic Acid on Cardiometabolic Risk Factors and Oxidative Stress in Healthy Rats

Type: Research article

Manuscript status: Submitted to Food & Function, May 18, 2021 (in peer review)

Title: Effects of a Fish Oil Rich in Docosahexaenoic Acid on Cardiometabolic Risk Factors and Oxidative Stress in Healthy Rats

Journal (Publisher): Food & Function (Royal Society of Chemistry)

Impact factor (JCR 2019): 4.171

Category and classification: Food Science & Technology 24/139 (Q1), and Biochemistry & Molecular Biology 91/297 (Q2)

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Citation: Not applicable

RESULTS

Effects of a Fish Oil Rich in Docosahexaenoic Acid on Cardiometabolic Risk Factors and Oxidative Stress in Healthy Rats

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Abstract

Omega-3 polyunsaturated fatty acids are associated with lower risk of cardiometabolic diseases. However, docosahexaenoic acid (DHA) is easily oxidized leading to cellular damage. The present study aimed to examine the effects of increasing concentrations of DHA in fish oil (80% of total fat) on cardiometabolic risk factors and oxidative stress compared to coconut oil, soybean oil, and fish oil containing both eicosapentaenoic acid (EPA) and DHA in a balanced ratio. Forty healthy male Sprague–Dawley rats were supplemented with the corresponding oil for 10 weeks by oral gavage. The supplementation with the fish oil containing 80% DHA decreased plasma total fat, plasma total cholesterol and muscle total fat compared to both the coconut oil and the soybean oil. Furthermore, the fish oil containing 80% DHA decreased blood glucose compared to the soybean oil. Increasing concentrations of DHA induced the incorporation of DHA and EPA in cell membranes and tissues along with a decrease in ω-6 arachidonic acid. The increase in DHA promoted lipid peroxidation, protein carbonylation and antioxidant response. Taken together, increasing concentrations of DHA in fish oil beneficially modulated several cardiometabolic risk factors in healthy rats compared to coconut oil and soybean oil. These benefits were accompanied by high lipid peroxidation and subsequent protein carbonylation, which may be an antioxidant mechanism in a healthy framework.

Keywords: docosahexaenoic acid; eicosapentaenoic acid; fish oil; soybean oil; coconut oil; cholesterol; glucose; inflammation; oxidative stress; lipid peroxidation; protein carbonylation

1. Introduction

Cardiovascular diseases (CVD) such as ischemic heart disease and stroke are major causes of death worldwide [1]. Risk factors for CVD include dyslipidemia, obesity, diabetes mellitus and increased blood pressure [2]. These CVD risk factors are highly related to dietary pattern. Observational data has shown that high intake of saturated fatty acids (SFA) increases the risk for CVD [3], whereas unsaturated fatty acids decrease that risk [4]. Therefore, the replacement of SFA to unsaturated fatty acids might be a useful nutritional strategy against metabolic alterations and CVD events [5,6].

Supplementation with omega-3 polyunsaturated fatty acids (ω-3 PUFA), specifically eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), exhibits cardioprotective effects by modulating lipid metabolism, vascular function, cell membrane dynamics as well as anti-inflammatory and antioxidant responses [7,8]. The use of fish oils supplements is prevalent in western societies [9]. Importantly, the supplements can differ in composition of ω-3 PUFA, providing them individually or in combination. Circulating and tissue lipids reflect the dietary fat intake. Thus, fish oil supplements increase the amount of ω-3 PUFA and related oxidized metabolites such as eicosanoids and docosanoids compared to soybean oil rich in linoleic acid (ω-6 LA; 18:2) [10,11]. Low ω-6/ω-3 ratio has been associated with a decrease in inflammation [12] and atherosclerotic lesions [13]. Furthermore, previous studies [10,11,14–18] have shown in rats that supplementation with fish oil decreases blood glucose, glycated hemoglobin, plasma total fatty acids, pro-inflammatory mediators and oxidative stress compared to soybean oil. Fish oil containing EPA/DHA in a balanced ratio (1:1) induces better health outcomes in blood glucose, pro-inflammatory mediators in tissues and oxidative damage to proteins than unbalanced EPA/DHA ratios 2:1 and 1:2 [16–18]. When provided individually in humans, DHA may promote greater decrease in pro-inflammatory mediators and circulating lipids than EPA [19].

Various mechanisms are involved in promoting the beneficial effects on cardiometabolic health resulting from fish oil supplements. First, ω-3 PUFA exert hypolipidemic effect through the activation of the peroxisome proliferator-activated receptor (PPAR)α, which in turn increases fatty acid β-oxidation and cholesterol uptake from plasma as well as inhibiting lipogenesis [15,20,21]. Second, ω-3 PUFA exert anti-inflammatory effect by binding to G-protein-coupled receptor 120 and increasing PPARγ, inhibiting the nuclear factor-kappa β (NF-κβ) pro-inflammatory signaling pathway [22,23]. The ω-3 and ω-6 PUFA—e.g., arachidonic acid (ARA)—may also compete for enzymes such as phospholipases A_2, cyclooxygenases (COX) and lipoxygenases (LOX) [24]. In this fashion, oxidized lipids derived from ω-3 PUFA by COX and LOX action, such as resolvins, protectins and maresins, exhibit anti-inflammatory and pro-resolving properties [24]. Furthermore, eicosanoids derived from EPA induce lower inflammatory responses than those from ω-6 PUFA [25]. And finally, ω-3 PUFA exert antioxidant effect through the induction of the nuclear factor-erythroid 2-related factor 2 (Nrf2) signaling pathway, increasing the endogenous antioxidant response [26].

Because PUFA are highly prone to oxidation by reactive oxygen species [27], supplementation with ω-3 PUFA may exhibit beneficial or deleterious effects in a dose-dependent manner [28,29]. Lipid peroxidation end products, such as 4-hydroxyhexenal (HHE), 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) derived from ω-3 and ω-6 PUFA, might form adducts with proteins leading to alterations in cell signaling and metabolic pathways [30]. In fact, accumulation of oxidized lipids is associated with increased liver triacylglycerol [29] inflammation [28] and CVD [31]. Lipid peroxidation is highly dependent on the degree of unsaturation in a fatty acid [27]. Thus, DHA might be more vulnerable to peroxidation than other fatty acids.

The aim of this study was to examine the effects of increasing concentrations of DHA in fish oil compared to coconut oil, soybean oil, and fish oil containing EPA/DHA (1:1) on cardiometabolic risk factors and oxidative stress in healthy male rats.

2. Material and Methods

2.1. Ethics Statement

All procedures on animals were adhered to the European Union (EU) guidelines for the care and handling of laboratory animals (Directive 2010/63/EU). The ethical approval was obtained from the Spanish National Research Council (CSIC) Subcommittee on Bioethical Issues and the regional Catalan authorities (reference number DAAM7921).

2.2. Animals and Experimental Design

A total of forty 22 weeks-old male Sprague–Dawley rats weighing about 400 g were purchased (Hsd:SD, Envigo, Indianapolis, IN, USA). The rats were housed in pairs in Makrolon cages (425 × 265 × 180 mm) under controlled conditions of temperature (22 ± 2 °C), humidity (60%), and 12-h artificial light/dark cycle.

After acclimatization, the rats were divided into four groups (10 per group) and fed a standard diet (Teklad Global 14% Protein Rodent Maintenance Diet; Envigo, Indianapolis, IN, USA), supplemented with coconut oil, soybean oil, EPA/DHA 1:1 or fish oil containing 80% DHA for 10 weeks. The rats were given free access to food and water throughout the study.

The oils were administered twice a week by oral gavage using a gastric probe at a dose of 0.8 mL oil/kg body weight. The coconut oil and the soybean oil were supplied by Fauser Vitaquellwerk KG (Hamburg, Germany) and Clearspring Ltd (London, United Kingdom), respectively. Fish oil containing EPA/DHA 1:1 ratio was obtained by combining fish oils AFAMPES 121 EPA (AFAMSA, Vigo, Spain) and Omega-3 RX (EnerZona, Milan, Italy). Fish oil containing 80% DHA was purchased from IFIGEN-EQUIP 98 S.L. (Barcelona, Spain). The dose of DHA supplement used in the present study was increased about 1.7-fold the safe dose suggested by the European Food Safety Authority for European general population [32]. The dose translation was performed as described in [33] based on body surface area.

2.3. Fatty Acid Composition and Peroxide Content of the Oils

Fatty acid composition of the soybean oil, the fish oil containing EPA/DHA 1:1 and the fish oil containing 80% DHA was measured according to the method developed by Lepage and Roy [34] using a gas chromatography coupled to a flame ionization detector technique (GC/FID; Clarus 500; PerkinElmer, Shelton, CT, USA), as previously described [18]. Because coconut oil is largely made up of medium-chain fatty acids (C6–12) and our GC/FID technique is not suitable for measuring this type of lipids, the fatty acid composition of coconut oil was assumed to be the one described by Lal et al. [35] . Fatty acid composition of the oils is described in the Table S1. Briefly, lauric acid (12:0) was the predominant fatty acid in the coconut oil, ω-6 LA in the soybean oil, both EPA and DHA in the fish oil containing EPA/DHA 1:1, and DHA in the fish oil containing 80% DHA.

In order to ensure that the oils were not oxidized prior to the nutritional intervention, amount of peroxide was measured according to the method developed by Chapman and Mackay [36] using a Beckman DU-640 UV-Vis spectrophotometer (Beckman Instruments Inc., Palo Alto, CA, USA). None of oils was oxidized before the beginning of the study—peroxide values below 10 mEq oxygen/kg oil.

2.4. Feed Intake and Biometric Data

Feed intake (g) and body weight (g) were monitored twice a week. At the end of the study, body weight gain, adiposity index (perigonadal white adipose tissue weight [g] / body weight [g] × 100) and hepatosomatic index (liver weight [g] / body weight [g] × 100) were calculated.

2.5. Measurement of Blood Glucose

Fasting blood glucose concentration was measured at week 0, 4, 8 and 10 of the study by means of the enzyme electrode method using an Ascensia ELITE XL blood glucometer (Bayer Consumer Care, Basel, Switzerland). Furthermore, oral glucose

tolerance tests (OGTT) were performed during week 9 of the study on fasting animals. Briefly, blood glucose was measured before the administration of a glucose solution (1 g/kg body weight) by oral gavage, and 15, 30, 45, 60, 90 and 120 min after glucose intake.

2.6. Sample Processing

After 10 weeks, the rats were fasted overnight, anesthetized intraperitoneally with ketamine and xylazine (80 and 10 mg/kg body weight, respectively) and sacrificed by exsanguination. Blood samples were taken by cardiac puncture. Subsequently, plasma was obtained by centrifugation at 850 × *g* for 15 min at 4 °C. After the removal of plasma, erythrocytes were obtained by washing twice with 154 mM sodium chloride solution and centrifugation at 1,300 × *g* for 5 min at 4 °C. Plasma and erythrocytes samples were aliquoted, and a portion of erythrocytes was mixed with 5 mM protease inhibitor phenylmethanesulfonyl fluoride for fatty acid analysis. All samples were stored at −80 °C until use, except one aliquot of erythrocytes that was washed five times with 5 mM sodium phosphate dibasic solution and centrifuged at 15,000 × *g* for 15 min at 4 °C for obtainment of erythrocyte membranes.

Perigonadal white adipose tissue, the liver and gastrocnemius muscle were collected, washed with 154 mM sodium chloride solution, weighted, and cut. After that, samples of perigonadal adipose tissue, the liver and gastrocnemius muscle were snap-frozen in liquid nitrogen and stored at −80 °C until use, except one part of the liver that was fixed for 24 h in 4% formaldehyde solution for histological study. Perigonadal adipose tissue samples were homogenized on ice in 200 mM sodium phosphate buffer (pH 6.25), sonicated for 1 min, and centrifuged at 1,000 × *g* for 10 min at 4 °C. Then, soluble fraction was carefully collected, and centrifuged at 129,000 × *g* for 1 h at 4 °C. Frozen liver samples were divided into three parts. One part of the liver sample was homogenized on ice in 154 mM sodium chloride solution containing 0.1% Triton X-100 and centrifuged at 3,000 × *g* for 5 min at room temperature for measurements of triacylglycerol (TG) and

total cholesterol (TC). The second part was homogenized on ice in 200 mM sodium phosphate buffer (pH 6.25) and centrifuged at 129,000 × *g* for 1 h at 4 °C for measurement of several oxidative stress biomarkers. The other part was homogenized on ice in 50 mM potassium phosphate buffer (pH 7.8) supplemented with 0.5 mM dithiothreitol and 1 mM EDTA, and centrifuged at 129,000 × g for 1 h at 4 °C for measurements of glutathione S-transferase (GST) and xanthine oxidase (XO). All tissue samples were aliquoted and stored at −80 °C until use.

2.7. Measurements of Total Fat and Fatty Acids in Blood and Tissues

Total fat content was extracted from erythrocytes, plasma, the liver and gastrocnemius muscle, and quantified as described in Dasilva et al. [11]. Fatty acid composition was measured in erythrocytes, perigonadal adipose tissue and the liver as previously described [11] by means of a GC/FID technique (Clarus 500; PerkinElmer, Shelton, CT, USA). Furthermore, activities of fatty acid desaturase were calculated as the ratio between product and substrate—i.e., SCD-16 = [16:1 ω-7/16:0], SCD-18 = [18:1 ω-9/18:0], Δ5D = [20:4 ω-6/20:3 ω-6], Δ6D = [20:3 ω-6/18:2 ω-6] and Δ5/6D = [20:5 ω-3/18:3 ω-3].

2.8. Measurement of Erythrocyte Membrane Fluidity

Erythrocyte membrane fluidity was measured as previously described [37]. Briefly, freshly isolated erythrocyte membranes were incubated with 1 μM 1,6-diphenyl-1,3,5-hexatriene for 1 h at room temperature under constant agitation. After that, the steady-state anisotropy (r) of the sample was measured using an LS55 fluorescence spectrophotometer (Perkin Elmer, Shelton, CT, USA). The membrane fluidity was calculated as the inverse value of anisotropy (1/r).

2.9. Measurements of Glycated Hemoglobin and Total hemoglobin

Blood glycated hemoglobin (HbA1c) was measured by means of spectrophotometry using the corresponding commercial kit (Spinreact, Girona, Spain) in a COBAS MIRA

autoanalyzer (Roche Diagnostics System, Madrid, Spain). Blood hemoglobin (Hb) concentration was measured according to the Drabkin's method [38] using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, Shelton, CT, USA).

2.10. Measurements of Lipid Profile, Transaminases and Inflammatory Biomarkers

Plasma TG, TC, high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) concentrations were measured by means of colorimetric enzymatic methods using the corresponding commercial kits (Spinreact, Girona, Spain) in a COBAS MIRA autoanalyzer (Roche Diagnostics System, Madrid, Spain). LDL/HDL ratio was also calculated. Liver TG and TC contents were measured in homogenates as described above.

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured by means of spectrophotometry using the corresponding commercial kits (Spinreact, Girona, Spain) in a COBAS MIRA autoanalyzer (Roche Diagnostics System, Madrid, Spain). AST/ALT ratio was also calculated as a biomarker of the liver function.

Plasma tumor necrosis factor α (TNFα) and C-reactive protein (CRP) concentrations were measured using the corresponding ELISA kits (Invitrogen, Waltham, MA, USA) in a PowerWave XS2 microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA).

2.11. Histological Analysis of the Liver

Fixed-liver samples were subjected to alcohol dehydration and paraffin infiltration-immersion at 52 °C. Subsequently, training paraffin block and successive 2-μm thickness sections were performed (Microm HM 355S; Thermo Fisher Scientific, Waltham, MA, USA). Sections were deposited above slides and subjected to automated hematoxylin-eosin staining (Shandon Varistain Gemini; Thermo Fisher Scientific, Waltham, MA,

USA). Histological examination was done using a Leica DM750 microscope (Leica Microsystems, Wetzlar, Germany) [39].

2.12. Measurements of Oxidative Stress Biomarkers

2.12.1. Plasma Antioxidant Capacity

Plasma non-enzymatic antioxidant capacity was assessed using the oxygen radical absorbance capacity (ORAC) [40] and the ferric reducing ability of plasma (FRAP) assays [41]. The ORAC was measured using a Fluoroskan Ascent microplate fluorimeter (Labsystems, Helsinki, Finland). The FRAP was measured using a PowerWave XS2 microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA).

2.12.2. Antioxidant Enzymes, Glutathione and Xanthine Oxidase

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) were assessed in erythrocytes, perigonadal adipose tissue and the liver, whereas GST was assessed in the liver. SOD and CAT activities were measured according to the methods developed by Mirsa and Fridovich [42] and Cohen et al. [43], respectively, using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, Shelton, CT, USA). GPx and GR activities were measured according to the method developed by Wheeler et al. [44] using a COBAS MIRA autoanalyzer (Roche Diagnostics System, Madrid, Spain). GST activity was measured according to the method developed by Habig et al. [45] using a COBAS MIRA autoanalyzer (Roche Diagnostics System, Madrid, Spain).

Reduced glutathione (GSH) and oxidized glutathione (GSSG) in plasma, erythrocytes, perigonadal adipose tissue and the liver were measured according to the method developed by Hissin and Hilf [46] using an LS55 fluorescence spectrophotometer (Perkin Elmer, Shelton, CT, USA). GSSG/GSH ratio was also calculated as a biomarker of the redox state.

XO activity in the liver was measured according to the method described by Maia and Mira [47] using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, Shelton, CT, USA).

2.12.3. Lipid Peroxidation and Protein Carbonylation

Plasma oxidized LDL concentration was measured using the corresponding ELISA kit (MyBioSource Inc., San Diego, CA, USA) in a PowerWave XS2 microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA).

Conjugated diene hydroperoxide amount in perigonadal adipose tissue and the liver was measured as previously described [48] using a Beckman DU-640 UV-Vis spectrophotometer (Beckman Instruments Inc., Palo Alto, CA, USA).

Thiobarbituric Acid-Reactive Substances (TBARS) amount in plasma, erythrocytes, perigonadal adipose tissue and the liver was measured according to the method developed by Buege and Aust [49] with modifications described by Richard et al. [50] using an LS55 fluorescence spectrophotometer (Perkin Elmer, Shelton, CT, USA).

Protein carbonylation in plasma and the liver was measured as previously described [48] by labeling carbonyl-modified proteins with fluorescein-5-thiosemicarbazide (FTSC) and resolving and quantified them using a one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2.13. Statistical Analysis

The statistical analysis was performed using SPSS v.26 software (IBM, Chicago, IL, USA). The results are expressed as mean and standard deviation, except the histological data that is expressed in frequencies (%). The Shapiro–Wilk test was used to test for normality of data. Groups were then compared by means of the one-way analysis of variance followed by the Scheffé *post-hoc* test or the non-parametric Kruskal–Wallis test followed by the Mann–Whitney U test. Frequencies were compared by means of

273 contingency tables using $\chi 2$ statistics. The level of statistical significance was set at *p*-

274 value < 0.05.

275

3. Results and Discussion

3.1. Biometric Data, Glucose Metabolism, Plasma Lipid Profile, Ectopic Fat and Transaminases

Fish oil containing 80% DHA as well as fish oil containing EPA/DHA 1:1 and coconut oil decreased fasting blood glucose in contrast to soybean oil at the end of the study (Table 1 and Table S2). Nevertheless, we observed no significant differences in either glucose tolerance—i.e., OGTT, feed intake or biometric data among the groups (Figure 1, Table S2). These findings agree with our previous study in healthy female Wistar rats [17]. In the present study, we administered a higher dose of fish oil than that used in our previous studies in healthy rats [16–18] with the aim of achieving a high incorporation of DHA into cell membranes and tissues. Other authors [21,51] have shown that supplementation with ω-3 PUFA decreases circulating glucose and enhances insulin sensitivity in tissues via action of adiponectin and PPARs. Interestingly, we observed no differences in parameters of glucose metabolism between rats supplemented with the fish oil containing either 80% DHA or EPA/DHA 1:1 and those supplemented with the coconut oil (Figure 1 and Table 1). A previous study [52] has shown that adaptive mechanisms such as increased fatty acid β-oxidation as well as decreased lipogenesis and gluconeogenesis might explain why glucose homeostasis is maintained after either high intake of SFA or PUFA under isocaloric conditions. Nevertheless, soybean oil increased blood glucose compared to the coconut oil (Table 1). Other authors [53] have suggested that supplementation with virgin coconut oil may beneficially modulate blood glucose probably via action of medium-chain FA lauric acid and polyphenols. Furthermore, the inhibition of the PI3K/AKT signaling pathway by supplementation with soybean oil [54] may contribute to explain the differences in glucose homeostasis among the groups.

The supplementation with fish oil containing either 80% DHA or EPA/DHA 1:1 clearly modulated total fat content in plasma (Table 1). Fish oil containing 80% DHA also decreased plasma TC mainly from HDL compared to both the coconut oil and the

soybean oil (Table 1). Furthermore, fish oil containing EPA/DHA 1:1 decreased plasma HDL compared to the coconut oil. In contrast, the rats supplemented with soybean oil had the most beneficial LDL/HDL ratio (Table 1). Hypercholesterolemia is a well-known risk factor for CVD [2]. Previous studies [55,56] have shown that ω-3 PUFA might decrease plasma cholesterol by stimulating of reverse cholesterol transport via an increase in scavenger receptor class B-1 expression and subsequent excretion of cholesterol by the liver. In the present study, fish oil containing 80% DHA even significantly decreased plasma HDL compared to EPA/DHA 1:1 (Table 1). In agreement with our results, it has been suggested that DHA is a more potent agent for lowering plasma lipids than EPA [7,19]. Other authors [57] have shown that a fish oil with EPA/DHA 1:2 might exert higher hypocholesterolemic effect than a balanced ratio in male apolipoprotein E knockout C57/BL6 mice on high-fat diet. Our previous studies [10,17,18] have shown no differences among distinct EPA/DHA ratios (2:1, 1:1 or 1:2) in healthy female Wistar rats. In the present study, the increase in LDL/HDL ratio after receiving the fish oil containing either 80% DHA or EPA/DHA 1:1 mainly compared to the soybean oil (Table 1) could be also explained by an increase in LDL particle size as suggested by other authors [58]. In fact, large LDL particles exhibit cardioprotective properties compared to small LDL particles [59]. Nevertheless, a recent study has evidenced an opposite effect of ω-3 PUFA supplements on LDL size [60]. Further investigation is required to understand the influence of ω-3 PUFA on lipoprotein properties. Although several studies [19,60] have shown that fish oil decreases plasma TG concentration, we observed no differences in plasma TG among the groups (Table 1). Our results are consistent with the observations of other studies in animal models [17,29].

In the present study, the supplementation with fish oil containing either 80% DHA or EPA/DHA 1:1 influenced ectopic fat accumulation (Table 1). Thus, fish oil containing 80% DHA decreased total fat content in gastrocnemius muscle compared to both coconut oil and soybean oil. On the other hand, fish oil containing 80% DHA as well as

the coconut oil increased total fat content in the liver compared to the fish oil containing EPA/DHA 1:1. Nevertheless, no significant differences in liver TG, TC and histological data were found among the groups (Table 1, Figure S1. A). A previous study [29] have shown that a high dose of DHA induces accumulation of TG in the liver of hypercholesterolaemic New Zealand White male rabbits, probably via an increase in lipoprotein receptors VLDLR and LDLR regulated by Farnesol X receptor. Interestingly, fish oil containing 80% DHA tended to increase plasma AST activity mainly compared to the soybean oil without affecting the ratio of AST to ALT (Table 1).

3.2. Fatty Acid Composition of Erythrocytes and Tissues

Numerous significant differences in fatty acid composition of erythrocytes, perigonadal adipose tissue and liver were found among the groups (Table 2, 3 and 4). Erythrocyte fatty acid composition reflects long-term dietary intake. In fact, erythrocytes are considered more robust markers of ω-3 PUFA intake than plasma [61]. In the present study, the supplementation with the fish oils containing either 80% DHA or EPA/DHA 1:1 increased the total amount of ω-3 PUFA and decreased the total amount of ω-6 PUFA in erythrocytes, perigonadal adipose tissue and liver compared to both coconut oil and soybean oil. In particular, fish oil containing 80% DHA induced the lowest ratio of ω-6 to ω-3 compared to all the other oils (Table 2, 3 and 4).

In accordance with the composition of the oils (Table S1), the supplementation with fish oil containing 80% DHA promoted higher incorporation of DHA in erythrocytes, perigonadal adipose tissue and liver than all the other oils (Table 2). Fish oil containing 80% DHA provided lower amount of EPA than fish oil containing EPA/DHA 1:1 (Table S1). In spite of this, the two types of fish oil induced similar incorporation of EPA in erythrocytes and in liver (Table 2 and 4). Our results are consistent with the observations of other authors [62]. Considering the pathways of EPA formation, fish oil containing 80% DHA seemed to promote elongation and desaturation (Δ5/6D) from α-linolenic acid (ALA, 18:3 ω-3) to EPA. Furthermore, the conversion from EPA to docosapentaenoic acid

(DPA, 22:5 ω-3) may be reduced by the supplementation of the fish oil containing 80% DHA. Thus, the modulation of these two steps after receiving the fish oil containing 80% DHA resulted in high incorporation of EPA in erythrocytes and liver. In agreement with these results, a previous study [63] showed that high dietary intake of ω-3 PUFA in the Inuit population decreased the conversion pathway from EPA to DPA, without significant effect on DHA. This fact has been considered a mechanistic adaptation to compensate for a high dietary intake of EPA [63]. The effects of the supplementation with the fish oil containing 80% DHA on EPA were not found in perigonadal adipose tissue (Table 3).

The supplementation with fish oils containing either 80% DHA or EPA/DHA 1:1 decreased the amount of ω-6 PUFA—i.e., ARA (20:4), docosatetraenoic acid (DTA, 22:4) and docosapentaenoic acid (DPA, 22:5)—in erythrocytes and in liver compared to the coconut oil and the soybean oil (Table 2 and 4) but not in the perigonadal adipose tissue (Table 3). Importantly, the two types of fish oil provided higher amounts of ARA, DTA and DPA than either coconut oil or soybean oil (Table S1). The decrease in these ω-6 PUFA in erythrocytes and in liver might be expected, given that the precursors of ω-3 and ω-6 PUFA pathways compete for the same desaturase enzymes [64]. In consonance with this, the supplementation with fish oil containing either 80% DHA or EPA/DHA 1:1 decreased the activity of Δ5-desaturase in the ω-6 PUFA pathway, resulting in a low conversion from dihomo-γ-linolenic acid (DGLA, 20:3 ω-6) to ARA. Furthermore, the activity of Δ6-desaturase = [20: 3 ω-6/18: 2 ω-6] remained unchanged (Table 2, 3 and 4). Taken together, these results showed that the supplementation with the fish oil containing either 80% DHA or EPA/DHA 1:1 modulate the ω-6 PUFA pathway, resulting in an accumulation of precursors—both LA and DGLA—along with a decrease in ARA, DTA and DPA (Table 2 and 4).

As regards to SCD-1 indexes, fish oil containing 80% DHA decreased values of SCD-16 in erythrocytes and in liver, but not those of SCD-18, compared to the coconut oil (Table

2 and 4). Low activities of SCD-1 may be associated with low accumulation of fat, low inflammation and prevention of obesity [65].

Finally, fish oil containing 80% DHA as well as fish oil containing EPA/DHA 1:1 and soybean oil induced lower incorporation of myristic acid (14:0) in perigonadal adipose tissue than coconut oil (Table 3). Despite the distinct fatty acid composition between coconut oil and soybean oil (Table S1), there were no other major differences in the fatty acid composition of their corresponding erythrocytes, perigonadal adipose tissue and liver (Table 2, 3 and 4).

3.3. Membrane Fluidity of Erythrocytes

Although highly distinct fatty acid composition of erythrocytes among the groups (Table 2), no significant differences in membrane fluidity were found (Table 1). It has been suggested that the fatty acid composition of membrane modulates fluidity, ion permeability and protein function [66]. In fact, it is assumed that increasing polyunsaturated fatty acids in membrane lead to an increase in its fluidity [66].

3.4. Inflammation and Oxidative Stress

The supplementation with fish oil containing either 80% DHA or EPA/DHA 1:1 slightly increased plasma TNFα concentration compared to both coconut oil and soybean oil (Table 1). Despite that, we observed no significant differences in either plasma CRP concentration (Table 1) or presence of portal chronic inflammation in the liver among the groups (Figure S1. B). TNFα is highly associated with CRP [67]. Nevertheless, it is important to note that concentration of plasma TNFα remained at normal, very low values in all the rats of the present study. Thus, secretion of CRP into plasma was not modified in our healthy framework. Concentration of CRP even tended to be higher in the rats supplemented with the soybean oil than in those supplemented with the other three oils (Table 1). Importantly, both low ω-6/ω-3 ratio and low SCD-16 activity are associated with a beneficial inflammatory status [12,65]. In agreement with our results, it has been

409 suggested that ω-3 PUFA supplements decrease TNFα inhibitor prostaglandin E_2 [8],
410 which is derived from ω-6 PUFA. The increase in plasma TNFα may be due to a low
411 TNFα expression in rats supplemented with the coconut oil and the soybean oil rather
412 than an increase in those supplemented with two type of fish oil. In contrast, other studies
413 have shown that ω-3 PUFA might increase secretion of TNFα from resident
414 macrophages in adipose tissue [68] and TNFα gene expression in adipocytes [28,69].

415 As far as oxidative stress is concerned, the fish oil containing 80% DHA markedly
416 increased the amount of TBARS in plasma, erythrocytes and perigonadal adipose tissue
417 (Tables 5 and 6) and tended to increase it in liver (Table 6). In fact, lipid peroxidation was
418 highly dependent on the degree of unsaturation of the oils—80% DHA > EPA/DHA 1:1
419 > soybean > coconut. Furthermore, fish oil containing 80% DHA increased the
420 abundance of protein carbonyls in plasma compared to both the coconut oil and the
421 soybean oil (Table 5), and in the liver compared to the soybean oil (Table 6).
422 Nevertheless, no significant differences were observed in either the oxidized LDL in
423 plasma (Table 5) or the conjugated diene hydroperoxides in perigonadal adipose tissue
424 and in the liver (Table 6). The TBARS assay estimates the amount of end products of
425 lipid peroxidation, which can highly react with proteins [30]. The most abundant protein in
426 plasma is albumin. Albumin exhibits antioxidant properties by enzymatic activity and by
427 scavenging of reactive compounds [70]. In the liver, a previous study [14] have shown that
428 actin is one of the main carbonylated proteins after receiving fish oil supplements. Like
429 albumin, actin may act as scavenger without undergoing significant functional
430 impairment [71]. Thus, high protein carbonylation—especially of albumin and actin—after
431 receiving the fish oil containing 80% may be an important antioxidant mechanism against
432 high lipid peroxidation in a healthy framework, preventing oxidative damage to other
433 critical proteins.

434 Concurrently, the group supplemented with fish oil containing 80% DHA had the highest
435 value of ORAC among the groups (Table 5). Importantly, the high value of ORAC may

436 be associated with a high antioxidant activity of albumin in blood samples [72]. This
437 observation was accompanied by an increase in erythrocyte GPx activity compared to
438 both soybean oil and fish oil containing EPA/DHA 1:1 (Table 5), and a decrease in
439 adipocyte GR activity compared to the soybean oil (Table 6). Furthermore, liver GSSG
440 and liver XO tended to decrease after receiving fish oil containing 80% DHA compared
441 to coconut oil and soybean oil, respectively (Table 6). Other authors [73] have shown that
442 high activity of XO in serum is associated with high risk for developing diabetes. We
443 observed no significant differences in either cytosolic SOD, CAT, GPx, GST or GSH in
444 perigonadal adipose tissue and in liver among the groups. However, we cannot exclude
445 differences in other critical enzymes related to antioxidant defense (e.g., heme
446 oxygenase-1), in other cell compartments and in other tissues. In fact, high content of
447 DHA in cell membranes may promote an increased antioxidant response through the
448 action of lipid peroxidation end products [74]. In agreement with our observations, previous
449 studies [29,62] have shown that supplementation with ω-3 PUFA increases the amount of
450 TBARS, 4-HHE and 4-HHE-protein adducts in a dose-dependent manner. Furthermore,
451 Takahashi et al. [75] have shown that a very high dose of fish oil induces gene expression
452 of enzymes related to antioxidant and xenobiotic-metabolizing activities. This
453 compensatory antioxidant response against increased lipid peroxidation could be
454 mediated via activation of Nrf2 [26]. A previous study [76] has shown that dietary oxidized
455 lipids induce the expression of the Nrf2 and subsequent increase in antioxidant enzymes
456 in intestinal mucosa. In particular, 4-HHE increases GPx2 expression in jejunum of male
457 C57BL/6 mice [77] involved in detoxification of lipid hydroperoxides. Interestingly, we found
458 no differences in erythrocyte GPx activity between the rats supplemented with the fish
459 oil containing 80% DHA and those supplemented with the coconut oil (Table 5). A
460 previous study [78] has shown that a supplementation with virgin coconut oil increases
461 antioxidant enzymes and decreases lipid peroxidation compared to copra oil, olive oil
462 and sunflower oil, probably related to its high amount of polyphenols and tocopherols.

4. Conclusions

The supplementation with fish oil rich in DHA beneficially modulated several cardiometabolic risk factors in healthy rats compared to coconut oil and soybean oil, especially total fat and cholesterol. The cardiometabolic benefits provided by high concentrations of DHA were greater than those obtained with fish oil containing EPA/DHA 1:1. The high dose of DHA increased the amount of DHA and EPA in erythrocytes and tissues, with a concomitant decrease in ARA. The accumulation of DHA promoted lipid peroxidation, which in turn induced protein carbonylation. In a healthy framework, high protein carbonylation (e.g., albumin and actin) after receiving fish oil rich in DHA may enhance the antioxidant response against end products of lipid peroxidation to prevent oxidative damage to other critical proteins.

Acknowledgments

This work was supported by the Spanish Ministry of Economy and Competitiveness (grant numbers AGL2013-49079-C2-1-R, AGL2013-49079-C2-2-R, AGL2017-83599-R and RTI2018-095659-B-I00). The Secretariat of Universities and Research of the Government of Catalonia and the European Social Fund are gratefully acknowledged for the doctoral fellowship to B.M.-P. (2018 FI_B 00919, 2019 FI_B1 00160, 2020 FI_B2 00119). Xunta de Galicia and Axencia Galega de Innovación (GAIN) are thankfully recognized by the financial support of the postdoctoral contract to L.M. (IN606B 2017/006).

Competing Interests

There are no conflicts to declare.

Author Contributions

M.R.N., I.M., and M.R. designed the research; B.M.-P., L.M., V.S.-M., A.F.-M., S.R.-R., and M.H. conducted the research; B.M.-P., L.M., M.R.N., and M.R. analyzed the data and wrote the manuscript. All authors reviewed and approved the final manuscript.

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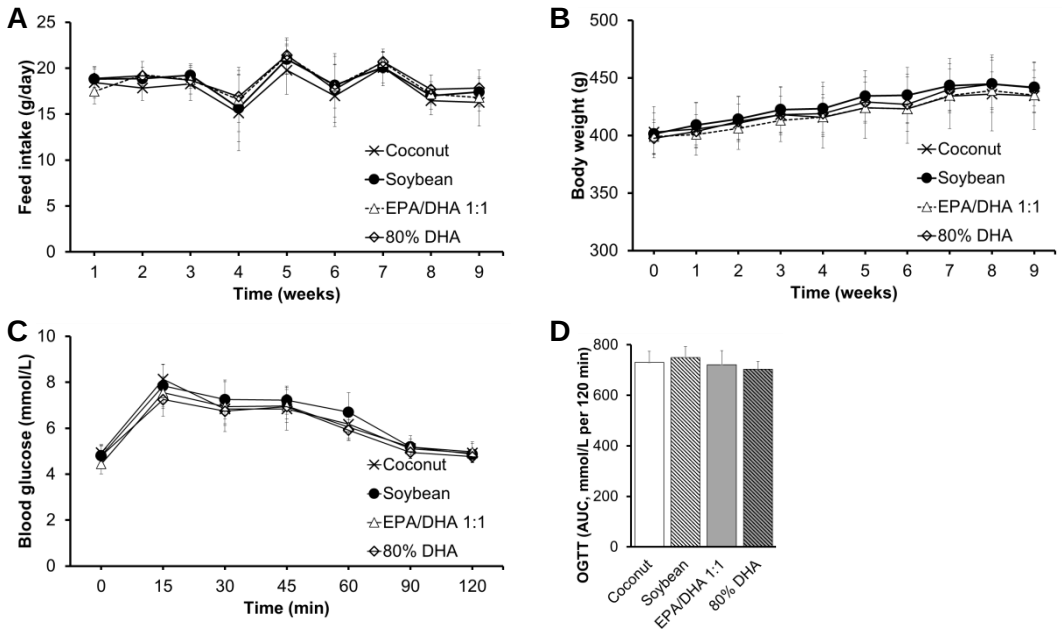

Figure 1. Feed intake (**A**), Biometric data (**B**) and oral glucose tolerance at week 9 (**C**, **D**). Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; OGTT, oral glucose tolerance test; AUC, area under the curve. OGTT was performed during week nine and the area under the curve (mmol/L per 120 min) was calculated using the Trapezium method. No significant differences were found.

Table 1. Glucose metabolism, plasma lipid profile, ectopic fat, transaminases, erythrocyte membrane properties and inflammatory biomarkers at the end of the study.

	Coconut	**Soybean**	**EPA/DHA 1:1**	**80% DHA**	***p*-value**
Glucose metabolism					
Blood glucose (mmol/L)	5.1 ± 0.3	5.5 ± 0.4[a]	5.0 ± 0.2[b]	5.0 ± 0.2[b]	0.008†
Blood HbA1c (%)	2.9 ± 0.1	3.0 ± 0.3	3.2 ± 0.2	3.2 ± 0.3	0.035*
Plasma lipid profile					
Fat content (%)	0.52 ± 0.04	0.45 ± 0.03	0.44 ± 0.03[a]	0.37 ± 0.04[a,b]	0.033*
TG (mmol/L)	0.7 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	0.7 ± 0.2	0.885*
TC (mmol/L)	2.7 ± 0.3	2.5 ± 0.4	2.4 ± 0.2	2.1 ± 0.2[a,b]	0.001*
HDL (mmol/L)	1.80 ± 0.21	1.69 ± 0.21	1.62 ± 0.12[a]	1.47 ± 0.15[a,b,c]	0.004†
LDL (mmol/L)	0.45 ± 0.08	0.35 ± 0.13[a]	0.45 ± 0.05[b]	0.39 ± 0.09	0.027†
LDL/HDL ratio	0.25 ± 0.03	0.20 ± 0.06[a]	0.28 ± 0.03[a,b]	0.27 ± 0.06[b]	0.003†
Ectopic fat					
Liver fat content (%)	5.36 ± 0.19	5.13 ± 0.35	4.92 ± 0.31[a]	5.12 ± 0.32[c]	0.019*
Liver TG (µmol/g tissue)	11.2 ± 1.1	10.9 ± 1.7	11.0 ± 1.3	10.9 ± 1.3	0.667†
Liver TC (µmol/g tissue)	4.7 ± 0.9	4.8 ± 1.1	5.8 ± 0.6	5.6 ± 0.6	0.014*
Muscle fat content (%)	2.13 ± 0.67	2.27 ± 0.53	2.17 ± 0.43	1.64 ± 0.17[a,b]	0.014*
Transaminases					
Plasma AST (U/L)	91.2 ± 29.4	85.8 ± 31.7	110.2 ± 15.5	120.7 ± 21.0	0.023*
Plasma ALT (U/L)	43.9 ± 15.9	43.8 ± 11.3	49.0 ± 12.2	53.8 ± 23.8	0.434†
Plasma AST/ALT ratio	2.13 ± 0.55	1.92 ± 0.34	2.39 ± 0.71	2.49 ± 0.88	0.348†
Erythrocyte membrane properties					
Fat content (%)	2.05 ± 0.19	2.04 ± 0.20	1.98 ± 0.26	1.98 ± 0.23	0.899*
Fluidity (AU)	4.6 ± 0.9	5.1 ± 0.9	4.9 ± 0.9	4.9 ± 0.8	0.455†
Inflammatory biomarkers					
Plasma TNFα (pg/mL)	21.6 ± 5.7	18.3 ± 8.8	27.9 ± 6.8[a,b]	30.6 ± 10.2[a,b]	0.004†
Plasma CRP (µg/mL)	390 ± 30	431 ± 56	380 ± 40	388 ± 47	0.059*

Values are expressed as mean ± standard deviation, n = 10 rats/group. Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; HbA1c, glycated hemoglobin; AU, arbitrary unit; TG, triacylglycerol; TC, total cholesterol; HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TNFα, tumor necrosis factor α; CRP, C-reactive protein. **p*-value was calculated by the one-way analysis of variance followed by the Scheffé post-hoc test. †*p*-value was calculated by the non-parametric Kruskal-Wallis test followed by the Mann–Whitney U test. The level of statistical significance was set at *p*-value < 0.05. a *vs*. Coconut, b *vs*. Soybean, c *vs*. EPA/DHA 1:1.

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Table 2. Fatty acids in erythrocytes at the end of the study.

FA	**Coconut**	**Soybean**	**EPA/DHA 1:1**	**80% DHA**	***p*-value**
14:0	0.31 ± 0.08	0.26 ± 0.05	0.34 ± 0.22	0.20 ± 0.02	0.079*
15:0	0.37 ± 0.03	0.32 ± 0.06	0.37 ± 0.04	0.34 ± 0.03	0.021*
16:0	30.02 ± 0.70	29.59 ± 0.86	30.89 ± 1.02[b]	30.79 ± 0.78[b]	0.003*
16:1 ω-7	0.51 ± 0.08	0.52 ± 0.11	0.53 ± 0.16	0.42 ± 0.06	0.086*
17:0	0.47 ± 0.05	0.46 ± 0.07	0.51 ± 0.04	0.39 ± 0.14[c]	0.033*
18:0	11.85 ± 0.34	12.08 ± 0.37	11.58 ± 0.28[b]	12.11 ± 0.33[c]	0.003*
18:1 ω-9	5.68 ± 0.30	5.79 ± 0.19	5.44 ± 0.77	5.60 ± 0.24	0.346*
18:1 ω-7	3.47 ± 0.16	3.30 ± 0.14	3.67 ± 1.02	2.95 ± 0.14[c]	0.034*
18:2 ω-6	8.91 ± 0.46	9.24 ± 0.19	10.22 ± 0.4[a,b]	10.94 ± 0.43[a,b,c]	<0.001*
18:3 ω-6	0.05 ± 0.04	0.06 ± 0.04	0.01 ± 0.03[b]	ND[a,b]	<0.001*
18:3 ω-3	0.04 ± 0.05	0.07 ± 0.04	0.07 ± 0.04	ND[b,c]	<0.001*
20:1 ω-9	0.17 ± 0.03	0.18 ± 0.04	0.18 ± 0.07	0.15 ± 0.02	0.240*
20:2 ω-6	0.33 ± 0.04	0.34 ± 0.04	0.33 ± 0.05	0.33 ± 0.04	0.992*
20:3 ω-6	0.41 ± 0.05	0.39 ± 0.06	0.47 ± 0.05[b]	0.54 ± 0.06[a,b]	0.015*
20:4 ω-6	22.71 ± 0.45	22.47 ± 0.74	19.69 ± 0.87[a,b]	18.55 ± 0.85[a,b,c]	<0.001*
22:1 ω-9	0.33 ± 0.10	0.34 ± 0.09	0.26 ± 0.06	0.33 ± 0.09	0.207*
20:5 ω-3	0.21 ± 0.04	0.22 ± 0.03	0.86 ± 0.33[a,b]	0.87 ± 0.21[a,b]	<0.001*
24:0	1.06 ± 0.11	1.23 ± 0.14[a]	1.16 ± 0.05	1.19 ± 0.12	0.013*
22:4 ω-6	2.68 ± 0.22	2.67 ± 0.15	1.40 ± 0.18[a,b]	1.07 ± 0.15[a,b,c]	<0.001*
24:1 ω-9	0.55 ± 0.04	0.68 ± 0.14[a]	0.60 ± 0.07	0.55 ± 0.06[b]	0.006*
22:5 ω-6	1.09 ± 0.11	1.04 ± 0.08	0.61 ± 0.05[a,b]	0.63 ± 0.06[a,b]	<0.001*
22:5 ω-3	1.54 ± 0.08	1.69 ± 0.17	2.56 ± 0.14[a,b]	1.86 ± 0.12[a,c]	<0.001*
22:6 ω-3	1.83 ± 0.19	1.86 ± 0.1	3.30 ± 0.2[a,b]	5.21 ± 0.56[a,b,c]	<0.001*
SAT	48.12 ± 0.85	47.87 ± 0.83	48.59 ± 1.09	48.87 ± 0.99	0.099*
MUFA	11.51 ± 0.37	11.4 ± 0.34	11.29 ± 0.42	10.6 ± 0.3[a,b,c]	<0.001*
PUFA	40.36 ± 0.9	40.72 ± 0.87	40.13 ± 1.35	40.53 ± 1.09	0.643*
ω-3	3.63 ± 0.22	3.85 ± 0.24	6.79 ± 0.51[a,b]	7.93 ± 0.8[a,b,c]	<0.001*
ω-6	36.18 ± 0.81	36.2 ± 0.73	32.73 ± 1.08[a,b]	32.05 ± 0.97[a,b]	<0.001*
ω-6/ω-3 ratio	10.01 ± 0.59	9.44 ± 0.58	4.84 ± 0.34[a,b]	4.08 ± 0.48[a,b,c]	<0.001*
SCD-16 = [16:1 ω-7/16:0]	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.017*
SCD-18 = [18:1 ω-9/18:0]	0.48 ± 0.02	0.48 ± 0.02	0.47 ± 0.07	0.46 ± 0.02	0.721*
Δ5D = [20:4 ω-6/20:3 ω-6]	56.14 ± 6.69	59.32 ± 9.01	42.35 ± 3.58[a,b]	35.16 ± 5.36[a,b]	<0.001*
Δ6D = [20:3 ω-6/18:2 ω-6]	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.084*
Δ5/6D = [20:5 ω-3/18:3 ω-3]	2.40 ± 0.78	2.62 ± 0.66	11.01 ± 4.65	ND	

Values are expressed as mean ± standard deviation (mg/100mg of total FA), n = 10 rats/group. Abbreviations: FA, fatty acid; ND, non-detected; SAT, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. **p*-value was calculated by the one-way analysis of variance followed by the Scheffé post-hoc test. The level of statistical significance was set at *p*-value < 0.05. a *vs*. Coconut, b *vs*. Soybean, c *vs*. EPA/DHA 1:1.

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Table 3. Fatty acids in perigonadal adipose tissue at the end of the study.

FA	**Coconut**	**Soybean**	**EPA/DHA 1:1**	**80% DHA**	***p*-value**
14:0	1.15 ± 0.17	0.94 ± 0.11[a]	0.98 ± 0.11[a]	0.87 ± 0.09[a]	<0.001*
15:0	0.33 ± 0.04	0.30 ± 0.03	0.33 ± 0.03	0.32 ± 0.04	0.206*
16:0	19.22 ± 1.78	19.25 ± 1.79	19.41 ± 1.75	18.52 ± 1.62	0.678*
16:1 ω-9	0.59 ± 0.05	0.59 ± 0.04	0.57 ± 0.03	0.62 ± 0.06	0.068*
16:1 ω-7	3.86 ± 1.21	3.41 ± 0.73	3.95 ± 1.52	3.28 ± 1.17	0.526*
17:0	0.24 ± 0.03	0.24 ± 0.02	0.25 ± 0.03	0.25 ± 0.03	0.519*
18:0	2.38 ± 0.30	2.45 ± 0.24	2.43 ± 0.38	2.47 ± 0.32	0.926*
18:1 ω-9	23.21 ± 0.40	23.44 ± 0.4	23.01 ± 0.46	22.88 ± 0.44	0.033*
18:1 ω-7	3.88 ± 0.32	3.77 ± 0.22	3.87 ± 0.19	3.6 ± 0.22	0.051*
18:2 ω-6	40.37 ± 2.02	41.15 ± 1.71	39.99 ± 1.5	41.38 ± 1.44	0.231*
18:3 ω-6	0.11 ± 0.01	0.10 ± 0.02	0.10 ± 0.01	0.09 ± 0.01 [a]	0.022*
20:0	0.07 ± 0.02	0.08 ± 0.02	0.10 ± 0.03	0.08 ± 0.01	0.066*
18:3 ω-3	1.99 ± 0.32	2.01 ± 0.27	1.93 ± 0.30	1.98 ± 0.18	0.923*
20:1 ω-9	0.31 ± 0.08	0.29 ± 0.04	0.32 ± 0.07	0.29 ± 0.05	0.521*
20:1 ω-7	0.37 ± 0.11	0.36 ± 0.07	0.42 ± 0.12	0.36 ± 0.08	0.523*
20:2 ω-6	0.31 ± 0.09	0.28 ± 0.08	0.29 ± 0.09	0.32 ± 0.1	0.745*
20:3 ω-6	0.18 ± 0.05	0.14 ± 0.05	0.16 ± 0.05	0.19 ± 0.07	0.326*
20:4 ω-6	0.80 ± 0.21	0.69 ± 0.20	0.69 ± 0.29	0.67 ± 0.24	0.590*
20:5 ω-3	0.02 ± 0.03	ND	0.2 ± 0.04[a,b]	0.12 ± 0.03[a,b,c]	<0.001*
22:4 ω-6	0.24 ± 0.06	0.20 ± 0.05	0.18 ± 0.07	0.19 ± 0.07	0.266*
22:5 ω-3	0.20 ± 0.08	0.16 ± 0.06	0.33 ± 0.08[a,b]	0.34 ± 0.09[a,b]	<0.001*
22:6 ω-3	0.19 ± 0.07	0.17 ± 0.06	0.5 ± 0.12[a,b]	1.18 ± 0.25[a,b,c]	<0.001*
SAT	23.38 ± 2.09	23.26 ± 2.11	23.49 ± 2.18	22.5 ± 2	0.715*
MUFA	32.22 ± 1.18	31.85 ± 0.73	32.13 ± 1.42	31.04 ± 1.41	0.136*
PUFA	44.40 ± 2.44	44.89 ± 2.30	44.37 ± 1.88	46.46 ± 1.65	0.104*
ω-3	2.39 ± 0.48	2.33 ± 0.37	2.96 ± 0.50[b]	3.63 ± 0.43[a,b,c]	0.003*
ω-6	42 ± 2.23	42.56 ± 1.98	41.42 ± 1.58	42.83 ± 1.58	0.347*
ω-6/ω-3 ratio	19.47 ± 3.43	19.94 ± 2.30	15.61 ± 2.28[a,b]	13.20 ± 1.66[a,b]	0.006*
SCD-16 = [16:1 ω-7/16:0]	0.20 ± 0.07	0.18 ± 0.05	0.21 ± 0.10	0.18 ± 0.07	0.745*
SCD-18 = [18:1 ω-9/18:0]	9.98 ± 1.24	9.73 ± 0.87	9.80 ± 1.84	9.51 ± 1.46	0.901*
Δ5D = [20:4 ω-6/20:3 ω-6]	4.66 ± 0.79	4.99 ± 0.57	4.28 ± 0.51	3.73 ± 0.60[a,b]	<0.001*
Δ6D = [20:3 ω-6/18:2 ω-6]	0.004 ± 0.001	0.003 ± 0.001	0.004 ± 0.001	0.004 ± 0.001	0.188*
Δ5/6D = [20:5 ω-3/18:3 ω-3]	0.008 ± 0.013	0.000 ± 0.000	0.103 ± 0.026[a,b]	0.062 ± 0.014[a,b,c]	<0.001*

Values are expressed as mean ± standard deviation (mg/100mg of total FA), n = 10 rats/group. Abbreviations: FA, fatty acid; ND, non-detected; SAT, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. **p*-value was calculated by the one-way analysis of variance followed by the Scheffé post-hoc test. The level of statistical significance was set at *p*-value < 0.05. a *vs*. Coconut, b *vs*. Soybean, c *vs*. EPA/DHA 1:1.

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Table 4. Fatty acids in the liver at the end of the study.

FA	**Coconut**	**Soybean**	**EPA/DHA 1:1**	**80% DHA**	***p*-value**
14:0	0.34 ± 0.12	0.24 ± 0.03	0.24 ± 0.06	0.23 ± 0.07	0.086*
15:0	0.22 ± 0.06	0.20 ± 0.02	0.19 ± 0.02	0.20 ± 0.02	0.221*
16:0	22.29 ± 1.10	21.58 ± 0.78	22.66 ± 0.58	23.07 ± 1.18[b]	0.009*
16:1 ω-7	1.19 ± 0.34	0.92 ± 0.16	0.87 ± 0.16	0.86 ± 0.29[a]	0.042*
17:0	0.39 ± 0.05	0.40 ± 0.06	0.38 ± 0.03	0.37 ± 0.04	0.326*
18:0	13.08 ± 1.28	14.26 ± 1.19	13.79 ± 0.81	13.72 ± 1.54	0.348*
18:1 ω-9	5.74 ± 2.26	5.61 ± 0.56	5.07 ± 0.48	4.81 ± 1.31	0.101*
18:1 ω-7	3.82 ± 0.45	3.52 ± 0.25	3.19 ± 0.23[a]	2.91 ± 0.38[a,b]	<0.001*
18:2 ω-6	20.59 ± 1.17	20.16 ± 1.29	20.94 ± 1.02	22.37 ± 1.15[b]	0.014*
18:3 ω-6	0.24 ± 0.05	0.25 ± 0.03	0.15 ± 0.03[a,b]	0.12 ± 0.02[a,b]	<0.001*
20:0	0.04 ± 0.04	0.08 ± 0.01	0.00 ± 0.00[a,b]	0.00 ± 0.00[a,b]	<0.001*
18:3 ω-3	0.38 ± 0.06	0.35 ± 0.05	0.31 ± 0.06	0.33 ± 0.11	0.607*
20:1 ω-9	0.12 ± 0.03	0.12 ± 0.02	0.10 ± 0.01	0.10 ± 0.04	0.1193*
20:1 ω-7	0.25 ± 0.04	0.26 ± 0.04	0.26 ± 0.07	0.23 ± 0.04	0.376*
20:2 ω-6	0.35 ± 0.07	0.36 ± 0.07	0.29 ± 0.04	0.30 ± 0.03	0.021*
20:3 ω-6	0.42 ± 0.09	0.41 ± 0.12	0.54 ± 0.08[b]	0.66 ± 0.09[a,b]	<0.001*
20:4 ω-6	22.01 ± 1.60	22.71 ± 0.94	19.26 ± 1.32[a,b]	16.15 ± 2.31[a,b]	<0.001*
22:1 ω-9	0.07 ± 0.04	0.1 ± 0.02	0.00 ± 0.00[a,b]	0.12 ± 0.02[c]	0.025*
23:0	0.14 ± 0.02	0.14 ± 0.02	0.13 ± 0.03	0.13 ± 0.02	0.441*
20:5 ω-3	0.23 ± 0.08	0.21 ± 0.03	0.91 ± 0.2[a,b]	1.09 ± 0.23[a,b]	<0.001*
24:0	0.43 ± 0.04	0.42 ± 0.06	0.46 ± 0.03	0.45 ± 0.06	0.357*
22:4 ω-6	0.61 ± 0.06	0.58 ± 0.03	0.30 ± 0.02[a,b]	0.21 ± 0.04[a,b,c]	<0.001*
24:1 ω-9	0.19 ± 0.17	0.13 ± 0.02	0.15 ± 0.01	0.15 ± 0.02	0.313*
22:5 ω-6	0.48 ± 0.15	0.41 ± 0.08	0.17 ± 0.02[a,b]	0.21 ± 0.06[a,b]	0.020*
22:5 ω-3	1.23 ± 0.14	1.25 ± 0.14	1.53 ± 0.19[a,b]	1.02 ± 0.13[b,c]	<0.001*
22:6 ω-3	5.14 ± 0.70	5.35 ± 0.29	8.10 ± 0.51[a,b]	10.19 ± 1.35[a,b,c]	<0.001*
SAT	36.93 ± 1.25	37.33 ± 1.06	37.84 ± 0.63	38.17 ± 1.04[a]	0.032*
MUFA	11.40 ± 2.60	10.65 ± 0.83	9.65 ± 0.75	9.18 ± 1.46[a,b]	0.012*
PUFA	51.67 ± 1.40	52.02 ± 1.08	52.51 ± 0.58	52.64 ± 0.80	0.204*
ω-3	6.97 ± 0.69	7.16 ± 0.32	10.86 ± 0.72[a,b]	12.63 ± 1.57[a,b,c]	<0.001*
ω-6	44.69 ± 0.88	44.87 ± 1.11	41.65 ± 0.87[a,b]	40.01 ± 1.78[a,b,c]	<0.001*
ω-6/ω-3 ratio	6.42 ± 0.53	6.06 ± 0.29	3.79 ± 0.29[a,b]	3.07 ± 0.46[a,b,c]	<0.001*
SCD-16 = [16:1 ω-7/16:0]	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01[a]	0.012*
SCD-18 = [18:1 ω-9/18:0]	0.50 ± 0.18	0.40 ± 0.07	0.37 ± 0.06	0.39 ± 0.13	0.213*
Δ5D = [20:4 ω-6/20:3 ω-6]	57.39 ± 13.78	59.20 ± 13.66	35.12 ± 6.98[a,b]	25.50 ± 5.64[a,b]	0.035*
Δ6D = [20:3 ω-6/18:2 ω-6]	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.053*
Δ5/6D = [20:5 ω-3/18:3 ω-3]	0.54 ± 0.17	0.63 ± 0.12	2.98 ± 0.81[a,b]	3.63 ± 0.83[a,b]	<0.001*

Values are expressed as mean ± standard deviation (mg/100mg of total FA), n = 10 rats/group. Abbreviations: FA, fatty acid; ND, non-detected; SAT, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. **p*-value was calculated by the one-way analysis of variance followed by the Scheffé post-hoc test. The level of statistical significance was set at *p*-value < 0.05. a *vs*. Coconut, b *vs*. Soybean, c *vs*. EPA/DHA 1:1.

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Table 5. Biomarkers of oxidative stress in blood at the end of the study.

	Coconut	**Soybean**	**EPA/DHA 1:1**	**80% DHA**	***p*-value**
Plasma					
ORAC (µmol TE/mL)	14.6 ± 2.7	12.5 ± 2.8	16.3 ± 2.6[b]	17.0 ± 1.9[a,b]	0.004†
FRAP (mmol TE/L)	0.12 ± 0.02	0.12 ± 0.04	0.11 ± 0.02	0.11 ± 0.03	0.734*
GSH (nmol/mL)	12.1 ± 3.0	10.8 ± 4.1	7.6 ± 3.0	9.7 ± 4.1	0.054*
GSSG (nmol/mL)	37.2 ± 4.1	37.2 ± 4.3	40.5 ± 4.6	38.9 ± 3.0	0.223*
GSSG/GSH ratio	3.24 ± 0.80	3.80 ± 1.08	5.25 ± 1.33[a,b]	4.72 ± 1.66	0.012†
Albumin carbonylation index	2.37 ± 0.29	2.14 ± 0.33	2.66 ± 0.65	2.93 ± 0.46[a,b]	0.002*
Oxidized LDL (µg/mL)	0.16 ± 0.03	0.15 ± 0.03	0.16 ± 0.03	0.18 ± 0.03	0.252†
TBARS (nmol MDA Eq/mL)	0.77 ± 0.18	0.99 ± 0.11	0.69 ± 0.20	1.04 ± 0.40[c]	0.011*
Erythrocytes					
SOD (U/g Hb)	2,631 ± 727	2,731 ± 971	2,415 ± 489	2,470 ± 389	0.730*
CAT (mmol/g Hb)	47.1 ± 11.5	43.0 ± 14.2	43.9 ± 7.2	52.5 ± 6.9	0.217*
GPx (U/g Hb)	132 ± 31	116 ± 36	113 ± 22	164 ± 45[b,c]	0.011*
GR (U/g Hb)	0.27 ± 0.12	0.26 ± 0.09	0.24 ± 0.07	0.21 ± 0.07	0.770†
GSH (µmol/g Hb)	1.85 ± 0.70	1.43 ± 0.67	1.50 ± 0.41	1.74 ± 0.63	0.401*
GSSG (µmol/g Hb)	1.18 ± 0.40	1.06 ± 0.47	1.11 ± 0.21	1.20 ± 0.33	0.824*
GSSG/GSH ratio	0.66 ± 0.19	0.78 ± 0.24	0.82 ± 0.34	0.74 ± 0.22	0.593*
TBARS (nmol MDA Eq/g Hb)	0.24 ± 0.11	0.32 ± 0.27	0.52 ± 0.28	0.73 ± 0.19[a,b]	<0.001*

Values are expressed as mean ± standard deviation, n = 10 rats/group. Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ORAC, oxygen radical absorbance capacity; TE, Trolox equivalent; FRAP, ferric reducing ability of plasma; GSH, reduced glutathione; GSSG, oxidized glutathione; oxidized LDL, oxidized low-density lipoprotein; TBARS, thiobarbituric acid-reactive substances; MDA Eq, malondialdehyde equivalent; SOD, superoxide dismutase; Hb, hemoglobin; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase. **p*-value was calculated by the one-way analysis of variance followed by the Scheffé post-hoc test. †*p*-value was calculated by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney U test. The level of statistical significance was set at *p*-value < 0.05. a *vs*. Coconut, b *vs*. Soybean, c *vs*. EPA/DHA 1:1.

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Table 6. Biomarkers of oxidative stress in tissues at the end of the study.

	Coconut	**Soybean**	**EPA/DHA 1:1**	**80% DHA**	***p*-value**
Perigonadal adipose tissue					
SOD (U/g tissue)	226 ± 45	237 ± 44	236 ± 61	225 ± 36	0.925*
CAT (mmol/g tissue)	0.11 ± 0.02	0.09 ± 0.01	0.11 ± 0.02	0.10 ± 0.03	0.132†
GPx (U/g tissue)	0.54 ± 0.20	0.58 ± 0.18	0.50 ± 0.16	0.41 ± 0.20	0.230*
GR (U/g tissue)	0.40 ± 0.07	0.46 ± 0.10	0.39 ± 0.11	0.35 ± 0.08[b]	0.047†
GSH (nmol/g tissue)	0.93 ± 0.23	1.05 ± 0.15	1.03 ± 0.19	0.91 ± 0.14	0.245*
GSSG (nmol/g tissue)	4.43 ± 0.58	4.56 ± 0.93	4.05 ± 0.52	4.53 ± 1.05	0.470*
GSSG/GSH ratio	5.10 ± 1.78	4.46 ± 1.26	4.03 ± 0.67	5.11 ± 1.44	0.152†
Conjugated dienes (mmol hydroperoxides/kg lipid)	9.87 ± 1.43	8.83 ± 2.03	10.33 ± 2.75	10.83 ± 2.86	0.425*
TBARS (nmol MDA Eq/g tissue)	2.41 ± 2.16	3.18 ± 2.51	4.95 ± 2.47[a]	4.99 ± 2.64[a]	0.046†
Liver					
SOD (U/g tissue)	6,411 ± 631	6,879 ± 1,046	6,577 ± 872	6,083 ± 829	0.231*
CAT (mmol/g tissue)	10.8 ± 1.3	11.1 ± 0.5	11.4 ± 1.2	11.8 ± 0.7	0.131*
GPx (U/g tissue)	29.5 ± 5.2	25.1 ± 2.9	26.8 ± 2.9	26.0 ± 3.2	0.065*
GR (U/g tissue)	6.80 ± 1.19	7.62 ± 2.34	7.78 ± 2.00	7.53 ± 1.73	0.656*
GST (U/g tissue)	113 ± 11	115 ± 22	104 ± 21	103 ± 16	0.358*
GSH (µmol/g tissue)	2.22 ± 0.55	2.22 ± 0.59	2.09 ± 0.52	2.08 ± 0.60	0.928†
GSSG (µmol/g tissue)	1.47 ± 0.20	1.38 ± 0.23	1.30 ± 0.16	1.23 ± 0.11	0.048*
GSSG/GSH ratio	0.71 ± 0.25	0.65 ± 0.20	0.66 ± 0.21	0.63 ± 0.16	0.859*
XO (mU/g tissue)	96 ± 26	121 ± 36	89 ± 15	88 ± 20	0.022*
Protein carbonylation index	2.73 ± 0.68	2.11 ± 0.57	2.43 ± 0.67	3.05 ± 0.35[b]	0.007*
Conjugated dienes (mmol hydroperoxides/kg lipid)	20.7 ± 2.3	20.6 ± 1.3	21.9 ± 1.5	21.0 ± 2.8	0.094*
TBARS (nmol MDA Eq/g tissue)	217 ± 72	229 ± 115	278 ± 90	320 ± 94	0.074*

Values are expressed as mean ± standard deviation, n = 10 rats/group. Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; TBARS, thiobarbituric acid-reactive substances; MDA Eq, malondialdehyde equivalent; GST, glutathione S-transferase; XO, xanthine oxidase. **p*-value was calculated by the one-way analysis of variance followed by the Scheffé post-hoc test. †*p*-value was calculated by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney U test. The level of statistical significance was set at *p*-value < 0.05. a *vs*. Coconut, b *vs*. Soybean, c *vs*. EPA/DHA 1:1.

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SUPPORTING INFORMATION

Effects of a Fish Oil Rich in Docosahexaenoic Acid on Cardiometabolic Risk Factors and Oxidative Stress in Healthy Rats

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Table S1. Fatty acid composition (mol %) of oils.

	Coconut*	**Soybean**	**Fish (EPA/DHA 1:1)**	**Fish (80% DHA)**
6:0	0.2–0.5			
8:0	5.4–9.5			
10:0	4.5–9.7			
12:0	44.1–51.0			
14:0	13.1–18.5	0.12 ± 0.03	1.40 ± 0.05	ND
15:0		ND	0.43 ± 0.06	ND
16:0	7.5–10.5	10.93 ± 0.13	8.39 ± 0.04	0.12 ± 0.02
16:1 ω-7		0.10 ± 0.00	1.92 ± 0.00	0.26 ± 0.01
17:0		0.16 ± 0.01	1.38 ± 0.01	0.16 ± 0.02
18:0	1.0–3.2	4.14 ± 0.08	3.74 ± 0.01	0.11 ± 0.02
18:1 ω-9	5.0–8.2	23.30 ± 0.18	8.09 ± 0.02	0.23 ± 0.03
18:1 ω-7		2.11 ± 0.01	1.88 ± 0.02	ND
18:2 ω-6	1.0–2.6	50.75 ± 0.36	1.29 ± 0.01	0.09 ± 0.02
20:00	0.2–1.5	0.33 ± 0.01	0.47 ± 0.00	ND
18:3 ω-3		7.02 ± 0.02	0.45 ± 0.03	ND
20:1 ω-9		0.20 ± 0.02	2.44 ± 0.01	ND
18:4 ω-3		ND	0.79 ± 0.01	ND
20:2 ω-6		ND	0.43 ± 0.02	ND
20:3 ω-6		ND	0.32 ± 0.01	ND
20:4 ω-6		0.49 ± 0.04	2.65 ± 0.04	0.75 ± 0.02
22:1 ω-11		ND	1.53 ± 0.00	0.10 ± 0.01
22:1 ω-9		ND	0.31 ± 0.02	ND
20:4 ω-3		ND	1.46 ± 0.03	0.26 ± 0.04
20:5 ω-3		0.10 ± 0.01	27.56 ± 0.04	3.87 ± 0.20
24:1 ω-9		ND	0.43 ± 0.01	ND
22:4 ω-6		0.25 ± 0.02	1.82 ± 0.00	1.15 ± 0.02
22:5 ω-6		ND	1.52 ± 0.10	6.18 ± 0.40
22:5 ω-3		ND	3.16 ± 0.01	4.09 ± 0.01
22:6 ω-3		ND	26.15 ± 0.03	82.63 ± 0.15

Abbreviations: ND, non-detected. Fatty acid composition of the soybean and the fish oils was measured according to the method described by Lepage and Roy [1]. *Fatty acid composition of coconut oil described by Lal et al. [2] was used.

1. Lepage, G.; Roy, C.C. Direct transesterification of all classes of lipids in a one-step reaction. J. Lipid Res. 1986, 27, 114–120.
2. Lal, J.J.; Sreeranjit Kumar, C.V.; Indira, M. Coconut Palm. In Encyclopedia of Food Sciences and Nutrition; Trugo, L., Finglas, P.M., Eds.; Elsevier Science Ltd: Amsterdam, Netherlands, 2003; Vol. 1, pp. 1464–1475.

Table S2. Feed intake, biometric data and blood glucose.

	Coconut	**Soybean**	**EPA/DHA 1:1**	**80% DHA**	***p*-value**
Feed intake					
Daily feed intake (g/rat)	18.4 ± 1.7	19.2 ± 1.3	19.1 ± 0.8	19.4 ± 1.1	0.362*
Biometric data					
Initial body weight (g)	403 ± 22	401 ± 18	399 ± 15	398 ±14	0.914*
Final body weight (g)	435 ± 29	442 ± 21	435 ± 15	441 ± 25	0.834*
Body weight gain (g)	31.8 ± 10.8	40.5 ± 12.5	35.8 ± 13.6	43.7 ± 12.3	0.164*
Perigonadal adipose tissue weight (g)	5.0 ± 1.4	5.2 ± 1.1	4.7 ± 0.7	4.8 ± 1.0	0.759*
Adiposity index (%)	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.2	0.711*
Liver weight (g)	10.9 ± 1.4	10.8 ± 0.8	10.8 ± 0.8	11.3 ± 1.3	0.750*
Hepatosomatic index (%)	2.5 ± 0.2	2.5 ± 0.1	2.5 ± 0.2	2.6 ± 0.2	0.687†
Blood glucose					
Glucose at week 0 (mmol/L)	4.5 ± 0.4	4.6 ± 0.9	4.3 ± 0.6	4.7 ± 0.7	0.679†
Glucose at week 4 (mmol/L)	5.1 ± 0.6	5.0 ± 0.4	4.7 ± 0.4	4.9 ± 0.4	0.247*
Glucose at week 8 (mmol/L)	4.6 ± 0.6	4.6 ± 0.5	4.4 ± 0.4	4.6 ± 0.2	0.674*

Values are expressed mean ± standard deviation, n = 10 rats/group. **p*-value was calculated by the one-way analysis of variance followed by Scheffé post-hoc test. †*p*-value was calculated by the non-parametric Kruskal–Wallis test followed by Mann–Whitney U test. The level of statistical significance was set at *p*-value < 0.05.

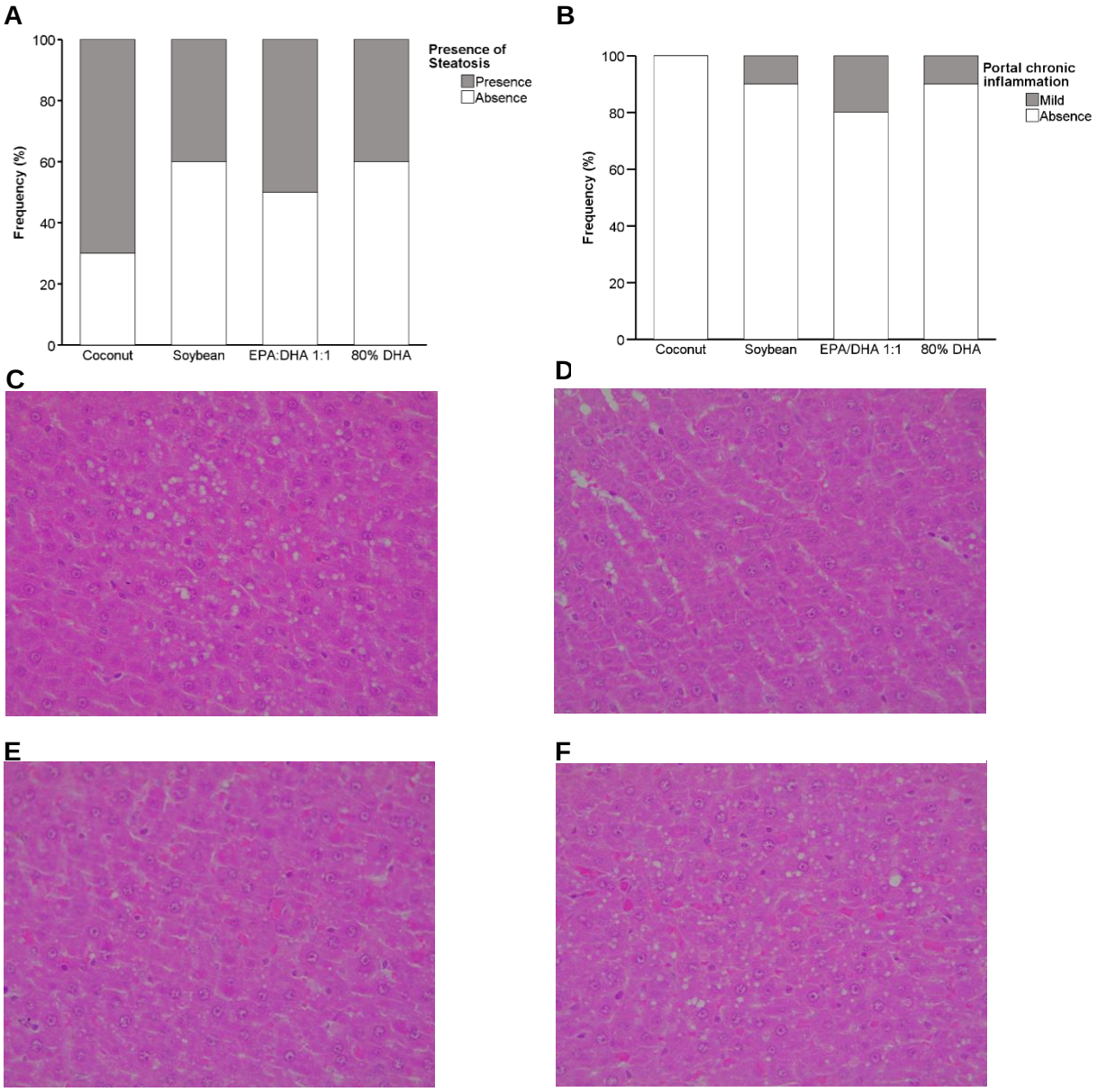

Figure S1. Histological analysis of the liver. Degree of steatosis (**A**); Portal chronic inflammation (**B**); Histological cuts of liver hematoxylin/eosin staining (400x) in rats supplemented with coconut oil (**C**) soybean oil (**D**) fish oil containing EPA/DHA 1:1 (**E**) or fish oil containing 80% DHA (**F**). Results are expressed as frequencies (%), n = 10 rats/group. No significant differences were found among the groups.

4.2. PAPER 2. Effects of Fish Oil and Grape Seed Extract Combination on Hepatic Endogenous Antioxidants and Bioactive Lipids in Diet-Induced Early Stages of Insulin Resistance in Rats

Type: Research article

Manuscript status: Published, June 16, 2020

Title: Effects of Fish Oil and Grape Seed Extract Combination on Hepatic Endogenous Antioxidants and Bioactive Lipids in Diet-Induced Early Stages of Insulin Resistance in Rats

Journal (Publisher): Marine Drugs (MDPI)

Impact factor (JCR 2019): 4.073

Category and classification: Chemistry, Medicinal 16/61 (Q2)

Authors: Núria Taltavull, Bernat Miralles-Pérez, Maria Rosa Nogués, Sara Ramos-Romero, Lucía Méndez, Isabel Medina, Josep Lluís Torres and Marta Romeu

Citation: Taltavull N, Miralles-Pérez B, Nogués MR, Ramos-Romero S, Méndez L, Medina I, Torres JL, Romeu M. Effects of Fish Oil and Grape Seed Extract Combination on Hepatic Endogenous Antioxidants and Bioactive Lipids in Diet-Induced Early Stages of Insulin Resistance in Rats. Mar Drugs. 2020;18:318. doi: 10.3390/md18060318

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Effects of Fish Oil and Grape Seed Extract Combination on Hepatic Endogenous Antioxidants and Bioactive Lipids in Diet-Induced Early Stages of Insulin Resistance in Rats

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Received: 21 May 2020; Accepted: 14 June 2020; Published: 16 June 2020

Abstract: Diacylglycerols (DAG) and ceramides have been suggested as early predictors of insulin resistance. This study was aimed to examine the combined effects of fish oil (FO) and grape seed extract (GSE) on hepatic endogenous antioxidants, DAG and ceramides in diet-induced early stages of insulin resistance. Thirty-five rats were fed one of the following diets: (1) a standard diet (STD group), (2) a high-fat high-sucrose diet (HFHS group), (3) an HFHS diet enriched with FO (FO group), (4) an HFHS diet enriched with GSE (GSE group) or (5) an HFHS diet enriched with FO and GSE (FO + GSE group). In the liver, endogenous antioxidants were measured using spectrophotometric and fluorometric techniques, and non-targeted lipidomics was conducted for the assessment of DAG and ceramides. After 24 weeks, the FO + GSE group showed increased glutathione peroxidase activity, as well as monounsaturated fatty acid and polyunsaturated fatty acid-containing DAG, and long-chain fatty acid-containing ceramides abundances compared to the STD group. The FO and GSE combination induced similar activation of the antioxidant system and bioactive lipid accumulation in the liver than the HFHS diet without supplementation. In addition, the FO and GSE combination increased the abundances of polyunsaturated fatty acid-containing DAG in the liver.

Keywords: omega-3; proanthocyanidins; polyphenols; antioxidants; diacylglycerol; ceramide

1. Introduction

Early stages of insulin resistance are characterized by increased insulin secretion from pancreatic β-cells for maintaining glucose homeostasis under overfeeding conditions [1]. Concretely, insulin resistance is a common feature in metabolic diseases such as obesity, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD), and is related to disturbances in lipid metabolism [2].

Several studies in both rodents and humans support the hypothesis that the aberrant accumulation of bioactive lipids such as diacylglycerol (DAG) and ceramide in the liver is a key step in the pathogenesis and progression of insulin resistance and NAFLD [3]. Indeed, DAG and ceramide species

have been suggested as early predictors of metabolic diseases [3,4]. DAG acts as a signal messenger in the cell [5], and ceramide is a signaling lipid and a precursor for molecules involved in the integrity of cell membranes [6]. The synthesis of DAG and ceramide species is stimulated during saturated fatty acid and sucrose overload [7–10]. At the same time, mitochondrial fatty acid oxidation is promoted to prevent lipid accumulation in the liver under fat overload [11]. This fact might lead to increased reactive oxygen species production and, as a consequence, oxidative damage to lipids, proteins and DNA [11].

Dietary ω-3 polyunsaturated fatty acids (ω-3 PUFA), specifically eicosapentaenoic (EPA, 20:5) and docosahexaenoic (DHA, 22:6) acids, exert antioxidant [12] and anti-inflammatory [13,14] effects on the organism. In addition, dietary supplementation of ω-3 PUFA-rich fish oil (FO) attenuates the development of insulin resistance in rodents under overfeeding conditions [15,16]. However, as the PUFA are highly prone to oxidation leading to irreversible cellular and tissue damage, the addition of antioxidants to PUFA-rich products may be required [17]. Concretely, a grape seed extract (GSE) rich in proanthocyanidins shows high-antioxidant capacity by scavenging free radicals [18]. In this context, the combined administration of PUFA and GSE prevents the oxidation of PUFA during digestion as well as in cell membranes [18,19].

Previously, we described the combined effects of FO and GSE on metabolic health in rats fed a high-fat high-sucrose (HFHS) diet [20–23]. Concretely, the combination of both ingredients attenuates plasma hyperinsulinemia and hyperleptinemia, decreases liver inflammation and improves blood antioxidant status better than individual supplementations under overfeeding conditions [20–23]. However, its effects on endogenous antioxidants, DAG and ceramide species in the liver have not been assessed yet.

The aim of this study was to examine the effects of FO and GSE combination on hepatic endogenous antioxidants, DAG and ceramides in diet-induced early stages of insulin resistance in rats.

2. Results

2.1. Biometric and Biochemical Data

As we previously described [23], the HFHS diet significantly increased the body weight, the perigonadal white adipose tissue weight, the fasting plasma insulin (FI) concentration and the Homeostatic Assessment Model of Insulin Resistance (HOMA-IR) value compared to the standard (STD) diet. Although the FO + GSE group showed increased biometric values compared to the STD group, the FI concentration and the HOMA-IR value were similar between the two groups. On the contrary, individual FO and GSE supplementations did not attenuate either hyperinsulinemia or increased HOMA-IR induced by the HFHS diet compared to the STD diet. Although the HFHS group showed a similar plasma triacylglycerol concentration than the STD group, the individual GSE supplementation increased triacylglycerol in plasma compared all the other groups. The liver weight and the FG concentration showed no significant differences between STD- and HFHS-fed animals (Table S1) [23].

2.2. Hepatic Endogenous Antioxidants

All groups fed an HFHS diet, with or without supplementation, presented increased glutathione peroxidase (GPx) activities compared to the STD group. Concretely, the GSE group showed the highest GPx activity. The superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and oxidized glutathione (GSSG) remained unchanged among the groups by the end of the study (Table 1).

Table 1. Hepatic endogenous antioxidants after 24 weeks of dietary intervention.

	STD n = 7	HFHS n = 7	FO n = 7	GSE n = 7	FO + GSE n = 7	*p*-Value
SOD (U/g)	3678.45 ± 663.32	4465.63 ± 609.73	3136.19 ± 813.26	4190.11 ± 820.02	4010.73 ± 943.69	0.033 *
CAT (nmol/g)	273.80 ± 152.39	408.22 ± 233.11	408.39 ± 119.98	357.27 ± 61.85	382.48 ± 98.63	NS †
GPx (U/g)	47.35 ± 7.11	61.41 ± 3.42 [a]	64.91 ± 10.33 [a]	83.47 ± 9.82 [a,b,c]	65.19 ± 16.74 [a,d]	<0.001 †
GSH (µmol/g)	0.86 ± 0.65	0.76 ± 0.55	0.95 ± 0.49	1.81 ± 0.34	1.41 ± 0.90	0.016 *
GSSG (µmol/g)	2.44 ± 0.75	2.74 ± 0.62	2.21 ± 0.57	2.00 ± 0.27	2.09 ± 0.54	NS *
GSSG/GSH	5.48 ± 4.57	6.72 ± 6.09	3.71 ± 3.59	1.15 ± 0.31	2.15 ± 1.38	NS †

Results were expressed as mean ± standard deviation. Abbreviations: STD, Standard; HFHS, High-Fat High-Sucrose; FO, Fish Oil; GSE, Grape Seed Extract; SOD, Superoxide Dismutase; CAT, Catalase; GPx, Glutathione Peroxidase; GSH, Reduced Glutathione; GSSG, Oxidized Glutathione. * *p*-value was calculated by a one-way ANOVA followed by a Scheffé post-hoc test. † *p*-value was calculated by the non-parametric Kruskal-Wallis test followed by or Mann–Whitney U test. [a]; vs. STD group, [b]; vs. HFHS group, [c]; vs. FO group, [d]; vs GSE group.

2.3. Hepatic Histological Analysis

The liver histological analysis showed that the HFHS diet, with or without supplementation, did not induce either steatosis or pro-inflammatory cell infiltration in the liver by the end of the study. However, FO supplementation, either individual or in combination with GSE, tended to promote fat accumulation compared to the individual GSE supplementation and the STD diet (Figure 1). Steatosis was found in no zonal localization in all cases.

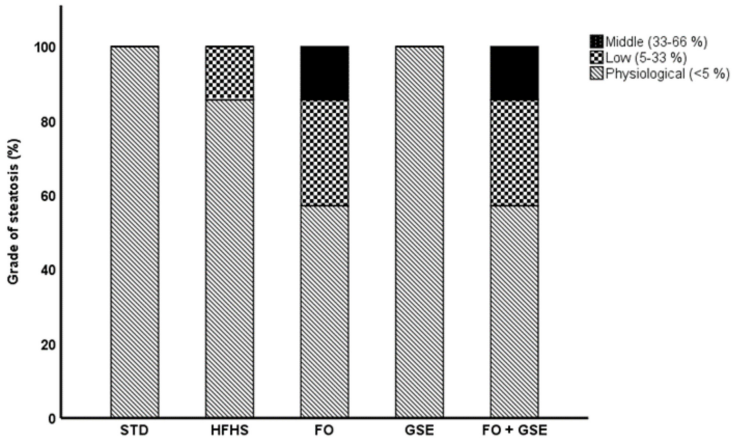

Figure 1. Hepatic steatosis. Results were expressed as frequencies. Abbreviations: STD, Standard; HFHS, High-Fat High-Sucrose; FO, Fish Oil; GSE, Grape Seed Extract. Steatosis was found in no zonal localization in all cases. No significant differences were found among groups.

2.4. Hepatic Bioactive Lipids

A total of 28 different lipid species were identified in the liver samples: 19 DAGs and nine ceramides (Table S2).

Regarding saturated fatty acid (SFA)-containing DAG species, 16:0,16:0-DAG (II) was significantly decreased in the FO + GSE group compared to the other groups. On the contrary, the FO + GSE group as well as GSE, HFHS and STD groups showed a significantly increase of 18:0,18:0—DAG compared to FO. No other significant differences were found in the abundance of the SFA-containing DAG species among the groups (Figure 2A).

Of the monounsaturated FA (MUFA)-containing DAG species detected, 16:0,16:1-DAG was higher in FO + GSE, FO and HFHS than in the STD group. In addition, 16:0,16:1-DAG was increased in the FO + GSE group compared to GSE. The FO + GSE and HFHS groups also increased 16:0,18:1—DAG and 16:0,18:1—DAG(II) compared to the STD group. Likewise, HFHS showed higher 16:0,18:1—DAG and 18:1,18:1—DAG than both the FO and GSE groups. 18:1,18:1—DAG was also increased in the HFHS group compared to the STD group (Figure 2A).

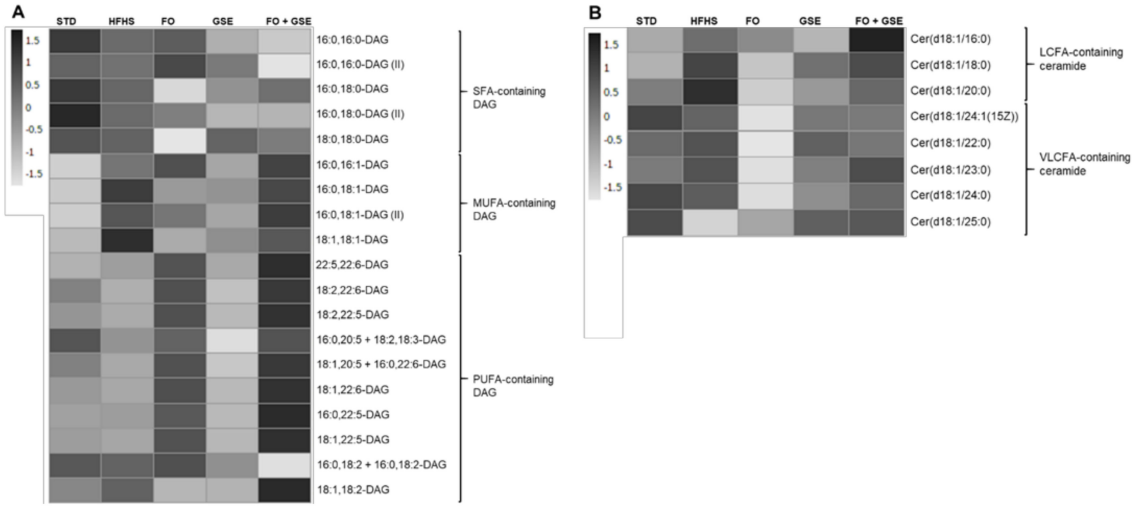

Figure 2. Hepatic bioactive lipids after 24 weeks of dietary intervention. A non-targeted lipidomic approach was carried out on six–seven rats of each group. Results were expressed as heatmaps where rows represent the individual lipid species and columns represent the dietary groups. Significant differences in individual species among groups were commented in the text. (**A**) Diacylglycerol species. (**B**) Ceramide species. Abbreviations: STD, Standard; HFHS, High-Fat High-Sucrose; FO, Fish Oil; GSE, Grape Seed Extract; DAG, Diacylglycerol; SFA, Saturated Fatty Acid; MUFA, Monounsaturated Fatty Acid; PUFA, Polyunsaturated Fatty Acid; Cer, ceramide; LCFA, Long-Chain Fatty Acid; VLCFA, Very Long-Chain Fatty Acid.

Regarding polyunsaturated FA (PUFA)-containing DAG species, 22:5,22:6—DAG was increased in FO + GSE, FO and GSE compared to STD. Moreover, FO supplementation, either individually or in combination with GSE, increased 22:5,22:6—DAG compared to the GSE and HFHS groups. However, the HFHS group decreased 22:5,22:6—DAG compared to the STD group. FO-supplemented groups showed higher 18:2,22:6—DAG and 18:2,22:5—DAG than the other groups. On the contrary, both GSE and HFHS groups showed lower 18:2,22:6—DAG and 18:2,22:5—DAG than the STD group. Furthermore, 18:2,22:6—DAG was decreased in the GSE group compared to the HFHS group. FO-supplemented groups also presented higher 18:1,20:5 + 16:0 22:6—DAG and 18:1,22:6—DAG than the other groups. In addition, 18:1,20:5 + 16:0 22:6—DAG was decreased in the GSE group compared to the STD group. 18:1,22:5—DAG and 16:0,22:5—DAG was increased in both FO-supplemented groups compared to the other groups. On the contrary, the GSE group showed lower 18:1,22:5—DAG and 16:0,20:5 + 18:2,18:3—DAG than the other groups. No other significant differences were found in abundance of PUFA-containing DAG species among the groups (Figure 2A).

As far as long-chain FA (LCFA)-containing ceramide species are concerned, Cer(18:1/16:0) was significantly increased in FO + GSE and HFHS groups compared to the STD group. Moreover, the FO + GSE group showed significantly higher abundance of Cer(18:1/16:0) than GSE, FO and HFHS groups. The FO and HFHS groups also increased the Cer(18:1/16:0) compared to the GSE group. Cer(18:1/18:0) was higher in the FO + GSE and HFHS groups than in the FO and STD groups (Figure 2B). Cer(18:1/18:1(9Z)) was detected in the liver of the rats fed an HFHS diet, but not in those rats fed an STD diet. No other significant differences were found in the abundance of LCFA-containing ceramide species among the groups.

All groups showed significantly increased very LCFA (VLCFA)-containing ceramides, Cer(d18:1/24:1(15Z)), Cer(d18:1/22:0), Cer(d18:1/23:0) and Cer(d18:1/24:0), compared to the FO group. In addition, the FO + GSE and HFHS groups showed higher Cer(d18:1/23:0) than the GSE group. No other significant differences were found in the abundance of VLCFA-containing ceramide species among the groups (Figure 2B).

3. Discussion

We previously described that HFHS diet induces early stages of insulin resistance in the same cohort of rats after 24 weeks, including increased perigonadal adipose tissue content, plasma hyperinsulinemia and plasma hyperleptinemia (Table S1) [23]. The combination of FO and GSE attenuates the metabolic features induced by an HFHS diet (Table S1), decreased liver inflammation, improved blood antioxidant status and increased plasma adiponectin under overfeeding conditions [20–23].

The present study examined the effects of an FO and GSE combination on hepatic endogenous antioxidants, DAG and ceramides as early biomarkers of insulin resistance. In the liver, HFHS diet induced, first, an early antioxidant response (Table 1) and, second, MUFA-containing DAG and LCFA-containing ceramide accumulation (Figure 2A and 2B) without neither significant steatosis (Figure 1) nor pro-inflammatory cell infiltration. Moreover, HFHS diet decreased several PUFA-containing DAG species compared to STD diet (Figure 2A).

FO + GSE group as well as all the other groups fed an HFHS diet showed moderately higher hepatic antioxidant response than STD (Table 1). This fact could be due to increased mitochondrial β-oxidation in all groups fed an HFHS diet, as previously reported [24]. Antioxidant response is mediated via activation of nuclear factor-erythroid 2-related factor 2 (NrF2) signaling pathway [25] to protect against increased reactive oxygen species production. In addition, both dietary ω-3 PUFA (FO) [26] and proanthocyanidins (GSE) [27] are well-known NrF2 activators. Concretely, the GSE group showed the highest GPx activity in the liver among the groups fed an HFHS diet (Table 1). Consistent with our observations, other authors have showed that the GSE supplementation enhances the expression of antioxidant enzymes in HepG2 cells [28]. Nevertheless, other studies have also suggested that the GSE supplementation reverts the obesity-induced endogenous antioxidant response in Zucker rats by acting as a scavenger of reactive oxygen species [29].

The FO + GSE and FO groups showed a tendency, even though not statistically significant, for lipid accumulation in the liver as assessed by the histological examination compared to the STD group (Figure 1). In accordance with our results, Feillet-Coudray et al. [30] showed no significant differences on hepatic lipid accumulation between fish oil- and lard oil-fed rats. However, other studies have shown that the FO supplementation decreased lipid accumulation in the liver by promoting fatty acid β-oxidation via activation of the peroxisome proliferator-activated receptor α [31,32]. On the other hand, the GSE group showed the lowest lipid accumulation under overfeeding conditions (Figure 1), but showing the highest plasma triacylglycerol concentration among all the groups (Table S1) [23]. In contrast, previous studies have showed that the GSE supplementation decreases the expression of hepatic lipogenic enzymes as well as the triacylglycerol secretion, lowering plasma lipids in Wistar rats fed high-fat diet [33,34].

The non-targeted lipidomic analysis on hepatic DAG and ceramide species showed clearly differentiated hepatic lipid profile between the HFHS and STD groups. As we have mentioned above, the HFHS diet increased the relative abundances of numerous MUFA-containing DAG and decreased several PUFA-containing DAG species compared to the STD diet (Figure 2A). These findings were similar to those of previous studies that had investigated rodents on a high-fat diet [10,35,36]. The hepatic accumulation of DAG species in the HFHS group was likely due to the lipid composition of the HFHS diet, mainly consisting of oleic acid (18:1 n-9), palmitic acid (16:0) and stearic acid (18:0). In contrast, the STD diet was largely made up of linoleic acid (18:2 n-6), which could explain the differences in the hepatic PUFA content between HFHS and STD groups. Additionally, according to Ciapaite et al. [24], adaptive responses (such as the fatty acid desaturation from SFA to MUFA after dietary saturated fat overload) may explain the differences observed in the hepatic MUFA content between HFHS and STD groups. The sucrose overload of HFHS diet might also be responsible for the accumulation of lipids in the liver by de novo synthesis of DAG species [37]. This accumulation of hepatic DAG species in HFHS group could lead to insulin resistance and the development of hepatic steatosis [2] via protein kinase C ε (PKCε) activation and the resulting inhibition of the insulin receptor, as previously described [5,38].

Individual FO and GSE supplementations attenuated the HFHS-diet-induced MUFA-containing DAG accumulation. Even though the combination of both FO and GSE promoted the accumulation of MUFA-containing DAG in the liver. In contrast, the combination of both ingredients markedly decreased the 16:0,16:0-DAG (II), an SFA-containing DAG. FO supplementation, either individually or in combination with GSE, increased PUFA-containing DAG compared to the other three groups (Figure 2A). As we previously described in the same cohort of rats [21], FO supplementation increases the total content of ω-3 PUFA in the liver in accordance with its lipid composition. On the contrary, the individual GSE supplementation significantly decreased several PUFA-containing DAG species in the liver compared to all the other groups (Figure 2A). As we have mentioned above, this fact could be related to the decreased hepatic lipogenesis [33,34] and the increased triacylglycerol secretion (Table S1) [23] induced by GSE.

Moreover, HFHS diet induced hepatic LCFA-containing ceramide accumulation, specifically Cer(d18:1/16:0) and Cer(d18:1/18:0) species, but not modified VLCFA-containing ceramide abundances compared to the STD diet (Figure 2B). These findings are similar to those of previous studies that had investigated rodents on a high-fat diet [10,35,36,39,40]. Nevertheless, other authors have not reported any changes in total hepatic ceramide content after a 3-day fat overload in rats [41] and an 8-week overload in mice [42]. In contrast, Holland et al. found that acute SFA infusions increase the total ceramide abundance in mice liver [43]. Total hepatic ceramide quantification may not reveal associations between individual ceramide species and metabolic diseases [35]. In this respect, recent studies have shown that several ceramide species have distinct roles in the progression of metabolic disorders and that this role depends on their acyl-chain length [35,44]. High ratios of LCFA-containing ceramides to VLCFA-containing ceramides in the liver are associated to the progression of metabolic disorders in rodents [35]. Indeed, a high liver content of Cer(d18:1/22:0) and Cer(d18:1/24:0) might prevent hepatic steatosis under fat overload conditions by reducing peroxisome proliferator-activated receptor γ2 content and, as a consequence, reducing CD36 and FSP27 gene expression [45].

Ceramides are generated by de novo synthesis from palmitoyl-CoA and serine or a salvage pathway from complex sphingolipids [6]. Concretely, HFHS diet-induced Cer(d18:1/16:0) and Cer(d18:1/18:0) accumulation could be, at least in part, due to increased ceramide synthase 6 activity [39]. High abundances of these two LCFA-containing ceramide species have been associated with body weight gain, impaired glucose tolerance or insulin resistance and hepatic steatosis in rodents [36,39,40,46,47]. In humans, increased de novo synthesis and accumulation of ceramides in the liver have been associated with severe obesity, hepatic insulin resistance and non-alcoholic steatohepatitis (NASH) [48–50]. Additionally, the activation of the salvage pathway may be positively associated with oxidative stress and inflammation in NASH conditions, suggesting that ceramide precursors contribute to the progression from fatty liver to NASH in humans [49]. These metabolic alterations may largely be due to serine/threonine kinase 1 inhibition [6] and the activation of PKC and CD36 gene expressions, which mediate hepatic fatty acid uptake [51]. In this respect, the promotion of LCFA-containing ceramide degradation by ceramidase improves glucose and lipid metabolism in liver and adipose tissue [51].

Although the individual FO supplementation decreased either LCFA- or VCFA-containing ceramides abundances under overfeeding conditions, the combination of FO and GSE did not attenuate the HFHS diet-induced LCFA-containing ceramide accumulation (Figure 2B). A previous study in humans showed that ω-3 PUFA derived from fatty fish decreased plasma ceramide concentration compared to control [52]. In addition, Dong et al. showed that ω-3 PUFA supplementation at 3:1 EPA:DHA ratio prevented hyperhomocysteinemia-induced ceramide accumulation in C57BL/6J mice [53]. Other authors also showed that FO supplementation decreased ceramide accumulation in adipose tissue under overfeeding conditions [9]. Nevertheless, mice fed a ω-3 PUFA-rich diet or a ω-3 PUFA-poor diet showed no difference either in hepatic or plasma ceramide content [54]. The GSE group also presented lower Cer(d18:1/16:0) and Cer(d18:1/23:0) abundances than HFHS one. In agreement with our results, Seo et al. [55] showed that a supplementation with Chardonnay grape seed flour may decrease de novo synthesis of ceramides.

In addition, Cer(18:1/18:1(9Z)) was only detected in the liver of the rats fed HFHS diet, suggesting that it could be directly derived from the milk fat included in this diet type [56].

The increased abundances of hepatic MUFA- and PUFA-containing DAG as well as LCFA-containing ceramide species observed in FO + GSE group (Figure 2A,B) were not accompanied with insulin resistance (Table S1). The DAG mediated-activation of the PKCε may depend on the DAG localization in the cell [57]. In this way, the accumulation of DAG in lipid droplets rather than plasma membrane prevents PKCε activation and, as a consequence, averting hepatic insulin resistance in CGI-58 ASO-treated mice [57]. In addition, the relationship between ceramide content and insulin resistance remains controversial [3]. Thus, in agreement with other authors [5,41], these results could suggest that ceramide accumulation in the liver is not involved in the development of insulin resistance.

4. Materials and Methods

4.1. Ethical Statement

All animal procedures respected the European Union guidelines (EU Directive 2010/63/EU) for the care and management of laboratory animals, and the relevant permission was obtained from the CSIC Subcommittee of Bioethical Issues (reference no.CEEA-12-007).

4.2. Animals and Diets

Thirty-five female Wistar Kyoto rats (147 ± 9 g body weight), 8–10 weeks old, were purchased from Charles Rivers Laboratories (WKY/NCrl, Wilmington, MA, USA). The rats were kept in an insulated room (two or three rats per Makrolon cage; 425 × 265 × 180 mm) with a constantly regulated temperature (22 ± 2 °C), and controlled humidity (50 ± 10%) in a 12-h artificial light cycle.

The rats were randomized into five groups (seven rats per group), and fed one of the following diets for 24 weeks: (1) an STD diet (STD group; based on Teklad Global 14% Protein Rodent Maintenance Diet [3.1 Kcal/g], Harlan Teklad Inc, IN, USA), (2) an HFHS diet (HFHS group; based on TD.08811 45% Kcal Fat Diet [4.8 Kcal/g], Envigo, IN, USA), (3) an HFHS diet enriched with FO (FO group), (4) an HFHS diet enriched with GSE (GSE group), or (5) an HFHS diet enriched with FO and GSE (FO + GSE group). The full composition of the diets is described in the supplementary materials (Tables S3 and S4). The rats were given free access to food and water (Ribes, Barcelona, Spain) throughout the study. FO and GSE contents were chosen as previously described [20,58].

FO with EPA (C20:5 ω-3) and DHA (C22:6 ω-3) in a balanced 1:1 ratio was obtained by mixing the appropriate quantities of the commercial fish oils AFAMPES 121 EPA (AFAMSA, Vigo, Spain), Omega-3 RX (EnerZona, Milan, Italy) and Oligen liquid DHA 80% (IFIGEN-EQUIP 98, S.L., Barcelona, Spain). FO mixture (24 mL/Kg feed) was added to the pellet of FO and FO + GSE groups. Soybean oil (Clearspring Ltd., London, UK) was added to the preparations of STD, HFHS and GSE groups. The GSE (Fine Grajfnol®, powder 98%) was purchased from JF-Natural Product (Tianjin, China), containing ≥ 95% oligomeric proanthocyanidins (UV), which 60% was procyanidin dimer B2 (HPLC) and ≤ 1.5% Ash. Loss on drying was ≤ 5.0%. GSE (1090 mg/Kg feed) was added to the pellet of GSE and FO + GSE groups.

After 24 weeks, the animals were fasted overnight, anesthetized intraperitoneally with ketamine and xylazine (80 mg/kg and 10 mg/kg body weight, respectively) and sacrificed by exsanguination. Blood samples were taken by cardiac puncture. Subsequently, plasma was obtained by centrifugation at 850 × g for 15 min at 4 °C from the blood samples. Perigonadal white adipose tissue and liver were collected, washed with 154 mM sodium chloride solution, cut and weighted. Then, adipose tissue and liver samples were quickly frozen in liquid nitrogen and stored at −80 °C until processing, except one part of liver that was fixed in 4% formaldehyde solution (v/v) for the histological study.

4.3. Biometric and Biochemical Data

Body weight (g) was measured daily throughout the study. Fasting blood glucose concentration (FG) was measured by applying the enzyme electrode method using an Ascensia Elite XL glucometer (Bayer Consumer Care AG, Basel, Switzerland). FI concentration was measured using an ELISA kit (Millipore Corporation, Billiberica, MA, USA) [23]. Subsequently, the HOMA-IR was calculated as: FI (mU/L) × FG (mmol/L) / 22.5 [59]. Plasma triacylglycerol was measured using the corresponding spectrophotometric kit (Spinreact, Barcelona, Spain) in a COBAS MIRA Autoanalyzer (Roche Diagnostics System, Madrid, Spain) [60].

4.4. Hepatic Histological Analysis

Formalin-fixed liver samples were dehydrated in alcohol and embedded in paraffin. Serial tissue sections (3 μm thick slices) were obtained using a steel knife mounted in a microtome (Microm HN 355S). Sections were stained with hematoxylin/eosin (Harris Hematoxylin, QCA). Hepatic histological examination was performed after hematoxylin/eosin staining and graded as detailed in (supplementary material Table S5).

4.5. Hepatic Endogenous Antioxidants

The liver was homogenized on ice in 200 mM sodium phosphate buffer (pH 6.25) and centrifuged at 129,000× *g* for 1 h at 4 °C. In the liver supernatant, total SOD, CAT and GPx activities were measured using spectrophotometric techniques, and GSH/GSSG contents were measured using fluorometric techniques as previously described [61].

4.6. Lipid Extraction from the Liver

The liver was lyophilized and homogenized in a porcelain mortar. The lipids were extracted using a method based on Bligh and Dyer [62]. Briefly, 2 ± 0.1 mg of lyophilized liver was mixed with 375 μL of $CHCl_3$/MeOH 1:2 (v/v) and 50 μL of 1 mg/L Cer(d18:1,17:0) and rac-1,2-Dipalmitoylglycerol-d5 as internal standards. After shaking in an ultrasonic bath, the lipid and aqueous phases were broken down by the addition of 125 μL of $CHCl_3$ and 125 μL of water. After vortex and centrifugation at room temperature, the organic phase (bottom phase) was carefully separated. Aliquots of 100 μL of the organic phase were diluted 1:10 in MeOH/2-propanol 60:40 (v/v). The sample was subjected to lipidomic analysis at the Centre for Omic Sciences, the joint unit of the Universitat Rovira i Virgili and the EURECAT Technology Centre of Catalonia.

4.7. HPLC-qTOF Analysis of DAG

The liver lipid extract was injected into a 1290 Infinity UHPLC system coupled to a 6545 qTOF (Agilent Technologies, Santa Clara, CA, USA). The chromatographic column was C8 BEH 150 × 2.1 mm, 1.7 μm from Waters (Milford, MA, USA.). The mobile phases were 0.1% aqueous HCOOH (A) and 0.1% HCOOH in CH_3CN/2-propanol 60:40 (v/v) (B). The injection volume was 2 μL. The column flow was set at 0.2 mL/min, and the gradients of the mobile phases were 0–1 min 40% B isocratic, 1–10 min 100% B, 10–19 min 100% B isocratic and 19–20 min 40% B. A post-run of 5 min was applied.

Source conditions were 250 °C and 11 mL/min of drying temperature and gas flow, respectively, a nebulizer pressure of 45 psi, a capillary voltage of 3000 V and a fragmentor voltage of 150 V. Acquisition was set in positive mode, and the *m/z* axis was internally calibrated throughout the run with reference masses 112.9855 *m/z* and 1033.9881 *m/z*. Scan data were recorded in a range between 100 and 1400 *m/z*, at an acquisition rate of 2 spec/s. The collision energy applied for targeted MS/MS analysis was 10, 20 and 40 V, and the product ion spectra were recorded in the range 50 to 800 *m/z*, at a scan range of 5 spec/s.

The compounds were identified using the exact mass of the molecular adducts $[M + NH_4]^+$ and $[M + Na]^+$ in the MS analysis and the observed product ions of $[M - R_1COOH + H]^+$, $[M - R_2COOH +$

$H]^+$, $[R_1COO + C_3H_5O + H]^+$, $[R_2COO + C_3H_5O + H]^+$, $[R_1CO]^+$ and $[R_2CO]^+$ in the targeted MS/MS analysis. The fragmentation rules for this kind of compound were taken into account. The DAG species identified were relatively quantified by comparison of the area under the chromatographic peak ratio corresponding to the $[M + NH_4]^+$ extracted ion chromatogram in the MS analysis [63–65].

DAG species were divided into three categories: (1) SFA-containing DAG, (2) MUFA-containing DAG and (3) PUFA-containing DAG (Table S2).

4.8. HPLC-QqQ Analysis of Ceramides

The liver lipid extract was injected into a 1290 Infinity UHPLC system was coupled to a 6490 QqQ mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The same chromatographic conditions were used as described above. Electrospray ionization was performed in positive mode by applying a gas temperature and flow of 150 °C and 11 mL/min, respectively. The nebulizer pressure was set at 20 psi, and the sheath gas temperature and flow were 350 °C and 12 L/min, respectively. Capillary voltage was 3000 V and nozzle voltage was 1000 V.

The acquisition was performed in precursor ion mode by scanning a range of *m/z* from 300 to 700 in the first quadrupole at a scan time of 200 ms, and monitoring the characteristic ceramide product ions of 282 and 264 *m/z* in the second quadrupole. The collision energy was set at 30 V for both transitions. The ceramide species were identified by observing both transitions from the parent ions $[M + H]^+$ and $[M - HO_2 + H]^+$. After ceramide specific transitions were identified, the samples were measured again using a Multiple Reaction Monitoring (MRM) method, and ceramides were relatively quantified by comparison of the area under the chromatographic peak ratio for the transitions corresponding to the $[M + H]^+ > 264$ *m/z* [66,67].

Ceramide species were divided into two categories: (1) LCFA-containing ceramide, and (2) VLCFA-containing ceramide (Table S2).

While DAG and ceramides were being analyzed by LC-MS, blank runs and quality control standards were injected alternately throughout the run sequence. This showed that there was no carryover among the samples, and that the compounds and/or instrumental drift did not significantly degrade during the sequence analysis.

4.9. Statistical Analysis

The statistical analysis was performed using the SPSS 25 statistical package (SPSS, Chicago, IL, USA). Biometric, biochemical and endogenous antioxidants data were expressed as mean ± standard deviation (SD), whereas histological results were expressed in frequencies (%). The Shapiro-Wilk test and Levene's test were used to test for normality and homoscedasticity of data, respectively. Then, the groups were statistically compared by the one-way analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis test followed by a Scheffé post-hoc test or Mann–Whitney U test, respectively. The contingency tables using $\chi 2$ statistics for categorical variables. Relative lipid abundances were log-transformed, auto-scaled and expressed as a heatmap using Metaboanalyst 4.0 free-web based tool [68]. The level of statistical significance was set at $p\text{-value} < 0.05$. Results of individual lipid species were corrected using the false discovery rate controlling procedure for multiple comparisons.

5. Conclusions

In conclusion, the FO and GSE combination enhanced antioxidant response and promoted MUFA-containing DAG and LCFA-containing ceramide accumulation in the liver. Additionally, FO and GSE combination increased PUFA-containing DAG mainly due to the FO composition. These findings suggested that the beneficial effects of FO and GSE combination on early stages of insulin resistance were not related to DAG or ceramide species. However, further powered studies are needed to determine the molecular and biochemical mechanisms that regulate DAG and ceramide abundances, including different cellular localizations and tissues, in a biological context.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/18/6/318/s1, Table S1: Biometric and biochemical data in rats after six months of dietary intervention, Table S2: Diacylglycerol and ceramide species identified in the rat liver, Table S3: Composition of diets, Table S4: Fatty acid composition of diets, Table S5: Characterization used for the liver histological study.

Author Contributions: M.R.N., I.M., J.L.T. and M.R. designed the research; N.T., S.R.-R. and L.M. conducted the research; N.T. and B.M.-P. analyzed the data; N.T., B.M.-P., M.R.N. and M.R. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Spanish Ministry of Economy and Competitiveness [grant numbers AGL2009-12374-C03-02, AGL2013-49079-C2-1-R and AGL2013-49079-C2-2-R].

Acknowledgments: The Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitat de Catalunya, the European Union (EU) and the European Social Fund (ESF) are gratefully acknowledged for the doctoral fellowship to B.M.-P.

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

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Supplementary data:

Effects of Fish Oil and Grape Seed Extract Combination on Hepatic Endogenous Antioxidants and Bioactive Lipids in Diet-Induced Early Stages of Insulin Resistance in Rats

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Table S1. Composition of diets

	STD	HFHS	FO	GSE	FO + GSE
Diet composition					
Flour (g)	1000[a]	1000[b]	1000[b]	1000[b]	1000[b]
TBHQ (g)	0.08	0.08	0.08	0.08	0.08
Porcine gelatin (g)	25	25	25	25	25
Soybean lecithin (g)	6	22	22	22	22
Soybean[c] or FO[d] oil (mL)	19	24	24	24	24
GSE[f] (mg)	-	-	-	1090	1090
Macronutrients (% weight)					
Protein	16.4	21.7	21.7	21.7	21.7
Fat	6.2	24.1	24.1	24.1	24.1
Carbohydrates	46.6	45.0	45.0	44.9	44.9
Macronutrients (% caloric value)[g]					
Protein	21.3	17.9	17.9	17.9	17.9
Fat	18.2	44.9	44.9	44.9	44.9
Carbohydrates	60.5	37.2	37.2	37.2	37.2
Total energy density (kcal/g)	3.1	4.8	4.8	4.8	4.8

Abbreviations: STD; Standard, HFHS; High-Fat High-Sucrose, FO; Fish Oil, GSE, Grape Seed Extract. [a]Standard flour (Teklad Global 2014), containing wheat middlings, ground wheat, ground corn, corn gluten meal, calcium carbonate, soybean oil, dicalcium phosphate, iodized salt, l-lysine, vitamin E acetate, DL-methionine, magnesium oxide, choline chloride, manganous oxide, ferrous sulphate, menadione sodium bisulphite complex (source of vitamin K activity), zinc oxide, copper sulphate, niacin, calcium pantothenate, calcium iodate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, vitamin B12 supplement, folic acid, cobalt carbonate, biotin and vitamin D3 supplement. [b]High-fat high-sucrose diet (Tekland TD 08811), containing sucrose (34 % of the total pellet weight), anhydrous milkfat, casein, maltodextrin, corn starch, cellulose, mineral mix AIN-93G-MX, soybean oil, vitamin mix AIN-93G-VX, L-cystine, choline, bitartrate, green food colour, tert-butylhydroquinone. [c]Soybean oil was purchased from Clearspring Ltd. (London, United Kingdom). [d]FO with eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) in a balanced 1:1 ratio was obtained by mixing the appropriate quantities of the commercial fish oils AFAMPES 121 EPA (AFAMSA, Vigo, Spain), Omega-3 RX (EnerZona, Milan, Italy) and Oligen liquid DHA 80% (IFIGEN-EQUIP 98, S.L., Barcelona, Spain). [f]GSE (Fine Grajfnol®, powder 98 %) was purchased from JF-Natural Product (Tianjin, China). [g]Energy density is estimated as metabolizable energy based on the Atwater factors, assigning 4 kcal/g to protein, 9 kcal/g to fat and 4 kcal/g to carbohydrate, including dietary fibre.

Table S2. Fatty acid composition of diets

	STD	HFHS and GSE	FO and FO + GSE
14:00	0.017	11.5	11.6
16:00	14.8	32.2	32.0
16:1 n-7	0.015	1.7	1.7
18:00	2.9	13.7	13.7
18:1n-9	20.5	26.4	26.2
18:1 n-7	0.026	0.67	0.68
18:2 n-6	58.6	9.7	8.7
20:00	n.d.	n.d.	0.0069
18:3 n-3	2.9	1.1	1.1
20:1 n-9	0.025	0.031	0.021
18:4 n-3	0.0026	0.0032	0.033
20:2 n-6	0.0034	0.0043	0.0045
20:3 n-6	n.d.	n.d.	0.0048
20:4 n-6	0.0069	0.0086	0.036
22:1 n-11	0.019	0.023	0.024
22:1 n-9	0.0043	0.0054	0.006
20:4 n-3	0.0034	0.0043	0.022
20:5 n-3	0.012	0.015	0.54
24:1 n-9	0.0048	0.0060	0.0082
22:5 n-3	0.0045	0.0056	0.093
22:6 n-3	0.02	0.024	0.56
Total SFAs	17.5	59.4	60.5
Total MUFAs	21.1	29.3	28.4
Total PUFAs	61.1	10.8	10.7
Total n-3	3.3	1.6	1.4
Total n-6	58.8	10.4	10.6

Results are expressed as a percentage of total fatty acids (mg/100 mg of total fatty acids). Abbreviations: STD; Standard, HFHS, High-fat High-sucrose, FO; Fish Oil, GSE; Grape Seed Extract, SFA; Saturated Fatty Acid, MUFA; Monounsaturated Fatty Acid, PUFA; Polyunsaturated Fatty Acid, n.d.; non-detected.

Table S3. Characterization used for the liver histological study	
Meaning Liver	**Grade**
Steatosis	
Physiological state	< 5%
Low-grade	5-33%
Middle-grade	33-66%
High-grade	> 66%
Localization of steatosis	
Centrilobular	0
Periportal	1
No zonal	2
Lobular inflammation	
No	0
< 2 focus	1
2-4 focus	2
> 4 focus	3
Portal inflammation	
No	0
Yes	1

Table S4. Diacylglycerol and ceramide species identified in the rat liver

	Lipid specie	m/z	RT (min)
DAG			
SFA	16:0,16:0-DAG	586.5409	13.18
	16:0,16:0-DAG (II)	586.5410	13.29
	16:0,18:0-DAG	614.5725	13.48
	16:0,18:0-DAG(II)	614.5723	13.60
	18:0,18:0-DAG	642.6039	13.81
MUFA	16:0,16:1-DAG	584.5247	12.99
	16:0,18:1-DAG	612.5560	13.19
	16:0,18:1-DAG(II)	612.5567	13.28
	18:1,18:1-DAG	638.5720	13.27
PUFA	22:5,22:6-DAG	732.5551	12.30
	18:2,22:6-DAG	682.5406	12.45
	18:2,22:5-DAG	684.5557	12.55
	16:0,20:5+18:2,18:3-DAG	632.5247	12.50
	18:1,20:5+16:0,22:6-DAG	658.5403	12.63
	18:1,22:6-DAG	684.5557	12.68
	16:0,22:5-DAG	660.5564	12.79
	18:1,22:5-DAG	686.5722	12.80
	16:0,18:2 + 16:0,18:2-DAG	610.5411	12.89 / 12.98
	18:1,18:2-DAG	636.5565	12.98
Ceramide			
LCFA	Cer(d18:1/16:0)	538.5194	12.96
	Cer(d18:1/18:1(9Z))	564.5350	13.00
	Cer(d18:1/18:0)	566.5507	13.28
	Cer(d18:1/20:0)	594.5820	13.63
VLCFA	Cer(d18:1/24:1(15Z))	648.6289	13.94
	Cer(d18:1/22:0)	622.6133	14.00
	Cer(d18:1/23:0)	636.6289	14.21
	Cer(d18:1/24:0)	650.6446	14.42
	Cer(d18:1/25:0)	664.6602	14.60

Abbreviations: RT, Retention time; DAG; Diacylglycerol, SFA; Saturated Fatty Acid, MUFA; Monounsaturated Fatty Acid, PUFA; Polyunsaturated Fatty Acid.

Table S5. Biometric and biochemical data in rats after 24 weeks of dietary intervention

	STD **n = 7**	**HFHS** **n = 7**	**FO** **n = 7**	**GSE** **n = 7**	**FO + GSE** **n = 7**	***p*-value***
Body weight (g)	256.0 ± 13.9	291.6 ± 23.9[a]	318.3 ± 41.8[a]	289.8 ± 19.6[a]	291.2 ± 25.7[a]	0.005
Perigonadal WAT (g)	4.9 ± 1.1	19.6 ± 12.2[a]	19.7 ± 9.5[a]	15.5 ± 6.3[a]	12.7 ± 5.0	0.002
Liver (g)	6.7 ± 1.2	7.2 ± 0.5	6.8 ± 0.3	7.4 ± 0.7	7.7 ± 0.7	NS
Blood glucose (mmol/L)	3.58 ± 0.15	3.65 ± 0.20	3.56 ± 0.38	3.63 ± 0.26	3.72 ± 0.22	NS
Plasma insulin (mU/L)	27.27 ± 9.55	58.38 ± 15.95[a]	57.66 ± 34.23[a]	74.20 ± 33.87[a]	43.34 ± 30.10	0.008
HOMA-IR	4.38 ± 1.63	9.50 ± 2.64[a]	9.32 ± 6.08[a]	12.11 ± 6.05[a]	7.30 ± 5.20	0.012
Plasma TAG (mmol/L)	1.53 ± 0.43	1.25 ± 0.20	1.57 ±0.35	2.19 ± 0.20[a,b,c]	1.46 ± 0.27[d]	<0.001

Results were expressed as mean ± standard deviation. Abbreviations: STD; Standard, HFHS; High-Fat High-Sucrose, FO; Fish Oil, GSE; Grape Seed Extract, WAT; White Adipose Tissue, HOMA- IR; Homeostatic Assessment, Model of Insulin Resistance, TAG; Triacylglycerol. **p*-value (<0.05) was calculated by the non-parametric Kruskal-Wallis test followed by Mann–Whitney U test. a; vs. STD group, b; vs. HFHS group, c; vs. FO group, d; vs GSE group. [a]These data have already been described in previous reports [1-4]

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4.3. PAPER 3. The Effects of the Combination of Buckwheat D-Fagomine and Fish Omega-3 Fatty Acids on Oxidative Stress and Related Risk Factors in Pre-Obese Rats

Type: Research article

Manuscript status: Published, February 4, 2021

Title: The Effects of the Combination of Buckwheat D-Fagomine and Fish Omega-3 Fatty Acids on Oxidative Stress and Related Risk Factors in Pre-Obese Rats

Journal (Publisher): Foods (MDPI)

Impact factor (JCR 2019): 4.092

Category and classification: Food Science & Technology 27/139 (Q1)

Authors: Bernat Miralles-Pérez, Maria Rosa Nogués, Vanessa Sánchez-Martos, Núria Taltavull, Lucía Méndez, Isabel Medina, Sara Ramos-Romero, Josep L. Torres and Marta Romeu

Citation: Miralles-Pérez B, Nogués MR, Sánchez-Martos V, Taltavull N, Méndez L, Medina I, Ramos-Romero S, Torres JL, Romeu M. The Effects of the Combination of Buckwheat D-Fagomine and Fish Omega-3 Fatty Acids on Oxidative Stress and Related Risk Factors in Pre-Obese Rats. Foods. 2021;10:332.

doi: 10.3390/foods10020332

RESULTS

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The Effects of the Combination of Buckwheat D-Fagomine and Fish Omega-3 Fatty Acids on Oxidative Stress and Related Risk Factors in Pre-Obese Rats

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Citation: Miralles-Pérez, B.; Nogués, M.R.; Sánchez-Martos, V.; Taltavull, N.; Méndez, L.; Medina, I.; Ramos-Romero, S.; Torres, J.L.; Romeu, M. The Effects of the Combination of Buckwheat D-Fagomine and Fish Omega-3 Fatty Acids on Oxidative Stress and Related Risk Factors in Pre-Obese Rats. *Foods* **2021**, *10*, 332. https://doi.org/10.3390/foods10020332

Academic Editor: Diana Ansorena Artieda
Received: 22 December 2020
Accepted: 1 February 2021
Published: 4 February 2021

Abstract: The combined supplementation of buckwheat D-fagomine (FG) and fish omega-3 polyunsaturated fatty acids (ω-3 PUFA) attenuates the development of insulin resistance in rats fed a high-fat (HF) diet. This study aimed to examine the effects of combined supplementation with FG and ω-3 PUFA on dyslipidemia, transaminases, interleukin-6, and oxidative stress. Forty-five male Sprague-Dawley rats were fed a standard diet, an HF diet, an HF diet supplemented with FG, an HF diet supplemented with ω-3 PUFA, or an HF diet supplemented with FG and ω-3 PUFA for 21 weeks. Triacylglycerol, cholesterol, aspartate aminotransferase, alanine aminotransferase, and interleukin-6 were measured. The assessment of oxidative stress included plasma antioxidant capacity, antioxidant enzyme activities, glutathione content, lipid peroxidation, and protein carbonylation. The combined supplementation with FG and ω-3 PUFA did not attenuate the slight accumulation of liver cholesterol induced by the HF diet but normalized the plasma alanine aminotransferase activity. Rats fed the HF diet supplemented with the combination showed a lower amount of plasma interleukin-6 than those fed a standard diet. The combination attenuated oxidative damage induced by the HF diet, decreased antioxidant enzyme activities, and enhanced glutathione status. The beneficial effects of the combination of FG and ω-3 PUFA on oxidative stress and related risk factors in pre-obese rats were mainly modulated by ω-3 PUFA.

Keywords: D-fagomine; fish oil; dyslipidemia; transaminases; inflammation; oxidative stress

1. Introduction

Obesity is characterized by the expansion of white adipose tissue content due to an increase in both the number and size of adipocytes [1]. Concretely, visceral obesity is a central component of metabolic syndrome, which also includes dyslipidemia, impaired glucose tolerance, and hypertension [2]. The past few decades have seen an alarming increase in the worldwide prevalence of obesity [3], which has been related to a Westernized dietary pattern that is rich in saturated animal fat and sucrose and high in overall energy amounts [4,5].

Long-term high-fat (HF) feeding promotes oxidative stress [6,7], which leads to a disturbance in redox signaling pathways and/or molecular damage to lipids and proteins [8]. Early oxidative stress in expanded adipose tissue plays a significant role in the

onset of obesity-related metabolic disorders such as insulin resistance, type 2 diabetes, and non-alcoholic fatty liver disease in rodents and humans [6,7,9–11]. Concretely, the aberrant accumulation of liver fat is also linked to oxidative stress, inflammation, and dysfunction of mitochondria, promoting the development of steatohepatitis and increasing the risk of fibrosis, cirrhosis, and hepatocellular carcinoma [12].

Dietary supplements and functional foods may prevent tissue dysfunction and the development of metabolic disorders by decreasing oxidative stress and inflammation. Individual D-fagomine (FG) and omega-3 polyunsaturated fatty acid (ω-3 PUFA) supplements attenuate the onset of insulin resistance in rats fed an HF diet [13–17]. FG is an iminocyclitol mainly present in buckwheat-based products [18]. Supplementation with FG improves glucose tolerance and reduces low-grade chronic inflammation, partially modulated by intestinal glycosidase inhibitory action and beneficial modifications in the gut microbiota [13,17,19]. The ω-3 PUFA, specifically eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), are present in large quantities in fish and seafood products. Supplementation with ω-3 PUFA in a balanced EPA/DHA 1:1 ratio improves systemic antioxidant status, decreases inflammatory parameters in tissues, and prevents plasma dyslipidemia and the accumulation of ceramides in the liver [14,16,20].

Combining bioactive nutrients targeting both oxidative stress and inflammatory pathways may be a better nutritional strategy to prevent the onset of obesity and related disorders than providing them individually [21,22]. We have recently shown that the combined supplementation of both FG and ω-3 PUFA attenuates the increase in visceral adipose tissue content and fasting glucose concentration, plasma hyperinsulinemia, plasma hyperleptinemia. as well as lobular inflammation in the liver induced by an HF diet [23]. Moreover, the combination of these two nutrients promotes the growth of beneficial populations of Lactobacilliales and Bifidobacteriales and the production of short-chain fatty acids in rats receiving the HF diet [23].

Although ω-3 PUFA has been found to exhibit antioxidant properties by enhancing the non-enzymatic antioxidant capacity and endogenous antioxidant defenses [24], the complementary or synergic effects of FG combined with ω-3 PUFA on these parameters have yet to be assessed.

This study therefore expands upon our previous work and aims to examine the effects of combined supplementation with FG and ω-3 PUFA on dyslipidemia, transaminases, interleukin-6, and oxidative stress in pre-obese rats.

2. Materials and Methods

2.1. Ethics Statement

All animal methods comply with the European Union guidelines for the care and handling of laboratory animals (EU Directive 2010/63/EU). The permission was obtained from the Bioethics subcommittee of the Spanish National Research Council and the regional Catalan authorities (reference number DAAM7921).

2.2. Animals and Experimental Design

We purchased 45 male Sprague-Dawley rats (324 ± 19 g body weight; 8–9 weeks old) (Hsd:SD, Envigo, Indianapolis, IN, USA). The rats were kept in an insulated room (three per Makrolon cage; 425 × 265 × 180 mm) at a controlled temperature (22 ± 2 °C), humidity (60%), and 12 h artificial light/dark cycle.

Prior to the dietary intervention, the rats were fed a standard diet (STD; Teklad Global 14% Protein Rodent Maintenance Diet [2.9 kcal/g], Envigo, Indianapolis, IN, USA). After two weeks of acclimatization, the rats were randomly allocated into five groups (nine per group) and fed one of the following diets for 21 weeks: (1) an STD diet (STD group), (2) an HF diet (TD.08811 45% kcal Fat Diet [4.7 kcal/g], Envigo, Indianapolis, IN, USA), (3) an HF diet supplemented with FG (FG group), (4) an HF diet supplemented with EPA/DHA in a balanced 1:1 ratio (ω-3 group), or (5) an HF diet supplemented with FG and EPA/DHA in a balanced 1:1 ratio (FG&ω-3 group). The rats had ad libitum access to food and water

(Ribes, Barcelona, Spain) throughout the study. The experimental diets are described in the Supplementary Material (Table S1).

The FG (>98%) was provided by Taihua Shouyue (HK) International Co. Ltd (Hong Kong, China) and was manufactured by Bioglane SLNE (Barcelona, Spain). It was included in the feed of the FG&ω-3 and FG groups at a proportion of 0.96 g/kg feed, as previously defined [19].

The EPA/DHA mixture in a balanced 1:1 ratio was obtained by combining the appropriate quantities of the commercial fish oils AFAMPES 121 EPA (AFAMSA, Vigo, Spain) and Omega-3 RX (EnerZona, Milan, Italy). Fish oil was administered once a week by oral gavage in the FG&ω-3 and ω-3 groups using a gastric probe at a dose of 0.8 mL oil/kg body weight. Soybean oil (Clearspring Ltd., London, UK) was administered by oral gavage in the STD, HF, and FG groups at the same time and at the same dose to compensate for the stress of probing and the excess of calories from fish oil in the FG&ω-3 and ω-3 groups. The fatty acid composition of the oils used is described in the Supplementary Material (Table S2).

Feed intake (g) was monitored daily. Subsequently, energy intake (kcal) was estimated using the Atwater conversion factors: 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrate.

Body weight (g) was monitored daily. Body weight gain (g), adiposity index (%, perigonadal adipose tissue weight (g)/body weight (g) × 100), and hepatosomatic index (%, liver weight (g)/body weight (g) × 100) were calculated at the end of the study.

2.3. Measurement of Glucose Tolerance

At week 18 of the study, an oral glucose tolerance test (OGTT) was performed on fasting animals. Before the test, glucose was administered by oral gavage (1 g/kg body weight), and blood glucose concentration was measured 15, 30, 45, 60, 90 and 120 min after glucose intake. Blood glucose was measured by means of the enzyme electrode method, using an Ascensia ELITE XL blood glucometer (Bayer Consumer Care, Basel, Switzerland). The results were expressed as area under the curve (AUC, mg/mL per 120 min), calculated using the Trapezium method.

2.4. Sample Processing

After 21 weeks of dietary intervention, the rats were fasted overnight, anesthetized intraperitoneally with ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight), and sacrificed by exsanguination. Blood samples were taken by cardiac puncture. Then, plasma was obtained by centrifugation at 850× *g* for 15 min at 4 °C. After the removal of plasma, erythrocytes were obtained by washing twice with 154 mM of sodium chloride solution and centrifugation at 1300× *g* for 5 min at 4 °C. Plasma and erythrocyte samples were aliquoted and stored at −80 °C until use.

Samples of perigonadal white adipose tissue as a biomarker of visceral adiposity and liver were collected, washed with 154 mM sodium chloride solution, weighed, and cut. After that, adipose tissue and liver samples were snap-frozen in liquid nitrogen and stored at −80 °C until use. Frozen adipose tissue samples were homogenized on ice in 200 mM of sodium phosphate buffer (pH 6.25), sonicated for 1 min, and centrifuged at 1000× *g* for 10 min at 4 °C. The soluble fraction was then carefully collected and centrifuged at 129,000× *g* for 1 h at 4 °C. Frozen liver samples were divided into two parts. One part of the liver sample was homogenized on ice in 154 mM of sodium chloride solution containing 0.1% Triton X-100 (*v*/*v*) and centrifuged at 3000× *g* for 5 min at room temperature for the measurement of lipid content. The other part was homogenized on ice in 200 mM of sodium phosphate buffer (pH 6.25) and centrifuged at 129,000× *g* for 1 h at 4 °C for the measurement of oxidative stress biomarkers. Tissue samples were aliquoted and stored at −80 °C until use.

2.5. Measurements of Insulin Resistance Biomarkers

At the end of the study, the fasting blood glucose concentration was measured as described above for the OGTT. Fasting plasma insulin concentration was measured using the corresponding ELISA kit (Millipore Corporation, Billerica, MA, USA).

2.6. Measurements of Lipid Profile, Transaminases and Interleukin-6

Plasma triacylglycerol (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL), and low-density lipoprotein cholesterol (LDL) concentrations were measured by means of colorimetric enzymatic methods using the corresponding commercial kits (Spinreact, Girona, Spain) in a COBAS MIRA autoanalyzer (Roche Diagnostics System, Madrid, Spain). In addition, the LDL/HDL ratio was calculated. Liver TG and TC contents were measured as described above for plasma.

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured by means of spectrophotometry using the corresponding commercial kits (Spinreact, Girona, Spain) in a COBAS MIRA autoanalyzer (Roche Diagnostics System, Madrid, Spain). In addition, the AST/ALT ratio was calculated as a biomarker of liver function.

Plasma interleukin-6 (IL-6) concentration was measured using magnetic bead Milliplex xMAP multiplex technology with the corresponding ELISA kit (Millipore Corporation, Billerica, MA, USA).

2.7. Measurements of Oxidative Stress Biomarkers

2.7.1. Plasma Non-Enzymatic Antioxidant Capacity

Plasma antioxidant capacity was assessed using the oxygen radical absorbance capacity (ORAC) [25] and the ferric reducing ability of plasma (FRAP) assays [26]. The ORAC was measured using a Fluoroskan Ascent microplate fluorimeter (Labsystems, Helsinki, Finland), and trolox as a standard (Sigma-Aldrich, Madrid, Spain). The FRAP was measured using a PowerWave XS2 microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA), and trolox as a standard.

2.7.2. Antioxidant Enzymes and Glutathione

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) were assessed in erythrocytes, adipose tissue, and liver. The total SOD and CAT activities were measured according to the methods developed by Mirsa and Fridovich [27] and Cohen et al. [28], respectively, using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, Shelton, CT, USA). GPx and GR activities were measured according to the method developed by Wheeler et al. [29] using a COBAS MIRA autoanalyzer (Roche Diagnostics System, Madrid, Spain).

Reduced glutathione (GSH) and oxidized glutathione (GSSG) in plasma, erythrocytes, adipose tissue, and liver were measured according to the method developed by Hissin and Hilf [30] using an LS55 fluorescence spectrophotometer (Perkin Elmer, Shelton, CT, USA) and the corresponding standard curves (Sigma-Aldrich, Madrid, Spain). In addition, the GSSG/GSH ratio was calculated as a biomarker of the redox state.

Measurements in erythrocyte samples were normalized to hemoglobin (Hb) concentration in total blood. Hb concentration was measured according to the Drabkin method [31] using a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, Shelton, CT, USA), and an Hb standard (Spinreact, Girona, Spain).

2.7.3. Lipid Peroxidation and Protein Carbonylation

Lipid peroxidation (conjugated dienes hydroperoxides) and total protein carbonylation in plasma and liver were measured as previously described [32]. Briefly, total lipids were extracted and quantified. After that, conjugated dienes hydroperoxides were measured in a Beckman DU-640 spectrophotometer (Beckman Instruments Inc., Palo Alto, CA, USA). Proteins were extracted and the protein carbonyl content was labeled with

fluorescein-5-thiosemicarbazide and detected by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2.8. Statistical Analysis

The statistical analysis was performed using IBM SPSS v.25 software (IBM, Chicago, IL, USA). Samples were assayed in duplicate. The results are expressed as mean and standard deviation and the Shapiro-Wilk test was used to test for the normality of data. Groups were then compared by means of the one-way analysis of variance, followed by Scheffé post-hoc test or the non-parametric Kruskal-Wallis test followed by the Mann–Whitney U test. The level of statistical significance was set at a p-value < 0.05.

3. Results

3.1. Feed Intake, Biometric Data and Insulin Resistance Biomarkers

Because these parameters have been partially described and discussed in previous work [23], we include these data here as Supplementary Material (Figure S1 and Table S3). All HF diet-fed groups presented a lower feed intake and higher energy intake than the STD group throughout the study. Although no significant differences were found in either final body weight or body weight gain among the groups, the HF diet increased the perigonadal white adipose tissue content after 21 weeks. Even so, both groups supplemented with FG, either individually or in combination with ω-3 PUFA, showed similar adipose tissue content to rats fed an STD diet. In contrast, the individual ω-3 PUFA supplementation did not attenuate the increase in adipose tissue content under HF-feeding conditions. No significant differences were found in liver weight among the groups.

At week 18, all the groups showed similar glucose tolerance in vivo. However, the HF group registered higher fasting blood glucose and fasting plasma insulin concentrations than the STD group by the end of the study. All the supplementations attenuated the increase in insulin concentration under HF-feeding. Nevertheless, the increase in fasting blood glucose was only attenuated by the combination of FG with ω-3 PUFA.

3.2. Lipid Profile, Transaminases and Interleukin-6

Both groups supplemented with ω-3 PUFA, either individually or in combination with FG, as well as the HF group registered lower plasma TC concentrations than the STD group, mainly due to the decreased HDL concentrations in these groups. Individual FG supplementation in HF-feeding conditions also decreased HDL compared to the STD diet. In addition, the FG group presented a higher LDL concentration than the ω-3 group, whereas the combination of both nutrients attenuated the increase in the concentration of plasma LDL induced by FG. Consistent with these results, the FG group showed the highest LDL/HDL ratio value among the groups, and the combination of FG with ω-3 PUFA significantly counteracted this increase. No significant differences were found in plasma TG concentrations among the groups (Table 1).

Isolated ω-3 supplementation attenuated lipid accumulation in the liver under HF-feeding conditions, showing similar TC and even decreased TG concentrations to those seen in the STD group. Conversely, the other groups showed higher TC concentrations than the STD group (Table 1).

No significant differences were observed in the ratio of AST to ALT in plasma among the groups. Interestingly, both transaminase activities tended to decrease in all the rats fed an HF diet compared to those fed a STD diet. The HF group even showed a significantly lower ALT activity than the STD group. The FG&ω-3 combination increased the ALT activity compared to both the FG and the HF groups, with values close to those observed in the STD group (Table 1).

The HF diet did not modify the plasma IL-6 concentration. Interestingly, the FG&ω-3 group showed lower IL-6 concentrations than the STD group (Table 1).

Table 1. Lipid profile, transaminases and interleukin-6 after 21 weeks of dietary intervention.

	STD	HF	FG	ω-3	FG&ω-3
Plasma lipid profile					
TG (mmol/L)	0.7 ± 0.2	0.5 ± 0.3	0.6 ± 0.1	0.6 ± 0.2	0.4 ± 0.1
TC (mmol/L)	3.6 ± 0.4	2.9 ± 0.5 [a]	3.1 ± 0.5	2.5 ± 0.4 [a]	2.6 ± 0.3 [a]
HDL (mmol/L)	1.15 ± 0.12	0.94 ± 0.16 [a]	0.94 ± 0.07 [a]	0.85 ± 0.12 [a]	0.93 ± 0.07 [a]
LDL (mmol/L)	0.43 ± 0.11	0.39 ± 0.09	0.47 ± 0.11	0.32 ± 0.07 [c]	0.36 ± 0.03
LDL/HDL ratio	0.37 ± 0.08	0.42 ± 0.06	0.50 ± 0.11 [a]	0.38 ± 0.07 [c]	0.37 ± 0.04 [c]
Liver lipid profile					
TG (μmol/g tissue)	17.6 ± 1.0	16.6 ± 2.6	14.6 ± 1.3	14.1 ± 2.3 [a]	16.7 ± 2.5
TC (μmol/g tissue)	5.9 ± 0.7	7.8 ± 1.8 [a]	7.8 ± 0.8 [a]	6.4 ± 1.0	7.6 ± 1.0 [a]
Transaminases and IL-6					
Plasma AST (U/L)	70.2 ± 21.9	57.1 ± 10.7	49.2 ± 8.4	53.6 ± 10.1	61.0 ± 14.4
Plasma ALT (U/L)	27.1 ± 8.2	19.0 ± 3.9 [a]	20.2 ± 4.7	22.8 ± 4.2	24.0 ± 3.6 [b,c]
Plasma AST/ALT ratio	2.64 ± 0.52	3.14 ± 0.92	2.49 ± 0.45	2.37 ± 0.29	2.53 ± 0.38
Plasma IL-6 (pg/mL)	522 ± 307	283 ± 167	452 ± 259	349 ± 184	154 ± 72 [a]

Values are expressed as mean ± standard deviation, nine rats per group. Abbreviations: STD, Standard group; HF, High-Fat group; FG, Fagomine group; ω-3, ω-3 PUFA group; FG&ω-3, Fagomine and ω-3 PUFA group; TG, Triacylglycerol; TC, Total Cholesterol; HDL, High-Density Lipoprotein cholesterol; LDL, Low-Density Lipoprotein cholesterol; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; IL-6, Interleukin-6. *p*-value was calculated by the one-way analysis of variance or the non-parametric Kruskal-Wallis test followed by Scheffé post-hoc test or Mann–Whitney U test, respectively. [a], vs. STD group; [b], vs. HF group; [c], vs. FG group.

3.3. Oxidative Stress Biomarkers in Blood

No significant differences were found in either the non-enzymatic antioxidant capacity or glutathione status in plasma among the groups. However, the HF diet increased plasma lipid peroxidation and albumin carbonylation compared to the STD diet. The combined supplementation with FG and ω-3 PUFA significantly attenuated the increase in the conjugated dienes and tended to decrease albumin carbonylation, mainly modulated by ω-3 PUFA (Table 2).

The HF diet did not modify either antioxidant enzyme activities or glutathione redox state in erythrocytes compared to the STD diet. Nevertheless, some endogenous antioxidant defenses were decreased in the supplemented groups compared to the non-supplemented groups by the end of the study. Although FG and ω-3 supplementations, either individually or combined, decreased the activities of the enzymes of the glutathione system (GPx and GR), the FG&ω-3 group showed a similar GSH content to those observed in the STD and HF groups. Moreover, a lower GSSG content was recorded in the FG&ω-3 group than in the other groups, and this group had the most beneficial glutathione redox state (Table 2).

Table 2. Oxidative stress biomarkers in blood after 21 weeks of dietary intervention.

	STD	HF	FG	ω-3	FG&ω-3
Plasma					
ORAC (μmol trolox Eq/mL)	18.3 ± 3.8	17.4 ± 4.7	18.4 ± 5.7	21.2 ± 6.1	21.1 ± 7.6
FRAP (μmol trolox Eq/mL)	0.16 ± 0.03	0.14 ± 0.03	0.12 ± 0.02	0.12 ± 0.01	0.13 ± 0.03
GSH (nmol/mL)	9.9 ± 1.4	11.2 ± 3.8	11.4 ± 6.2	10.4 ± 1.9	9.9 ± 3.2
GSSG (nmol/mL)	32.4 ± 2.3	30.5 ± 3.3	29.6 ± 4.9	29.7 ± 4.9	29.3 ± 1.9
GSSG/GSH ratio	3.24 ± 0.57	2.96 ± 0.85	3.19 ± 1.57	2.88 ± 0.41	3.22 ± 1.00
Conjugated dienes (mmol hydroperoxides/kg lipid)	0.13 ± 0.03	0.21 ± 0.06 [a]	0.17 ± 0.03 [a]	0.14 ± 0.01 [b]	0.14 ± 0.02
Albumin carbonylation index	0.17 ± 0.04	0.42 ± 0.14 [a]	0.36 ± 0.08[a]	0.26 ± 0.05 [a,b,c]	0.31 ± 0.06 [a]
Erythrocytes					
SOD (U/g Hb)	1155 ± 410	1022 ± 279	1156 ± 245	1250 ± 367	968 ± 289
CAT (mmol/g Hb)	77.5 ± 11.4	86.6 ± 22.3	71.4 ± 12.8	64.1 ± 6.9	70.1 ± 18.6
GPx (U/g Hb)	85.4 ± 14.7	83.4 ± 13.1	76.7 ± 13.2	71.3 ± 6.8 [a,b]	61.3 ± 14.4 [a,b,c]
GR (U/g Hb)	0.31 ± 0.06	0.24 ± 0.04	0.21 ± 0.07 [a]	0.18 ± 0.03 [a]	0.19 ± 0.05 [a]

Table 2. *Cont.*

	STD	HF	FG	ω-3	FG&ω-3
GSH (μmol/g Hb)	1.51 ± 0.49	1.16 ± 1.06	0.69 ± 0.22 [a]	0.78 ± 0.57 [a]	1.69 ± 0.53 [c,d]
GSSG (μmol/g Hb)	0.85 ± 0.26	0.77 ± 0.28	0.82 ± 0.30	0.93 ± 0.44	0.43 ± 0.08 [a,b,c,d]
GSSG/GSH ratio	0.60 ± 0.25	1.29 ± 0.99	1.21 ± 0.34	1.56 ± 0.87	0.29 ± 0.11 [d]

Values are expressed as mean ± standard deviation, nine rats per group. Abbreviations: STD, Standard group; HF, High-Fat group; FG, Fagomine group; ω-3, ω-3 PUFA group; FG&ω-3, Fagomine and ω-3 PUFA group; ORAC, Oxygen Radical Absorbance Capacity; FRAP, Ferric Reducing Ability of Plasma; SOD, Superoxide Dismutase; Hb, Hemoglobin; CAT, Catalase; GPx, Glutathione Peroxidase; GR, Glutathione Reductase; GSH, Reduced Glutathione; GSSG, Oxidized Glutathione. *p*-value was calculated by the one-way analysis of variance or the non-parametric Kruskal–Wallis test followed by Scheffé post-hoc test or Mann-Whitney U test, respectively. [a], vs. STD group; [b], vs. HF group; [c], vs. FG group; [d], vs. ω-3 group.

3.4. Oxidative Stress Biomarkers in Tissues

No significant differences were found in antioxidant enzyme activities in adipose tissue between rats fed HF and STD diets. However, the ω-3 group exhibited a higher GSH content than the FG and both non-supplemented groups and had the lowest GSSG/GSH ratio value among the groups. Its combination with FG attenuated the increase in the GSSG/GSH ratio value induced by FG compared to the HF diet without supplementation (Table 3).

Table 3. Oxidative stress biomarkers in tissues after 21 weeks of dietary intervention.

	STD	HF	FG	ω-3	FG&ω-3
Perigonadal white adipose tissue					
SOD (U/g tissue)	83.9 ± 13.1	75.5 ± 7.0	80.8 ± 28.8	63.0 ± 18.2	74.7 ± 24.4
CAT (mmol/g tissue)	0.11 ± 0.01	0.07 ± 0.04	0.10 ± 0.04	0.10 ± 0.03	0.08 ± 0.06
GPx (U/g tissue)	0.25 ± 0.23	0.07 ± 0.06	0.15 ± 0.20	0.05 ± 0.04	0.05 ± 0.05
GR (U/g tissue)	0.15 ± 0.09	0.10 ± 0.08	0.14 ± 0.05	0.14 ± 0.07	0.12 ± 0.07
GSH (nmol/g tissue)	6.79 ± 1.23	6.71 ± 2.79	5.75 ± 2.84	10.92 ± 0.93 [a,b,c]	8.24 ± 3.76
GSSG (nmol/g tissue)	28.3 ± 6.7	24.2 ± 6.4	26.2 ± 4.5	27.3 ± 4.3	23.5 ± 5.1
GSSG/GSH ratio	4.32 ± 1.49	3.87 ± 0.86	5.31 ± 1.82 [b]	2.52 ± 0.54 [a,b,c]	3.13 ± 0.96 [c]
Liver					
SOD (U/g tissue)	4523 ± 271	2896 ± 883 [a]	4122 ± 1556	3714 ± 917	2956 ± 379 [a]
CAT (mmol/g tissue)	17.7 ± 0.9	15.5 ± 1.4	16.1 ± 1.7	15.9 ± 2.5	17.2 ± 2.4
GPx (U/g tissue)	40.7 ± 6.1	34.0 ± 5.2	37.9 ± 2.3	38.3 ± 8.0	36.3 ± 5.6
GR (U/g tissue)	7.86 ± 1.37	5.46 ± 1.18	6.05 ± 1.49	6.34 ± 1.34	7.84 ± 2.64
GSH (μmol/g tissue)	0.86 ± 0.37	0.47 ± 0.29	0.62 ± 0.31	0.58 ± 0.37	0.57 ± 0.40
GSSG (μmol/g tissue)	1.56 ± 0.26	1.29 ± 0.14	1.22 ± 0.07	1.21 ± 0.26 [a]	1.08 ± 0.28 [a]
GSSG/GSH ratio	2.02 ± 0.65	3.46 ± 1.55	2.45 ± 1.15	2.97 ± 2.04	2.90 ± 2.10
Conjugated dienes (mmol hydroperoxides/kg lipid)	15.8 ± 0.5	23.3 ± 1.1 [a]	23.8 ± 1.3 [a]	21.7 ± 1.1 [a]	22.6 ± 1.6 [a]
Total protein carbonylation index	0.74 ± 0.11	0.94 ± 0.16 [a]	0.91 ± 0.16 [a]	0.75 ± 0.10 [b,c]	0.63 ± 0.08 [b,c]

Values are expressed as mean ± standard deviation, nine rats per group. Abbreviations: STD, Standard group; HF, High-Fat group; FG, Fagomine group; ω-3, ω-3 PUFA group; FG&ω-3, Fagomine and ω-3 PUFA group; SOD, Superoxide Dismutase; CAT, Catalase; GPx, Glutathione Peroxidase; GR, Glutathione Reductase; GSH, Reduced Glutathione; GSSG, Oxidized Glutathione. *p*-value was calculated by the one-way analysis of variance or the non-parametric Kruskal–Wallis test followed by Scheffé post-hoc test or Mann–Whitney U test, respectively. [a], vs. STD group; [b], vs. HF group; [c], vs. FG group.

In the liver, the FG&ω-3 and HF groups presented a lower SOD activity than the STD group. However, both groups supplemented with ω-3 PUFA, either individually or in combination with FG, had a decreased GSSG content compared to the STD group. The HF diet increased lipid peroxidation and protein carbonylation compared to the STD diet. However, the combined supplementation with FG and ω-3 PUFA attenuated the increase in total protein carbonylation, mainly modulated by ω-3 PUFA (Table 3).

4. Discussion

We previously showed that the combined supplementation with FG and ω-3 PUFA attenuates the increase in perigonadal adipose tissue content and the onset of insulin

resistance induced by an HF diet [23]. The combination of ω-3 PUFA with another bioactive nutrient may be a useful nutritional strategy for the prevention of obesity and insulin resistance by enhancing anti-inflammatory and antioxidant status [22]. Thus, the present study aimed to examine, for the first time, the combined effects of FG and ω-3 PUFA on dyslipidemia, transaminases, interleukin-6, and oxidative in pre-obese rats.

We observed that the HF diet decreased the TC as well as HDL concentrations in plasma and promoted a slight TC accumulation in the liver compared to the STD diet. Nevertheless, the plasma and liver TG concentrations were unchanged after 21 weeks under HF feeding. This may be because the cholesterol content of the HF diet is too low for the induction of plasma hypercholesterolemia (0.05% of feed), as previously suggested [33]. In addition, the fatty acid overload leads to adaptive responses such as increased fatty acid desaturation and β-oxidation [10,34,35], which may explain why the plasma and liver TG concentrations remained unchanged.

Although none of the supplements counteracted the decrease in plasma HDL induced by the HF diet, the groups supplemented with ω-3 PUFA, either individually or combined with FG, showed lower LDL/HDL values than those found in the FG group. The supplementation with the combination of FG and ω-3 PUFA did not mitigate the slight TC accumulation in the liver induced by the HF diet. On the contrary, individual ω-3 PUFA supplementation prevented TC accumulation, with the rats in that group registering a similar TC and even a lower TG content than the rats fed an STD diet. Other authors have shown that the ω-3 PUFA supplementation exerts plasma hypocholesterolemic effects under HF-feeding conditions [34,36,37]. Thus, ω-3 PUFA supplementation may decrease lipid accumulation in the liver by increasing the ω-3 PUFA content [22,38], which may stimulate β-oxidation [39,40] as well as cholesterol uptake from plasma, bile acid synthesis, and its extraction [41]. In addition, ω-3 PUFA supplementation may decrease cholesterol and TG synthesis [40].

Even though the HF diet induced a slight TC accumulation in the liver, we found no differences in the AST/ALT ratio in plasma among the groups. Increased circulating transaminases are indicators of liver injury and liver metabolic functioning [42]. Interestingly, the HF group exhibited a lower plasma ALT concentration than the STD group. This difference may be associated with decreased muscle mass [43], which is a common characteristic of obesity [44]. Thus, the combination of FG and ω-3 PUFA counteracted the reduction in ALT in plasma with a concomitant decrease in the perigonadal adipose tissue content. Moreover, other studies have shown in rodents that ω-3 PUFA may improve skeletal muscle metabolic function by activating the AMPK/PGC-1α signaling pathway [45] and may decrease proteolysis in the liver by the down-regulation of l-serine dehydratase/l-threonine deaminase, dipeptidyl peptidase 1 light chain, and proteasome subunit beta type-8 [40]. The ω-3 PUFA supplementation may also increase protein synthesis in older adults by activating the mTOR-p70s6k signaling pathway [46].

In the present study, the HF diet did not modify the plasma IL-6 concentration, as observed in other studies in rodents [47]. Low-grade chronic inflammation is a characteristic of obesity [48]. Furthermore, inflammation, rather than hypercholesterolemia, has been identified as a key player in the onset of chronic diseases [21,49]. In fact, other authors have shown in rodents that an HF diet increases the plasma IL-6 concentration [9,38]. Nevertheless, it is possible to observe increased circulating inflammatory biomarkers once obesity and insulin resistance are fully established [9]. Interestingly, the combination of FG and ω-3 PUFA decreased IL-6 compared to the STD group. Previous studies have shown that FG prevents the dysbiosis of gut microbiota induced by excessive fat consumption, maintaining intestinal barrier integrity [13,23]. Furthermore, ω-3 PUFA and its oxidized metabolites exert anti-inflammatory effects [22,50–52] by binding to G-protein-coupled receptor 120 and increasing peroxisome proliferator-activated receptor γ, inhibiting the nuclear transcription factor-kappa β pro-inflammatory signaling pathway [37,51,53,54]. Nevertheless, in the present study we observed no significant effects of individual supplementations on plasma IL-6. The decrease in plasma IL-6 in rats receiving the combined

supplementation compared to those fed the STD diet might be explained by the distinct fatty acid composition of the dietary backgrounds (STD or HF) and the use of fish oil instead of soybean oil. As we previously reported in the same cohort of rats [32], the STD diet increases the ω-6 PUFA content in the liver compared to the HF diet. The soybean oil is rich in linoleic acid (LA, 18:2 ω-6) (Table S3), which is a precursor of arachidonic acid (ARA, 20:4 ω-6) [55]. Non-esterified ARA can be oxidized producing a large number of lipid mediators related to the induction of pro-inflammatory pathways [55]. Hence, when fish oil is used instead of soybean oil, FG may participate in modulating inflammatory parameters such as plasma IL-6, which is mainly derived from adipose tissue.

We observed that the HF diet induced early stages of insulin resistance, accompanied by increased lipid peroxidation and protein carbonylation in plasma and the liver compared to the STD diet. In agreement with our findings, Ciapaite et al. [35] have shown that an HF diet increases the content of thiobarbituric acid reactive substances and total protein carbonyls in the liver. A previous study found that an HF diet induces an early antioxidant response against the excessive production of reactive oxygen species (ROS) in both adipose tissue and the liver in mice before an advanced insulin-resistant state [10]. It is important to note that, whereas physiological ROS production may enhance insulin sensitivity in the onset of insulin resistance [56], long-term ROS overproduction leads to oxidative stress and inflammation, promoting irreversible oxidative damage and subsequent metabolic alterations [7,11,57].

The combined supplementation with FG and ω-3 PUFA attenuated the lipid peroxidation in plasma and total protein carbonylation in the liver induced by the HF diet. Furthermore, the FG&ω-3 group showed the lowest GSSG content in erythrocytes, accompanied by decreased GPx and GR activities. In fact, the GSSG content in erythrocytes has been suggested as the best oxidative stress biomarker to describe the redox status of tissues [58]. The combined supplementation also decreased the SOD activity as well as the GSSG content in the liver compared to the STD diet, which could suggest decreased ROS production and enhanced antioxidant status in this group, as observed in a previous study [22]. In the present study, the individual supplementation with ω-3 PUFA significantly decreased oxidative damage under HF-feeding conditions. On the contrary, the individual supplementation with FG did not attenuate high-fat diet-induced oxidative stress. Furthermore, the combination of the two supplements attenuated the redox imbalance (i.e., GSSG/GSH) in the adipose tissue induced by FG. Concretely, GSH is a low-molecular weight thiol containing glutamic acid, cysteine, and glycine, ubiquitously present in mammalian tissues but especially in the liver [59]. It is the primary reducing agent both in mitochondria and in the endoplasmic reticulum [60], maintaining a reduced state by either directly or indirectly scavenging ROS [61]. As the GPx and GR activities in the perigonadal adipose tissue were unchanged, the increase in the content of GSH in both ω-3 PUFA-supplemented groups could be provided thought de novo synthesis via the activation of the γ-glutamylcysteine synthetase instead of the recycle process [59]. Taken together, these findings suggest that the beneficial effects of the combination of FG and ω-3 PUFA on oxidative stress were mainly modulated by ω-3 PUFA. It is well-known that dietary ω-3 PUFA and its oxidized metabolites exert antioxidant effects in humans [24], probably by activating the nuclear factor-erythroid 2-related factor 2 signaling pathway [62,63]. Indeed, previous studies have shown that ω-3 PUFA supplementation attenuates the increase in the GSSG/GSH ratio and the content of protein carbonyls in the liver induced by excessive fat intake in rodents [32,38], which is accompanied by enhanced insulin sensitivity [38]. Furthermore, Selenscig et al. [37] have shown that replacing corn oil as a dietary fat source with fish oil improves the epididymal adipose tissue function in rats fed a diet rich in sucrose by increasing antioxidant defenses and reducing ROS production. As far as FG is concerned, other authors have reported that FG attenuates oxidative stress induced by high glucose contents in human umbilical vein endothelial cells by activating the AMPK/SIRT1/PGC-1α pathway [64]. Nevertheless, no previous studies have assessed the effects of FG on antioxidant status in vivo under an HF diet.

5. Conclusions

The combined supplementation with FG and ω-3 PUFA partially attenuated oxidative damage to lipids and proteins induced by the HF diet. Furthermore, the combination decreased antioxidant enzyme activities and enhanced glutathione status. The combination did not attenuate the slight accumulation of cholesterol in the liver induced by the HF diet but normalized the plasma alanine aminotransferase activity. The beneficial effects of the combination of FG and ω-3 PUFA on oxidative stress in pre-obese rats were mainly modulated by ω-3 PUFA. Therefore, the increase in the dietary intake of ω-3 PUFA from fish, either individually or in combination with FG from buckwheat, may be a useful nutritional strategy against oxidative stress attenuating the onset of insulin resistance.

Supplementary Materials: The following are available online at https://www.mdpi.com/2304-8158/10/2/332/s1: Table S1: Diet composition; Table S2: Fatty acid composition (mol %) of soybean and fish oils; Figure S1: (A) Energy intake and (B) body weight throughout the study; Table S3: Feed intake, biometric data and insulin resistance biomarkers after 21 weeks of dietary intervention.

Author Contributions: M.R.N., J.L.T., I.M. and M.R. designed the research; B.M.-P., V.S.-M., N.T., L.M. and S.R.-R. conducted the research; B.M.-P., M.R.N. and M.R. analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Spanish Ministry of Economy and Competitiveness (grant numbers AGL2013-49079-C2-1-R, AGL2013-49079-C2-2-R and AGL2017-83599-R).

Institutional Review Board Statement: The study was conducted according to the guidelines of the European Union for the care and handling of laboratory animals (EU Directive 2010/63/EU), and approved by the Bioethics subcommittee of the Spanish National Research Council and the regional Catalan authorities (reference number DAAM7921).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The Secretariat of Universities and Research of the Government of Catalonia, the European Union (EU), and the European Social Fund (ESF) are gratefully acknowledged for the doctoral fellowship of B.M.-P. (2018 FI_B 00919, 2019 FI_B1 00160, 2020 FI_B2 00119). Xunta de Galicia and Axencia Galega de Innovación (GAIN) are thankfully recognized by the financial support of the postdoctoral contract to L.M. (IN606B 2017/006).

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

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Electronic Supplementary Information

The Effects of the Combination of Buckwheat D-Fagomine and Fish Omega-3 Fatty Acids on Oxidative Stress and Related Risk Factors in Pre-Obese Rats

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Table S1. Diet composition.

	STD	HF	FG	ω-3	FG&ω-3
Diet composition					
Flour (g)	1,000[a]	1,000[b]	1,000[b]	1,000[b]	1,000[b]
Fagomine (g)	-	-	0.96	-	0.96
Oil	0.8 mL SBO/kg b.w.	0.8 mL SBO/kg b.w.	0.8 mL SBO/kg b.w.	0.8 mL FO/kg b.w.	0.8 mL FO/kg b.w.
Macronutrients (% weight)					
Protein	14.3	17.0	17.0	17.0	17.0
Fat	4.0	23.0	23.0	23.0	23.0
Carbohydrates	48.0	47.6	47.6	47.6	47.6
Macronutrients (% caloric value)					
Protein	20.0	14.7	14.7	14.7	14.7
Fat	13.0	40.6	40.6	40.6	40.6
Carbohydrates	67.0	40.7[c]	40.7[c]	40.7[c]	40.7[c]
Total energy density (kcal/g)[d]	2.9	4.7	4.7	4.7	4.7

Abbreviations: STD, Standard Group; HF, High-Fat Group; FG, Fagomine Group; ω-3, ω-3 PUFA Group; FG&ω-3, Fagomine and ω-3 PUFA Group; SBO, Soybean Oil; FO, Fish Oil; b.w., Body Weight. [a]Teklad Global 14% Protein Rodent Maintenance Diet (Envigo, IN, USA), [b]TD.08811 45% kcal Fat Diet (Envigo, IN, USA), [c]Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates, [d]Energy density was calculated as estimates of metabolizable energy based on the Atwater conversion factors, assigning 4 kcal/g of protein, 9 kcal/g of fat, and 4 kcal/g of available carbohydrate.

Table S2. Fatty acid composition (mol %) of soybean and fish oils.

	SBO	**FO**
14:00	0.96 ± 0.02	4.37 ± 0.05
15:00	0.15 ± 0.01	0.29 ± 0.02
16:00	17.78 ± 0.10	10.15 ± 0.16
16:1 ω-7	0.90 ± 0.03	4.99 ± 0.04
17:00	0.21 ± 0.01	0.45 ± 0.004
18:00	2.07 ± 0.01	2.94 ± 0.03
18:1 ω-9	18.75 ± 0.03	6.41 ± 0.06
18:1 ω-7	1.52 ± 0.02	1.91 ± 0.03
18:2 ω-6	47.55 ± 0.01	0.65 ± 0.01
20:00	n.d	0.32 ± 0.01
18:3 ω-3	4.00 ± 0.04	0.36 ± 0.01
20:1 ω-9	1.43 ± 0.09	0.98 ± 0.03
18:4 ω-3	0.15 ± 0.004	1.51 ± 0.02
20:2 ω-6	0.20 ± 0.05	0.21 ± 0.003
20:3 ω-6	n.d	0.22 ± 0.01
20:4 ω-6	0.40 ± 0.02	1.68 ± 0.04
22:1 ω-11	1.08 ± 0.01	1.14 ± 0.01
22:1 ω-9	0.25 ± 0.02	0.28 ± 0.03
20:4 ω-3	0.20 ± 0.03	1.02 ± 0.02
20:5 ω-3	0.70 ± 0.02	25.09 ± 0.10
24:1 ω-9	0.28 ± 0.05	0.38 ± 0.003
22:5 ω-3	0.26 ± 0.01	4.30 ± 0.05
22:6 ω-3	1.15 ± 0.03	25.70 ± 0.21
ω-3	6.47 ± 0.14	58.84 ± 0.16
SFA	21.17 ± 0.10	18.52 ± 0.22
MUFA	24.21 ± 0.11	17.22 ± 0.12
PUFA	54.62 ± 0.03	64.26 ± 0.33
EPA+DHA	1.85 ± 0.06	50.79 ± 0.31

Abbreviations: SBO, Soybean Oil; FO, Fish Oil; SFA; n.d., non-detected; SFA, Saturated Fatty acids; MUFA, Monounsaturated Fatty Acids; PUFA, Polyunsaturated Fatty Acids; EPA, Eicosapentaenoic; DHA, Docosahexaenoic.

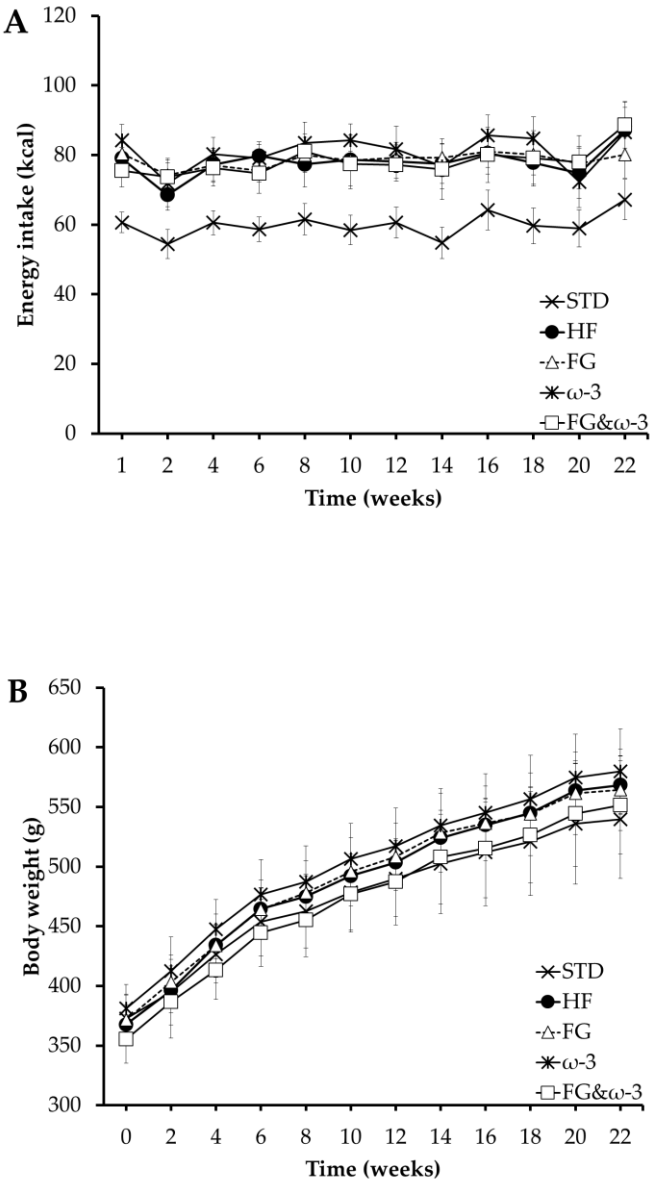

Figure S1. (**A**) Energy intake and (**B**) body weight throughout the study. Values are expressed as mean ± standard deviation, nine rats per group. Abbreviations: STD, Standard group; HF, High-Fat group; FG, Fagomine group; ω-3, ω-3 PUFA group; FG&ω-3, Fagomine and ω-3 PUFA group. Groups fed a HF diet either with supplement or without supplement showed significantly higher energy intake than the STD group (p-value < 0.05). Nevertheless, no significant differences were observed in body weight among the groups.

Table S3. Feed intake, biometric data and insulin resistance biomarkers after 21 weeks of dietary intervention*

	STD	HF	FG	ω-3	FG&ω-3
Intake data					
Feed intake (g/day)	20.3 ± 1.6	16.3 ± 0.9[a]	16.4 ± 1.2[a]	16.8 ± 1.0[a]	15.9 ± 1.2[a]
Energetic intake (kcal/day)	58.9 ± 4.6	76.5 ± 4.3[a]	77.2 ± 5.7[a]	79.0 ± 4.7[a]	74.8 ± 5.7[a]
Biometric data					
Initial body weight (g)	373 ± 20	368 ± 17	370 ± 22	381 ± 20	355 ± 20
Final body weight (g)	540 ± 49	568 ± 24	564 ± 34	580 ± 36	552 ± 41
Body weight gain (g)	167 ± 36	201 ± 29	194 ± 21	199 ± 30	196 ± 24
Perigonadal adipose tissue (g)	8.1 ± 1.7	13.1 ± 3.9[a]	10.8 ± 1.3	13.3 ± 4.4[a]	9.9 ± 2.1
Adiposity index (%)	1.5 ± 0.3	2.3 ± 0.7[a]	1.9 ± 0.3	2.3 ± 0.6[a]	1.8 ± 0.3
Liver (g)	14.0 ± 1.7	14.0 ± 1.1	14.6 ± 1.0	14.1 ± 1.0	14.6 ± 2.3
Hepatosomatic index (%)	2.6 ± 0.1	2.4 ± 0.2	2.6 ± 0.1	2.5 ± 0.1	2.6 ± 0.3
Insulin resistance biomarkers					
OGTT[#] (AUC, mg/mL per 120 min)	10,350 ± 739	10,335 ± 282	10,623 ± 475	10,864 ± 625	10,338 ± 604
Glucose (mmol/L)	3.5 ± 0.4	3.9 ± 0.3[a]	3.9 ± 0.3[a]	3.9 ± 0.3[a]	3.8 ± 0.2
Insulin (mU/L)	16.2 ± 9.4	52.1 ± 23.9[a]	39.3 ± 19.5	42.1 ± 20.9	40.9 ± 16.5

Values are expressed as mean ± standard deviation, nine rats per group. Abbreviations: STD, Standard group; HF, High-Fat group; FG, Fagomine group; ω-3, ω-3 PUFA group; FG&ω-3, Fagomine and ω-3 PUFA group; OGTT, Oral Glucose Tolerance Test; AUC, Area Under the Curve. *These parameters have partially been published in a previous report [1]. [#]OGTT was performed during week 18.

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UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF FISH OIL AND ITS COMBINATION WITH GRAPE SEED POLYPHENOLS OR BUCKWHEAT D-FAGOMINE ON CARDIOMETABOLIC RISK FACTORS AND OXIDATIVE STRESS IN RATS
Bernat Miralles Pérez

5. Summary of Results

SUMMARY OF RESULTS

SUMMARY OF RESULTS

Table 3 describes the abbreviations used for the groups throughout the corresponding articles and the result section of this thesis to facilitate the reading of the results.

Table 3. Group by experiment, diet and supplement.

Diet	Supplement	Abbreviation *
Experiment 1		
Standard diet	Coconut oil	–
Standard diet	Soybean oil	–
Standard diet	Fish oil containing EPA/DHA 1:1	–
Standard diet	Fish oil containing 80% DHA	–
Experiment 2 †		
Standard diet	–	STD
High-fat diet	–	HFHS
High-fat diet	Fish oil containing EPA/DHA 1:1	FO
High-fat diet	Grape seed extract	GSE
High-fat diet	Fish oil containing EPA/DHA 1:1 and GSE	FO + GSE
Experiment 3 †		
Standard diet	–	STD
High-fat diet	–	HF
High-fat diet	D-Fagomine	FG
High-fat diet	Fish oil containing EPA/DHA 1:1	ω-3
High-fat diet	FG and fish oil containing EPA/DHA 1:1	FG&ω-3

* En dash (–) indicates “not applicable”.
† Despite the distinct abbreviations, high-fat diets used in the Experiment 2 and in the Experiment 3 were based on the same type of commercial diet.
Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

The main results of this thesis by experiment are summarized below:

5.1. EXPERIMENT 1. Fish Oil Rich in Docosahexaenoic Acid

The main objective of the Experiment 1 was to evaluate the effects of increasing concentrations of DHA in fish oil on cardiometabolic risk factors and oxidative stress in heathy rats.

The following paragraph summarizes the effects of the supplementations in healthy conditions on biometric data and glucose metabolism to put into context the Experiment 1. These parameters are partially included in **Paper 1** as supplementary materials.

The supplementation with fish oil containing 80% DHA did not affect either feed intake, body weight or perigonadal adipose tissue weight compared to the other three oils. Nevertheless, the supplementation with fish oil containing 80% DHA as well as fish oil containing EPA/DHA 1:1 and coconut oil decreased blood glucose at the end of the study compared to soybean oil, but with no significant changes in oral glucose tolerance (**Paper 1**).

Effect of increasing concentrations of DHA in fish oil on plasma lipid profile in healthy rats.

The supplementation with fish oil containing 80% DHA decreased total fat and total cholesterol in plasma compared to both coconut oil and soybean oil. The decrease in total cholesterol after receiving fish oil containing 80% DHA was mainly achieved by reducing HDL-cholesterol. Fish oil containing 80% DHA provided greater effects on plasma lipids than the obtained with fish oil containing EPA/DHA 1:1 (**Paper 1**).

SUMMARY OF RESULTS

Effects of increasing concentrations of DHA in fish oil on lipids in erythrocytes and in tissues of healthy rats.

The supplementation with fish oil containing 80% DHA decreased total fat in gastrocnemius muscle compared to both coconut oil and soybean oil. Nevertheless, fish oil containing 80% DHA as well as coconut oil increased total fat in the liver compared to fish oil containing EPA/DHA 1:1 (**Paper 1**).

As far as fatty acid composition is concerned, the supplementation with fish oil containing 80% DHA markedly decreased values of ω-6/ω-3 ratio in erythrocytes and in tissues. Fish oil containing 80% increased amount of DHA compared to the other three oils. Furthermore, fish oil containing either 80% DHA or EPA/DHA 1:1 increased EPA compared to both coconut oil and soybean oil, together with a decrease in ARA, especially in erythrocytes and in liver. Fish oil containing 80% also decreased activity of stearoyl CoA desaturase 16 (SCD-16) compared to coconut oil, especially in the liver (**Paper 1**).

Effect of increasing concentrations of DHA in fish oil on inflammation in healthy rats.

The supplementation with fish oil containing either 80% DHA and EPA/DHA 1:1 increased concentration of TNFα in plasma compared to both coconut oil and soybean oil, with no changes in plasma CRP or in presence of portal chronic inflammation in the liver (**Paper 1**).

Effect of increasing concentrations of DHA in fish oil on oxidative stress in healthy rats.

The supplementation with fish oil containing 80% DHA induced the highest increase in quantity of TBARS in blood and in tissues compared to the other three oils. Furthermore, fish oil containing 80% DHA increased abundance of protein carbonyls in plasma and in the liver, especially compared to soybean oil (**Paper 1**).

Simultaneously, fish oil containing 80% DHA enhanced the antioxidant capacity of plasma, as assessed by the ORAC assay, compared to both coconut oil and soybean oil. Furthermore, fish oil containing 80% DHA increased activity of GPx in erythrocytes compared to both soybean oil and fish oil containing EPA/DHA 1:1. Fish oil containing EPA/DHA 1:1 increased the value of GSSG/GSH ratio in plasma compared to both coconut oil and soybean oil, but fish oil containing 80% DHA attenuated the increase (**Paper 1**).

5.2. EXPERIMENT 2. Fish Oil and Grape Seed Extract

The main objective of Experiment 2 was to evaluate the effects of combined supplementation with fish oil containing EPA/DHA 1:1 and GSE on oxidative stress and bioactive lipids in the liver of rats fed an HFHS diet.

Table 4 summarizes the main effects of the supplementations, either individually or in combination, under HFHS diet conditions on biometric data and glucose metabolism compared to STD diet to put into context the Experiment 2. These parameters are included in **Paper 2** as supplementary materials.

Table 4. Brief scheme of the main effects of the supplementations, either individually or in combination, under HF diet conditions on biometric data and glucose metabolism compared to STD diet in Experiment 2 *.

	HFHS	FO	GSE	FO + GSE
Final body weight	↑	↑	↑	↑
PWAT weight	↑	↑	↑	–
Blood glucose	–	–	–	–
Plasma insulin	↑	↑	↑	–

* These parameters are included in Paper 2 as supplementary materials.
Abbreviations: HFHS, high-fat high-sucrose diet; FO, fish oil; GSE, grape seed extract; PWAT, perigonadal white adipose tissue.
En dash (–) indicates no significant differences.

Effect of the combined supplementation with fish oil containing EPA/DHA 1:1 and GSE on oxidative stress in the liver of rats fed an HFHS diet.

The supplementation with FO + GSE induced similar increase in GPx in liver than the induced by HFHS diet without supplementation, but tended to increase the quantity of GSH and then tended to decrease the value of GSSG/GSH ratio. The combination of FO + GSE attenuated the increase in activity of GPx caused by GSE alone (**Paper 2**).

Effects of the combined supplementation with fish oil containing EPA/DHA 1:1 and GSE on bioactive lipids in the liver of rats fed an HFHS diet.

The supplementation with FO + GSE clearly increased abundance of several PUFA-containing DAG species, mainly due to FO. Nevertheless, the combination of FO + GSE increased abundance of several ceramide species either LCFA- or VLCFA-containing ceramides compared to FO alone. Simultaneously, the combination of FO + GSE promoted accumulation of DAGs in liver, whereas GSE alone markedly decreased abundance of DAGs. The accumulation of MUFA-containing DAG and LCFA-containing ceramide species after receiving FO + GSE was similar than the induced by HFHS diet, with no significant changes in the degree of steatosis (**Paper 2**).

5.3. EXPERIMENT 3. Fish Oil and Buckwheat D-Fagomine

The main objective of Experiment 3 was to evaluate the effects of combined supplementation with fish oil containing EPA/DHA 1:1 and FG on oxidative stress and related cardiometabolic risk factor in rats fed an HF diet.

Table 5 summarizes the main effects of the supplementations, either individually or in combination, under HF diet conditions on biometric data and glucose metabolism compared to STD diet to put into context the Experiment 3. These parameters are included in **Paper 3** as supplementary materials.

Table 5. Brief scheme of the main effects of the supplementations, either individually or in combination, under HF diet conditions on biometric data and glucose metabolism compared to STD diet in Experiment 3 *.

	HF	FG	ω-3	FG&ω-3
Final body weight	–	–	–	–
PWAT weight	↑	–	↑	–
Blood glucose	↑	↑	↑	–
Plasma insulin	↑	–	–	–

* These parameters are included in Paper 3 as supplementary materials.
Abbreviations: HF, High-fat diet; FG, D-fagomine; ω-3, omega-3 polyunsaturated acids from fish oil; PWAT, perigonadal white adipose tissue.
En dash (–) indicates no significant differences.

Effect of the combined supplementation with fish oil containing EPA/DHA 1:1 and FG on plasma lipid profile in rats fed an HF diet.

The supplementation with FG&ω-3 attenuated the increase in values of LDL/HDL ratio induced by FG alone. Nevertheless, the combination of FG&ω-3 did not modify the decrease in concentration of total cholesterol in plasma induced by HF diet compared to STD diet. The decrease in total cholesterol under HF diet conditions was mainly achieved via reducing HDL-cholesterol (**Paper 3**).

Effects of the combined supplementation with fish oil containing EPA/DHA 1:1 and FG on hepatic fat accumulation and hepatic function in rats fed an HF diet.

The supplementation with FG&ω-3 did not modify the accumulation of total cholesterol in liver induced by HF diet compared to STD diet. In contrast, individual supplementation with ω-3 from fish oil attenuated the accumulation of total cholesterol induced by the HF diet, and even significantly decreased triacylglycerol compared to STD diet. Interestingly, the combination of FG&ω-3 attenuated the decrease in plasma activity of ALT induced by the HF diet (**Paper 3**).

Effect of the combined supplementation with fish oil containing EPA/DHA 1:1 and FG on inflammation in rats fed an HF diet.

The supplementation with FG&ω-3 decreased concentration of IL-6 in plasma compared to STD diet. The HF diet tended to decrease plasma IL-6 by itself, but the addition of FG&ω-3 to the HF diet significantly potentiated this effect (**Paper 3**).

Effect of the combined supplementation with fish oil containing EPA/DHA 1:1 and FG on oxidative stress in rats fed an HF diet.

HF diet increased quantity of conjugated diene hydroperoxides and protein carbonylation in plasma and in liver. The supplementation with FG&ω-3 attenuated the increase in conjugated diene hydroperoxides in plasma, and even significantly prevented protein carbonylation in liver. Simultaneously, the combination of FG&ω-3 decreased activity of GPx in erythrocytes compared to both the FG alone and the HF diet. Furthermore, the combination of FG&ω-3

increased quantity of GSH in erythrocytes compared to both individual supplementations, together with the lowest quantity of GSSG among the groups. In perigonadal adipose tissue, the combination of FG&ω-3 also attenuated the increase in value of GSSG/GSH ratio induced by the FG alone. The combination of FG&ω-3 under HF diet conditions even significantly decreased several biomarkers of oxidative stress in erythrocytes (GSSG, GPx and GR) and in liver (GSSG and SOD) compared to STD diet. The ω-3 from fish oil were responsible for most of these effects, except for the beneficial changes in GSH and GSSG in erythrocytes that were clearly potentiated by the combination of FG&ω-3 (**Paper 3**).

6. General Discussion

GENERAL DISCUSSION

GENERAL DISCUSSION

The aim of the present thesis was to explore the influence of fish oil and its combination with GSE or FG on cardiometabolic risk factors and oxidative stress in rats. This thesis was divided into three parts. The first part showed that increasing concentrations of DHA in fish oil beneficially affects several cardiometabolic risk factors but increases oxidative damage to biomolecules in healthy rats, compared to soybean oil and coconut oil (**Paper 1**). Soybean oil and coconut oil were used as control groups because they are rich in linoleic acid (ω-6 PUFA, 18:2) and in lauric acid (SFA, 12:0), respectively. The second part showed that the combined supplementation with fish oil containing EPA/DHA 1:1 and GSE tends to attenuate oxidative stress, and significantly modulate abundances of several bioactive lipid species in the liver of rats fed an HFHS diet (**Paper 2**). The final part of this thesis showed that the combined supplementation with fish oil containing EPA/DHA 1:1 and FG exerts beneficial influence on oxidative stress and on some related cardiometabolic risk factors in rats fed an HF diet (**Paper 3**). Male Sprague–Dawley rats were used in Experiments 1 and 3, whereas female Wistar–Kyoto rats were used in Experiment 2. As far as strain is concerned, previous studies have shown that Wistar–Kyoto rats are more prone to accumulate fat in adipose tissue and develop faster insulin resistance than Sprague–Dawley rats [135,136]. Sprague–Dawley rats seems to be the appropriate laboratory model to achieve early stages in the onset of insulin resistance under HF diet conditions, which was the background desired to test the effects of FG. As far as gender is concerned, numerous studies have assessed the effects of polyphenols on metabolic health in male rats, but few studies have used female rats due to hormonal changes. For these reasons, we

decided to use male Sprague–Dawley rats to test the effects of fish oil rich in DHA and those of FG, and to use female Wistar–Kyoto rats to test the GSE.

Effects of fish oil and its combination with GSE or FG on biometric data and glucose metabolism in rats

The individual supplementation with fish oil either containing 80% DHA or EPA/DHA 1:1 exerts no influence on weight of perigonadal white adipose tissue of the healthy rats (**Paper 1**) or that of the rats fed an HF diet (**Papers 2** and **3**). Although no differences were found in blood glucose at weeks 4 and 8, fish oil either containing 80% DHA or EPA/DHA 1:1 as well as coconut oil decreases blood glucose in healthy rats after about 10 weeks of dietary intervention compared to soybean oil (**Paper 1**). This fact proves that length of time is an important factor for assessing the potential benefits of fish oil supplements on health, as previously suggested [137]. The results on adipose tissue and glucose homeostasis agree with our previous study [138] in healthy female Wistar rats supplemented with a lower dose of fish oil containing EPA/DHA 1:1 for 13 weeks. Unlike the beneficial effect on glucose homeostasis in a healthy framework, when provided individually, fish oil containing EPA/DHA 1:1 did not attenuate the increase in blood glucose (**Paper 3**) or the increase in plasma insulin under HF diet conditions (**Papers 2**). Previous studies [139–141] have shown that supplementation with fish oil prevents the development of insulin resistance in rodents under HF diet conditions. Nevertheless, distinct stages in the development of insulin resistance among the studies may explain why these parameters were unchanged under our experimental conditions. In this respect, our HF diet induced early stages in the onset of insulin resistance (**Papers 1** and **2**), which are characterized by an increase in secretion of insulin from pancreatic

β-cells as a compensatory mechanism for maintaining glucose homeostasis but with no changes in oral glucose tolerance [67]. Other studies [94,139,140] have shown that supplementation with fish oil enhances insulin signaling in target tissues and maintains glucose homeostasis by increasing PPARs and adiponectin with a concomitant decrease in leptin. We previously reported [142] that the fish oil containing EPA/DHA 1:1 tends to increase circulating concentration of adiponectin along with a significant decrease in leptin under HF diet conditions, when compared to soybean oil. The combined supplementation with fish oil containing EPA/DHA 1:1 with GSE significantly enhanced the effect of fish oil on circulating adiponectin and increased other important hormones such as glucagon and ghrelin [142]. Interestingly, the combination of fish oil containing EPA/DHA 1:1 and GSE or FG effectively delays both the increase in perigonadal adipose tissue weight and the onset of insulin resistance (**Papers 2** and **3**). These facts evidence that the combination of bioactive compounds potentially has considerable benefits for prevention of cardiometabolic disorders.

Effect of fish oil and its combination with GSE or FG on plasma lipid profile in rats

The individual supplementation with fish oil containing either 80% DHA or EPA/DHA 1:1 decreased total cholesterol mainly from HDL particles in plasma of the healthy rats (**Paper 1 and Paper A4**) and that of the rats fed an HF diet (**Paper A2**; also in [142]). Fish oil containing 80% DHA had greater effect on plasma cholesterol in healthy rats than the induced by fish oil containing EPA/DHA 1:1 (**Paper 1**). Other studies [143] have shown in humans that DHA is a more potent agent for lowering plasma lipids than EPA. Nevertheless, our previous work [138,144,145] has shown in healthy female Wistar rats no

differences among distinct EPA/DHA ratios tested (2:1, 1:1 or 1:2). The discrepancy in plasma lipids between the two studies on healthy rats may be, at least in part, attributed to distinct doses used. We provided higher doses of fish oils in **Paper 1** than those previously used [138,144,145] with the aim of achieving a high incorporation of DHA into cell membranes and tissues. The ω-3 PUFA have beneficial influence on lipids in plasma and in tissues by increasing the fatty acid β-oxidation, the cholesterol uptake from plasma, the bile acid synthesis and its extraction as well as by inhibiting lipogenesis [92–94]. In particular, previous studies [146,147] have shown that a supplementation with fish oil decreases plasma cholesterol by stimulating of reverse cholesterol transport by means of an increase in scavenger receptor class B-1 expression in liver.

Early stages in the onset of insulin resistance induced by HF diet were accompanied by no aberrant lipid profile in plasma (**Paper 3**). The HF diet even promoted a decrease in total cholesterol compared to STD diet mainly by reducing HDL-cholesterol, with no changes in plasma triacylglycerol (**Papers 3** and **A2**; also in [142]). The HF diet may lead to adaptive responses such as high fatty acid desaturation and high β-oxidation [105,148,149], which may explain why the plasma lipids remained unchanged or even decreased in early stages of insulin resistance. The supplementation with fish oil containing EPA/DHA 1:1, either individually or in combination with GSE, significantly decreases total cholesterol in rats fed an HF diet compared to the other rats not supplemented with fish oil (**Paper A2**; also in [142]). Furthermore, the combination of fish oil containing EPA/DHA 1:1 and GSE attenuated the increase in plasma triacylglycerol induced by GSE alone (**Papers 2** and **A2**; also in [142]). In agreement with our observations, other authors [105] have shown that fish oil

decreases plasma lipids under HF diet conditions. Nevertheless, the supplementation with fish oil containing EPA/DHA 1:1, either individually or in combination with FG, did not modify concentration of plasma cholesterol compared to HF diet without supplementation (**Paper 3**). The use of distinct rat model and distinct doses may explain the discrepancy in the effect of fish oil on plasma lipid profile between our two experiments in rats fed an HF diet (**Papers 2 and 3**). We provided higher doses of fish oil to the rats of the **Paper 2** than those of the **Paper 3**. The supplementation with FG alone increased values of LDL/HDL ratio but the combination of fish oil containing EPA/DHA 1:1 and FG attenuated the increase (**Paper 3**).

Effects of fish oil and its combination with GSE or FG on ectopic fat, fatty acids, and bioactive lipids in rats

The individual supplementation with fish oil containing either 80% DHA or EPA/DHA 1:1 also exerted influence on the amount of lipids in tissues of the healthy rats (**Paper 1**) and in those of the rats fed an HF diet (**Papers 2** and **3**). Fish oil containing 80% DHA preferentially decreased total fat in gastrocnemius muscle of healthy rats, whereas fish oil containing EPA/DHA 1:1 decreased total fat in liver, but with no changes in degree of steatosis or in amount of triacylglycerol and total cholesterol (**Paper 1**). A previous study [150] has shown that a supplementation with fish oil rich in DHA enhances fatty acid β-oxidation in skeletal muscle of mice resulting in better insulin signaling and lower plasma glucose. Conversely, other authors [106] have shown that a fish oil rich in DHA increases the amount of triacylglycerol in liver of rabbits in a dose-dependent manner probably by an increase in VLDL and LDL receptors and without affecting expression of genes related to β-oxidation. Taken together, the findings

suggested that increasing concentrations of DHA preferentially activates β-oxidation of fatty acids in muscle over that in the liver.

The HF diet induced no major changes in accumulation of lipids in liver compared to STD diet (**Papers 2, 3** and **A3**), showing no significant changes in the degree of steatosis as assessed by histological examination (**Papers 2** and **A3**). These results on liver steatosis agree with our previous studies in rats fed an HF diet [126], suggesting that the amount of fat provided by our HF diet is too low for induction of steatosis in rats. Despite that, the HF diet induced a slight increase in total cholesterol in liver, which was attenuated by the individual supplementation with fish oil containing EPA/DHA 1:1 (**Paper 3**). In agreement with our observations, other authors [151] have shown that a supplementation with fish oil decreases hepatic fat, either triacylglycerol or cholesterol, compared to an HF diet without the supplement. Nevertheless, in our study, the combination of fish oil containing EPA/DHA 1:1 and FG showed no influence on total cholesterol in the liver (**Paper 3**) and even increased degree of steatosis compared to the HF diet (**Paper A3**).

The individual supplementation with fish oil either containing 80% DHA or EPA/DHA 1:1 clearly promote incorporation of ω-3 PUFA in erythrocytes and in tissues of the healthy rats (**Paper 1**) and in those of the rats fed an HF diet (**Paper 2**). Interestingly, fish oil containing 80% DHA increases the amount of EPA probably due to modulation of elongases and deaturases to prevent further accumulation of DHA. Our results are consistent with the observations of other studies in humans on high intake of oily fish [152] or DHA supplements [107]. The increase in ω-3 PUFA in erythrocytes and in tissues after receiving fish oil alone

promoted a decrease in total abundance of ω-6 PUFA (**Paper 1**) and ceramide species (**Paper 2**).

The supplementation with fish oil containing EPA/DHA 1:1 alone increased PUFA-containing DAG (probably ω-3) and decreased ceramides in liver, whereas GSE decreased either DAGs or ceramides (**Paper 2**). These observations agree with those from the histological examination (**Paper 2**) and those from other authors [153–157]. Unlike individual supplementations, the combination of fish oil containing EPA/DHA 1:1 and GSE induced similar accumulation of MUFA-containing DAGs and LCFA-containing ceramides in liver than the induced by the HF diet without supplement (**Paper 2**). Intracellular accumulation of DAG and ceramide species can lead to insulin resistance via the activation of certain protein kinase C isoforms and then inhibiting insulin signaling cascade [75]. Despite that, the combined supplementation with FO and GSE effectively delays the onset of insulin resistance under HF diet conditions. Because the activation of protein kinase C by bioactive lipids may depend on the localization of lipids in the cell, the accumulation of them in lipid droplets rather than in cytosol or in plasma membrane could prevent the activation of protein kinase C [158]. Therefore, these results suggested that the combination of fish oil containing EPA/DHA 1:1 and GSE promotes storage of fat in lipid droplets, and subsequently maintains insulin signaling under HFHS diet conditions.

Effects of fish oil and its combination with GSE or FG on inflammation in rats

The supplementation with fish oil containing either 80% DHA and EPA/DHA 1:1 increased concentration of TNFα in plasma of healthy rats compared to both coconut oil and soybean oil (**Paper 1**). When we assessed another important marker of inflammation such as CRP, we observed that the supplementations with two types of fish oil induced no changes in concentration of plasma CRP (**Paper 1**). The decrease in ω-6/ω-3 ratio in tissues after receiving the fish oils may explain the increase in TNFα observed (**Paper 1**). In this respect, oxidation of ARA generates prostaglandin E_2 that in turn inhibits production of TNFα [159]. We (**Paper A5**) have recently reported that the supplementation with fish oil containing EPA/DHA 1:1 decreases prostaglandin E_2 in perigonadal adipose tissue of healthy rats, when compared to soybean oil. The increase in TNFα observed is consistent with the observations of other authors in resident peritoneal macrophages [160] and in adipocytes [161]. Nevertheless, in the liver, fish oil containing EPA/DHA 1:1 decreased amount of both the TNFα and the CRP in liver of heathy rats compared to soybean oil (**Paper A2**), suggesting that other tissues such as adipose tissue may be especially relevant on the increase in plasma TNFα observed in **Paper 1**.

The HF diet did not affect concentration of IL-6 in plasma (**Paper 3**). Because significant changes in markers of inflammation may appear once obesity is fully established [73,162], the results suggest that our rat models reached a moderate degree of obesity under HF diet conditions (**Papers 2**, **3 and A3**). Interestingly, unlike individual supplementations, the combination of fish oil containing EPA/DHA 1:1 and FG under HF diet conditions decreased plasma IL-6 compared to STD diet (**Paper 3**). Previous studies have shown that two types of

supplements can exert beneficial influence on inflammation in rats fed an HF diet. On one hand, FG prevents dysbiosis of gut microbiota induced by HF diet (**Paper A3** and [126]), maintaining intestinal barrier integrity. On the other hand, the ω-3 PUFA from fish oil generates lipids with anti-inflammatory and pro-resolving properties and inhibits pro-inflammatory NF-κB signaling pathway [98,99,163]. The discrepancies of the effects of individual supplementations on inflammation between **Paper 3** and previous studies may be attributed to distinct backgrounds. In this respect, the STD diet used in **Paper 3** provides higher amount of ω-6 PUFA than the provided by the HF diet. Furthermore, soybean oil was used to compensate for both the stress of probing and the excess of energy provided by fish oil. A previous report on the same cohort of rats [164] evidenced higher incorporation of ω-6 PUFA in liver samples of rats fed the STD diet than in those of the rats fed the HF diet. Once again, oxidation of ARA produces pro-inflammatory lipid mediators including prostaglandin E_2 that in turn, in this case, can stimulate secretion of IL-6 [159]. Therefore, when provided together under these experimental conditions, fish oil and FG can potentially have considerable lowering effect on plasma IL-6.

Locally in the liver, the HF diet induced a significant increase in lobular inflammation as assessed by histological examination in **Paper A3** but not in portal chronic inflammation in **Paper 2**. Other authors [162] have also shown a slight increase in abundance of IL-6 in liver but with no changes in circulating markers of inflammation in rats fed a high-fat high-fructose diet. The combined supplementation with fish oil containing EPA/DHA 1:1, either individually or in combination with FG, attenuated the lobular inflammation induced by the HF diet (**Paper A3**). Furthermore, the supplementation with fish oil containing EPA/DHA

1:1, either individually or in combination with GSE, decreased the amount of TNFα in liver compared to the HF diet without supplement (**Paper A2**).

Effects of fish oil and its combination with GSE or FG on oxidative stress in rats

The individual supplementation with fish oil containing either 80% DHA or EPA/DHA 1:1 enhanced endogenous antioxidants in healthy rats (**Paper 1**) and in rats fed an HF diet (**Paper 3**). Nevertheless, the supplementation with fish oil containing 80% DHA markedly promoted lipid peroxidation in healthy rats, which in turn induced protein carbonylation in plasma and in liver (**Paper 1**). These observations are consistent with those observed in other studies [106,107] in which supplementation with DHA increases TBARS, 4-HHE and 4-HHE-protein adducts in a dose-dependent manner. Simultaneously, fish oil containing 80% DHA induced the highest NEAC of plasma—i.e., in ORAC but not in FRAP (**Paper 1**). The results of NEAC assays may differ among them because they measure distinct antioxidant activities [12]. In this respect, the ORAC assay mainly measures antioxidant activity of thiols (i.e., albumin and GSH), whereas the FRAP assay mainly measures action of uric acid [165]. Because ORAC assay mainly measures antioxidant activity of albumin [165], our results suggested that oxidation of certain cysteine residues in albumin may enhance its antioxidant activity in a healthy context [25], and then preventing oxidative damage to other circulating proteins including LDL particles. Fish oil containing 80% DHA also increased protein carbonylation in liver (**Paper 1**). We have previously reported (**Paper A2**) that the supplementation with a lower dose of fish oil containing EPA/DHA 1:1 mainly increases carbonylation of actin in liver. Like albumin, actin may act as a scavenger of end products of lipid peroxidation without undergoing significant functional impairment [166]. Within this context, carbonylation of albumin and actin after receiving fish oil containing 80% may be an important

antioxidant mechanism in a healthy framework to protect against high lipid peroxidation.

HF diet increased quantity of conjugated diene hydroperoxides **(Paper 3)**, abundance of protein carbonyls (**Paper 3**) and activity of GPx (**Paper 2**) compared to STD diet, but not significantly affected GSSG/GSH ratio (**Papers 2** and **3**). The increase in GPx was probably a compensatory antioxidant mechanism mediated via activation of Nrf2 signaling pathway [36] to protect against high production of ROS derived from mitochondrial β-oxidation of fatty acids under HF diet conditions [148,149]. Interestingly, **Paper 2** and **Paper 3** differ in the effect of HF diet on endogenous antioxidants in liver. On one hand, in **Paper 2**, the HF diet increased GPx and tended to increase SOD compared to STD diet. On the other hand, in **Paper 3**, the HF diet significantly decreased SOD compared to STD diet, but with no significant changes in GPx. Distinct gender may explain, at least in part, why endogenous antioxidant response is different between the two experiments [167]. In this respect, female rats (**Paper 2**) have better redox status than their male counterparts (**Paper 3**) probably due to estrogens [168].

The combined supplementation with fish oil containing EPA/DHA 1:1 and GSE under HF diet conditions attenuated the increase in activity of GPx in the liver caused by GSE alone (**Paper 2**). Enhanced fatty acid β-oxidation and the resulting increase in ROS after receiving GSE alone may explain the increase in GPx. These observations agree with low abundances of DAGs and ceramides recorded in those rats (**Paper 2**). Furthermore, other authors have shown that GSE activates Nrf2 [118] and enhances expression of antioxidant enzymes [11,118]. On the contrary, fish oil containing EPA/DHA 1:1 alone did no affected

GPx (**Paper 2**). Because ω-3 PUFA through their oxidized metabolites are also capable to activate the Nrf2 signaling pathway [101] that may result in enhanced endogenous antioxidants [101,103], the present results suggest that GSE is a more potent activator of Nrf2 than fish oil under our experimental conditions (**Paper 2**). On the other hand, when compared to FG, fish oil containing EPA/DHA 1:1 clearly exerts greater beneficial influence on oxidative stress than FG (**Paper 3**). The beneficial effects of fish oil on oxidative stress are similar than those observed by other authors in rodents on an HF diet [151]. Even so, the combination of fish oil containing EPA/DHA 1:1 and FG significantly decreased activities of several antioxidant enzymes and induced the lowest values of GSSG/GSH ratio, suggesting potential benefits of the combination on oxidative stress (**Paper 3**).

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EFFECTS OF FISH OIL AND ITS COMBINATION WITH GRAPE SEED POLYPHENOLS OR BUCKWHEAT D-FAGOMINE ON CARDIOMETABOLIC RISK FACTORS AND OXIDATIVE STRESS IN RATS
Bernat Miralles Pérez

7. Conclusions

CONCLUSIONS

7.1. CONCLUSIONS BY EXPERIMENT

7.1.1. EXPERIMENT 1. Fish Oil Rich in Docosahexaenoic Acid

- Increasing concentrations of DHA in fish oil have no effects on feed intake or biometric data in healthy rats compared to coconut oil, soybean oil, and fish oil containing EPA/DHA 1:1.
- Increasing concentrations of DHA in fish oil decreases fasting blood glucose in healthy rats compared to soybean oil, but with no differences compared to coconut oil or fish oil containing EPA/DHA 1:1.
- Increasing concentrations of DHA in fish oil decreases total fat and total cholesterol in plasma mainly from HDL in healthy rats compared to both coconut oil and soybean oil. The effect on plasma lipid profile provided by high concentrations of DHA in fish oil are greater than the obtained with fish oil containing EPA/DHA 1:1.
- Increasing concentrations of DHA in fish oil decreases total fat in gastrocnemius muscle in healthy rats compared to both coconut oil and soybean oil, but increases total fat in the liver compared to fish oil containing EPA/DHA 1:1. Simultaneously, increasing concentrations of DHA in fish oil promotes an increase in DHA and EPA along with a decrease in ARA in erythrocytes and in tissues.
- Increasing concentrations of DHA in fish oil slightly increases plasma TNFα in healthy rats compared to both coconut oil and soybean oil, without affecting plasma CRP or infiltration of inflammatory cells in the liver. The increase in TNFα in plasma may be probably due to low amounts of prostaglandin E_2 derived from ARA after receiving two types of fish oil, when compared to both coconut oil and soybean oil.

- Increasing concentrations of DHA in fish oil promotes lipid peroxidation in healthy rats compared to the other three oils, increasing protein carbonylation of plasma albumin and probably liver actin that in turn may be related to an enhanced antioxidant response in a healthy context.

7.1.2. EXPERIMENT 2. Fish Oil and Grape Seed Extract

- The combined supplementation with fish oil containing EPA/DHA 1:1 and GSE attenuated the increase in perigonadal adipose tissue weight and in concentration of plasma insulin induced by HF diet, showing greater benefits than individual supplementations.
- The combined supplementation with fish oil containing EPA/DHA 1:1 and GSE tends to decrease oxidative stress (GSSG/GSH ratio) in the liver of rats fed an HFHS diet. Nevertheless, the combination of fish oil containing EPA/DHA 1:1 and GSE attenuates the increase in hepatic antioxidant response induced by GSE alone.
- The combined supplementation with fish oil containing EPA/DHA 1:1 and GSE not only increases abundance of several MUFA- and PUFA-containing DAG species compared to GSE alone, but also increases abundance of several LCFA- and VLCFA-containing ceramide species compared to FO alone. Unlike individual supplementations under HFHS diet conditions, the combination of fish oil containing EPA/DHA 1:1 and GSE has no major effects on abundance of bioactive lipids compared to the HFHS diet, except for those of PUFA-containing DAG species. Because the combination of fish oil containing EPA/DHA 1:1 and GSE effectively attenuates the onset of insulin resistance induced by the HFHS diet, these findings suggest that the combined supplementation may promote accumulation of fat in lipid droplets.

7.1.3. EXPERIMENT 3. Fish Oil and Buckwheat D-Fagomine

- The combined supplementation with fish oil containing EPA/DHA 1:1 and FG attenuated the increase in perigonadal adipose tissue weight as well as in concentrations of blood glucose and plasma insulin induced by HF diet, showing greater benefits than individual supplementations.
- The combined supplementation with fish oil containing EPA/DHA 1:1 and FG has no effect on plasma lipid profile in rats fed an HF diet but attenuates the increase in LDL/HDL induced by FG alone.
- The combined supplementation with fish oil containing EPA/DHA 1:1 and FG has no effect on hepatic fat accumulation in rats fed an HF diet but attenuates the decrease in activity of plasma ALT induced by the HF diet. This last fact seems to be related to a lower gain of adipose tissue weight after receiving the combined supplementation under HF feeding rather than to any change in hepatic function.
- The combined supplementation with fish oil containing EPA/DHA 1:1 and FG under HF feeding decreases plasma IL-6 compared to STD diet. This fact may be explained by differences in fatty acid composition between the HF diet and the STD diet, in addition to the use of fish oil instead of soybean oil to compensate for both the stress of probing and the excess of energy.
- The combined supplementation with fish oil containing EPA/DHA 1:1 and FG partially attenuates oxidative damage to lipids and proteins induced by HF diet. Furthermore, the combination of fish oil containing EPA/DHA 1:1 and FG enhances glutathione status and decreases antioxidant enzyme activities, suggesting lower oxidative stress. These benefits are mainly modulated by ω-3 PUFA, even so potentiated in erythrocytes by the combination.

7.2. GENERAL CONCLUSIONS

The findings of this thesis suggest that the supplementation with ω-3 PUFA from fish oil beneficially affects several cardiometabolic risk factors, especially modulating lipids such as ω-6 PUFA, ceramides and cholesterol, in healthy rats and in rats fed an HF diet. In a healthy context, increasing concentrations of DHA in fish oil provides greater benefits than the obtained with fish oil containing EPA/DHA 1:1 but markedly promotes oxidative damage to lipids and proteins, with a concomitant increase in antioxidant response. Under HF feeding conditions, fish oil containing EPA/DHA 1:1 is the main responsible for the beneficial effects on oxidative damage to biomolecules observed, when compared to FG. In contrast, GSE seems to be a more potent activator of the endogenous antioxidant response than fish oil containing EPA/DHA 1:1. Individual supplementations are not as effective as combined supplementations to prevent the onset of insulin resistance under fat overload conditions. The combination of bioactive compounds can result in greater benefits for prevention of cardiometabolic disorders than providing them individually by acting on distinct pathways, such us oxidative stress, inflammation and abundance of bioactive lipids, and even by cooperating on them. Therefore, the increase in dietary intake of ω-3 PUFA from fish oil supplements containing EPA/DHA 1:1, alone or combined with GSE or FG, may be a useful nutritional strategy to protect against the mechanisms underlying the onset of cardiometabolic disorders in healthy individuals and in individuals at high risk of disease.

8. References

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UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF FISH OIL AND ITS COMBINATION WITH GRAPE SEED POLYPHENOLS OR BUCKWHEAT D-FAGOMINE ON CARDIOMETABOLIC RISK FACTORS AND OXIDATIVE STRESS IN RATS
Bernat Miralles Pérez

9. Appendix

APPENDIX

9.1. OTHER PUBLICATIONS

9.1.1. PAPER A1. The Buckwheat Iminosugar D-Fagomine Attenuates Sucrose Induced Steatosis and Hypertension in Rats

Type: Research article

Manuscript status: Published, October 28, 2019

Title: The Buckwheat Iminosugar D-Fagomine Attenuates Sucrose-Induced Steatosis and Hypertension in Rats

Journal (Publisher): Molecular Nutrition & Food Research (Wiley)

Impact factor (JCR 2019): 5.309

Category and classification: Food Science & Technology 8/139 (Q1)

Authors: Sara Ramos-Romero, Mercè Hereu, Lidia Atienza, Susana Amézqueta, Josefina Casas, Silvia Muñoz, Isabel Medina, Bernat Miralles-Pérez, Marta Romeu and Josep L. Torres

Citation: Ramos-Romero S, Hereu M, Atienza L, Amézqueta S, Casas J, Muñoz S, Medina I, Miralles-Pérez B, Romeu M, Torres JL. The Buckwheat Iminosugar D-Fagomine Attenuates Sucrose-Induced Steatosis and Hypertension in Rats. Mol Nutr Food Res. 2020;64:e1900564.
doi: 10.1002/mnfr.201900564

9.1.2. PAPER A2. Modulation of the Liver Protein Carbonylome by the Combined Effect of Marine Omega-3 PUFAs and Grape Polyphenols Supplementation in Rats Fed an Obesogenic High Fat and High Sucrose Diet

Type: Research article

Manuscript status: Published, December 30, 2019

Title: Modulation of the Liver Protein Carbonylome by the Combined Effect of Marine Omega-3 PUFAs and Grape Polyphenols Supplementation in Rats Fed an Obesogenic High Fat and High Sucrose Diet

Journal (Publisher): Marine Drugs (MDPI)

Impact factor (JCR 2019): 4.073

Category and classification: Chemistry, Medicinal 16/61 (Q2)

Authors: Lucía Méndez, Silvia Muñoz, Bernat Miralles-Pérez, Maria Rosa Nogués, Sara Ramos-Romero, Josep L.Torres and Isabel Medina

Citation: Méndez L, Muñoz S, Miralles-Pérez B, Nogués MR, Ramos-Romero S, Torres JL, Medina I. Modulation of the Liver Protein Carbonylome by the Combined Effect of Marine Omega-3 PUFAs and Grape Polyphenols Supplementation in Rats Fed an Obesogenic High Fat and High Sucrose Diet. Mar Drugs. 2019;18:34.
doi: 10.3390/md18010034

9.1.3. PAPER A3. Effects of combined D-fagomine and omega-3 PUFAs on gut microbiota subpopulations and diabetes risk factors in rats fed a high-fat diet

Type: Research article

Manuscript status: Published, November 12, 2019

Title: Effects of combined D-fagomine and omega-3 PUFAs on gut microbiota subpopulations and diabetes risk factors in rats fed a high-fat diet

Journal (publisher): Scientific Reports (Nature)

Impact factor (JCR 2019): 3.998

Category and classification: Multidisciplinary Sciences 17/71 (Q1)

Authors: Mercè Hereu, Sara Ramos-Romero, Cristina Busquets, Lidia Atienza, Susana Amézqueta, Bernat Miralles-Pérez, Maria Rosa Nogués, Lucía Méndez, Isabel Medina and Josep L. Torres

Citation: Hereu M, Ramos-Romero S, Busquets C, Atienza L, Amézqueta S, Miralles-Pérez B, Nogués MR, Méndez L, Medina I, Torres JL. Effects of combined D-fagomine and omega-3 PUFAs on gut microbiota subpopulations and diabetes risk factors in rats fed a high-fat diet. Sci Rep. 2019;9:16628.
doi: 10.1038/s41598-019-52678-5

9.1.4. PAPER A4. Combined Buckwheat D-Fagomine and Fish Omega-3 PUFAs Stabilize the Populations of Gut Prevotella and Bacteroides While Reducing Weight Gain in Rats

Type: Research article

Manuscript status: Published, October 31, 2019

Title: Combined Buckwheat D-Fagomine and Fish Omega-3 PUFAs Stabilize the Populations of Gut Prevotella and Bacteroides While Reducing Weight Gain in Rats

Journal (Publisher): Nutrients (MDPI)

Impact factor (JCR 2019): 4.546

Category and classification: Nutrition & Dietetics 17/89 (Q1)

Authors: Mercè Hereu, Sara Ramos-Romero, Roser Marín-Valls, Susana Amézqueta, Bernat Miralles-Pérez, Marta Romeu, Lucía Méndez, Isabel Medina and Josep L. Torres

Citation: Hereu M, Ramos-Romero S, Marín-Valls R, Amézqueta S, Miralles-Pérez B, Romeu M, Méndez L, Medina I, Torres JL. Combined Buckwheat D-Fagomine and Fish Omega-3 PUFAs Stabilize the Populations of Gut Prevotella and Bacteroides While Reducing Weight Gain in Rats. Nutrients. 2019;11:2606. doi: 10.3390/nu11112606

9.1.5. PAPER A5. Fish Oil Improves Pathway-Oriented Profiling of Lipid Mediators for Maintaining Metabolic Homeostasis in Adipose Tissue of Prediabetic Rats

Type: Research article

Manuscript status: Published, April 21, 2021

Title: Fish Oil Improves Pathway-Oriented Profiling of Lipid Mediators for Maintaining Metabolic Homeostasis in Adipose Tissue of Prediabetic Rats

Journal (Publisher): Frontiers in Immunology (Frontiers)

Impact factor (JCR 2019): 5.085

Category and classification: Immunology 39/159 (Q1)

Authors: Gabriel Dasilva, Salomé Lois, Lucía Méndez, Bernat Miralles-Pérez, Marta Romeu, Sara Ramos-Romero, Josep L. Torres and Isabel Medina

Citation: Dasilva G, Lois S, Méndez L, Miralles-Pérez B, Romeu M, Ramos-Romero S, Torres JL, Medina I. Fish Oil Improves Pathway-Oriented Profiling of Lipid Mediators for Maintaining Metabolic Homeostasis in Adipose Tissue of Prediabetic Rats. Front Immunol. 2021;12:608875.
doi: 10.3389/fimmu.2021.608875.

9.2. ORAL COMMUNICATIONS TO CONFERENCES

- Miralles-Pérez B, Nogués MR, Sánchez-Martos V, Taltavull N, Hereu M, Ramos-Romero S, et al. Effects of D-fagomine and omega-3 combination on hepatic oxidative stress in high-fat high-sucrose diet-fed rats [Presentation]. XII Spanish Group of Free Radical Research (GEIRLI) Meeting. Barcelona, Spain. July 4, 2019

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