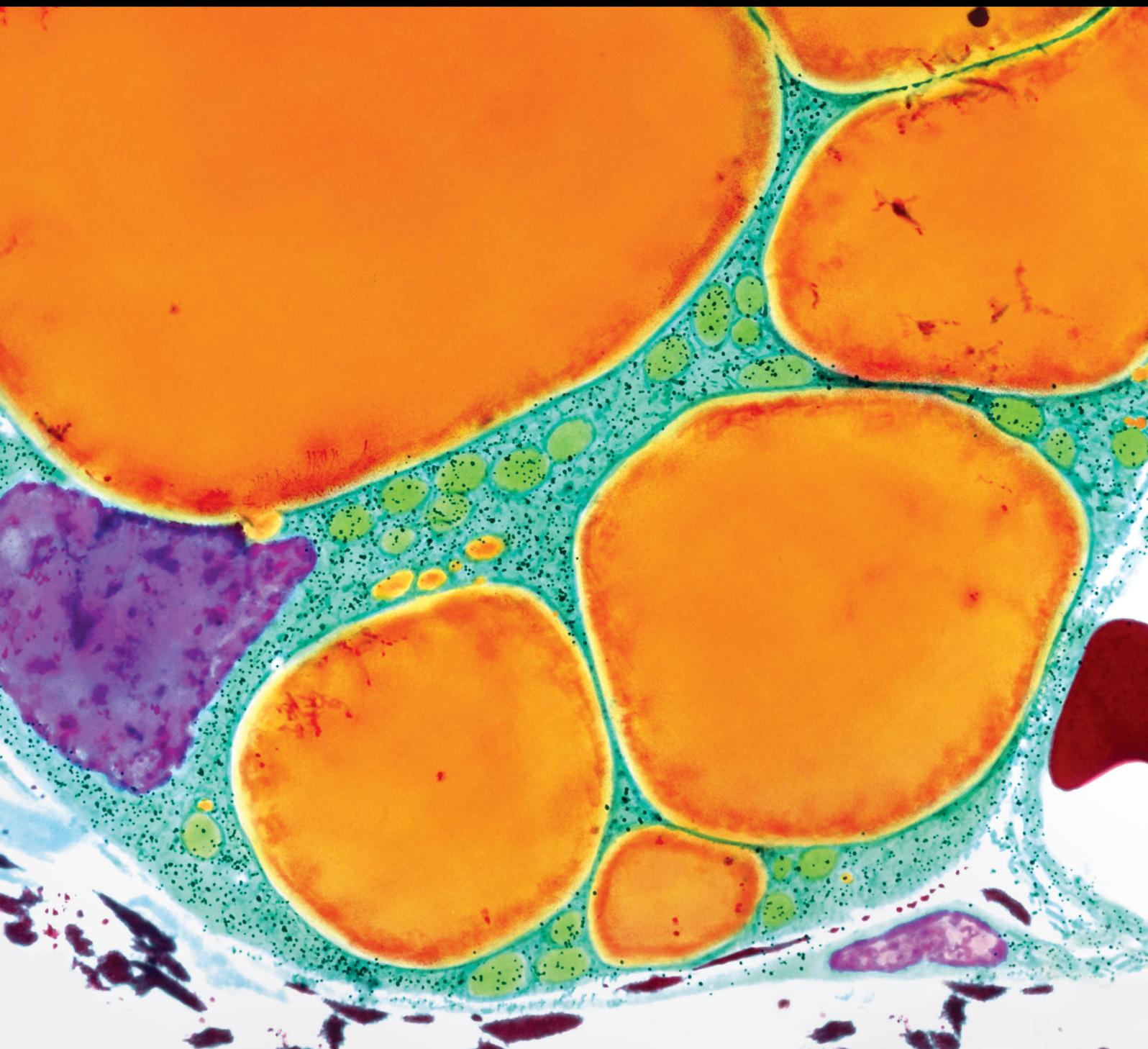


# Dietary Lipids in Health and Disease

Lead Guest Editor: Kamal A. Amin

Guest Editors: Mahdi Garelnabi, Abdelgadir M. Homeida, Reda El Mazoudy,  
and Khalid S. Hashim





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Journal of Lipids

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## Editorial

# Dietary Lipids in Health and Disease

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Lipids are important cellular and extracellular molecules. They are critical for cell structure, function, and energy, as well as organs and body insulation and protection. In addition, lipids metabolites are extremely essential for a wide range of cellular communication and metabolism. However, defective lipids metabolism is well known to modulate a wide range of chronic diseases such as cardiovascular diseases and cancer and several other genetically defective lipids pathways with severe health implications.

Major lipids in human health and diseases may broadly be classified as saturated and unsaturated fatty acids, sterols, phospholipids, sphingosine derivatives, and various other lipids metabolites such as eicosanoids

The Genome Wide Association Studies (GWAS) helped identify several genetically defective lipids pathways and linked them to causes of morbidity and mortality around the globe. This GWAS emerging technology has unraveled many metabolic defects associated with dietary lipids and causes of many health conditions such as obesity, cardiovascular neurodegenerative defects, and cancer. GWAS studies have demonstrated that many of the lipids disorders mechanisms are associated primarily with oxidative stress and inflammation. It is unclear how the environmental modulators and lifestyle are linked to these disorders, which prompt further investigations to determine the initial causes and possible intervention approaches.

This special issue covers some original research articles and reviews that seek to provide insight into the role of lipids in health and disease highlighting some

critical links to lipoprotein metabolism and atherosclerosis.

The review by H. Takeuchi and M. Sugano described the complex linkages of industrial TFAs to cardiovascular which concluded that the relationship between dietary industrial TFAs and concentration of plasma cholesterol should be evaluated from the viewpoint of dietary patterns rather than TFAs alone. On the other hand, E. Derbyshire has demonstrated the role of omega-3/6 fatty acids in the treatment and management of ADHD suggesting that omega-3/6 fatty acids offer great promise as a suitable therapy for this childhood condition. However, F. Drobnic et al. assessed the omega-3 index response, in RBC, to supplemental EPA + DHA intake in the form of high purity and stable composition gums in elite summer athletes and concluded that supplementation of omega-3 FA helps improve the content of EPA + DHA in the RBC at 4 months in a dose-dependent manner.

M. Garelnabi et al. determined the effects of longer-term supplementation of mouse oxidized linoleic acid (OxLA) on plasma triglycerides on normal C57BL/6 mice. The study reported a 39% decrease in hepatic PPAR- $\alpha$  and a significant decrease in the plasma HDL levels compared to the mice that were fed diets of plain and linoleic acid supplemented chow, suggesting that the longer-term consumption of oxidized linoleic acid may predispose to atheropathogenesis. The other interesting study was authored by R. Ariyanti and B. Besral on the link between dyslipidemia associated with hypertension and incidence of CHD in Harapan Kita Hospital, National Cardiovascular Center, Jakarta. The study has reported that,

after controlling for age, in hypertensive respondents, those with dyslipidemia were 18.1 times more likely to develop CHD compared with those without dyslipidemia, whereas in nonhypertensive subjects, those with dyslipidemia were 2.5 times more likely to develop CHD compared with those without dyslipidemia.

Certainly, these interesting articles point towards the need for additional studies to elaborate on the role of dietary lipids on health and diseases. We are confident this special issue will enrich our current understanding and further interest in lipids in health and disease.

### **Conflicts of Interest**

The editors declare that they have no conflicts of interest regarding the publication of this Special Issue.

*Kamal A. Amin*  
*Abdelgadir M. Homeida*  
*Reda H. El Mazoudy*  
*Khalid S. Hashim*  
*Mahdi Garelnabi*

## Research Article

# Dyslipidemia Associated with Hypertension Increases the Risks for Coronary Heart Disease: A Case-Control Study in Harapan Kita Hospital, National Cardiovascular Center, Jakarta

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**Background.** Coronary Heart Disease (CHD) is the main highlight of the major cardiovascular diseases. In Indonesia, CHD is the leading cause of death from all deaths, with rates reaching 26.4%, which is four times greater than cancer mortality rates. **Objective.** This study aims to determine whether dyslipidemia associated with hypertension increases the risks for the incidence of CHD in Harapan Kita Hospital, National Cardiovascular Center, Jakarta, or does not. **Methods.** The study design was case control. The sample was 163 respondents, 82 respondents in the case group and 81 respondents in the control group. The data were analyzed by using logistic regression. **Results.** In the CHD group, the percentage of respondents with dyslipidemia was 50%, while in the control group, the percentage of respondents with dyslipidemia was 17.3%. The relationship of dyslipidemia with the incidence of CHD differed according to hypertension status. After being controlled for age, in hypertensive respondents, those with dyslipidemia was 18.1 times more likely to develop CHD compared with those nondyslipidemic, whereas in nonhypertensive respondents, those with dyslipidemia was 2.5 times more likely to develop CHD compared with those nondyslipidemic. **Recommendation.** It is recommended that the community have medical checkup regularly and change lifestyles by taking healthy diet to control lipid profile and blood pressure.

## 1. Introduction

Coronary Heart Disease (CHD) is one of the largest contributors to mortality and morbidity worldwide. Globally, CHD accounts for 17.5 million deaths in 2012, with over 75% of deaths occurring in developing countries [1, 2]. By 2015, 16% of all female and male deaths were caused by CHD [3]. In Indonesia, CHD is the leading cause of death from all deaths, with rates reaching 26.4%, which is four times higher than cancer death rates [4]. Basic Health Research (Riskesdas) in 2013 showed that the prevalence of CHD in Indonesia was 0.5% based on doctor-diagnosed interviews and 1.5% based on physicians' diagnosis with symptoms similar to CHD [5].

Broadly speaking, the cause of CHD is multifactorial in which some of them can be modified [6]. One of the modifiable risk factors is dyslipidemia. Dyslipidemia is defined

as a lipid metabolic disorder characterized by an increase or decrease in lipid fraction in plasma [7]. Low-density lipoprotein cholesterol, and triglyceride, and low levels of high-density lipoprotein cholesterol are major risk factors of atherosclerosis affecting arteries of large and medium size and consequently causing ischemia in the heart [8].

Dyslipidemia is thought to be a primary risk factor for CHD and may play a role before other risk factors appear [9]. Dyslipidemia in Indonesia currently has a high prevalence rate. The prevalence of dyslipidemia based on Riskesdas Report of Biomedical Field in 2007 was 39.8% when viewed from total cholesterol >200 mg/dl. Report of Riskesdas in 2013 showed that there are 35.9% of Indonesian population aged ≥15 years with cholesterol levels [3].

Harapan Kita Hospital, National Cardiovascular Center, is a special hospital which is the National Referral Center

for handling of heart and blood vessel disease. In addition, Harapan Kita Hospital is also one of the existing hospitals in Indonesia that serves as a Center for Cardiovascular Training and Education as well as a Center for Cardiovascular Research. Based on data from Harapan Kita Hospital, as many as 144,820 patients with heart and vascular disease (cardiovascular) came to visit in 2012. Of the total number of patients, most cases or about 3000 cases are coronary heart disease, as many as 2500 CHD patients without surgery and the remaining with surgery. This study aims to determine the relationship between dyslipidemia and the incidence of coronary heart disease in Harapan Kita Hospital year 2017. The novelty in this study is a factor dyslipidemia in this research assessed on three aspects such as HDL, LDL, and Triglyceride. This is different from previous study, where dyslipidemia just was assessed based on one of the three aspects.

## 2. Methods

This case-control study used secondary data from medical record data from Harapan Kita Hospital. The weakness design of the study is susceptible to selection bias. To reduce the risk of selection bias in this study is use of simple randomization. The dependent variable was incidence of coronary heart disease (CHD), and the main independent variable was dyslipidemia status. The potential confounding variables were age, gender, family history of CHD, smoking habit, hypertension or history of hypertension, diabetes or history of diabetes, and obesity.

The population in this study was all patients in Harapan Kita Hospital. The sample in this study was patients who visited in January 2016 until December 2017. The sample of case group was patients diagnosed with CHD by the doctor, randomly selected a total of 82 respondents, while the control group sample was a patient diagnosed with Atrial Fibrillation and Flutter (AFF) by a physician, randomly selected 81 respondents. Ethical approval number from the hospital is LB.02.01/VII/222/KEP.065/2017.

The data were analyzed using a binomial regression statistical test where an interaction assessment and confounding test were conducted. The interaction between dyslipidemia status variable and potential confounding variables was assessed using the forward method, in which the interaction variables were entered one by one into logistic regression model. Variables were considered to interact if they had a  $p$  value  $< 0.05$ . The assessment of confounders was done by removing candidate confounding variables one by one, starting from the variable with the highest Wald  $p$  value. If the variable after being issued from the model caused on *odds ratio* (OR) of dyslipidemia status variable change greater than 10%, the variable was considered a confounder and remained in the model.

## 3. Results

In Table 1, the data showed the following: the percentage of respondents with CHD who mostly suffered from dyslipidemia (50%), aged  $< 60$  years (67.1%), male (74.4%), did not

have family history of CHD (75.6%), did not have smoking habit (53.7%), did not have hypertension or hypertension history (62.2%), having diabetes or history of diabetes (53.7%), and nonobese (62.2%).

The results showed that respondents with dyslipidemia had odds to suffer CHD 4.8 times higher compared with nondyslipidemic respondents. Respondents aged  $\geq 60$  years had odds to suffer CHD 1.4 times higher than respondents  $< 60$  years old. Male respondents had odds to suffer CHD 3.5 times higher compared to female respondents. Respondents who had family history of CHD had odds to have CHD 2.1 times higher compared with respondents who did not have family history of CHD. Respondents who have smoking habit had odds to suffer CHD 1.7 times higher compared to respondents who do not have smoking habit. Respondents who were hypertensive or have history of hypertension had odds to suffer CHD 1.9 times higher compared to nonhypertensive respondents. Respondents with diabetes or history of diabetes had odds to suffer CHD 1.3 times higher compared with nondiabetic respondents. Furthermore, respondents with obesity had odds to suffer CHD 0.9 times lower compared to nonobese respondents.

In Table 2, the results showed that the mean of age in CHD group was older than non-CHD group, which was 56.5 years with standard deviation of 9.5, where the youngest age was 37 years and the oldest was 83 years. The mean of SBP in CHD group was higher than non-CHD group, which was 126.2 mmhg with standard deviation of 21.0, where the lowest SBP was 100 mmhg and the highest was 200 mmhg. The average of DBP in CHD group was higher than non-CHD group, which was 85.6 mmhg with standard deviation of 15.5, where the lowest DBP was 60 mmhg and the highest was 120 mmhg.

Furthermore, before the multivariable analysis, stratification tests were conducted to determine the effect of a control variable on the main variables, i.e., dyslipidemia and CHD. In Table 3, full model of dyslipidemia with CHD (hierarchically well-formulated model), it appears that the interaction between dyslipidemia and hypertension has  $p$  value = 0.038.

In Table 4, final model of dyslipidemia connection with CHD shows that there is interaction between dyslipidemia and hypertension and age was found as confounder.

Table 5 shows the relationship of dyslipidemia with CHD according to hypertension. Once controlled for age, in hypertensive respondents or having history of hypertension, those with dyslipidemia were 18 times more likely to develop CHD compared to those nondyslipidemic. Whereas in respondents who were not hypertensive or have no history of hypertension, those with dyslipidemia had chance 2.5 times higher to suffer CHD than those nondyslipidemic.

## 4. Discussion

The results showed that in CHD group, the percentage of respondents with dyslipidemia was 50%, while in the non-CHD group, the percentage of respondents with dyslipidemia was 17.3%. The results of this study are in accordance with the results of previous studies [10]. Dyslipidemia is considered to have an important role in cardiovascular events, especially

TABLE 1: Relationship of dyslipidemia and covariates with coronary heart disease.

Variables	Control (%)	Case (%)	OR	95% CI
Dyslipidemia				
(i) No	82.7	50.0		
(ii) Yes	17.3	50.0	4.8	2.2 – 10.3
Age				
(i) < 60 years	74.1	67.1		
(ii) ≥ 60 years	25.9	32.9	1.4	0.7 – 2.8
Gender				
(i) Female	54.3	25.6		
(ii) Male	45.7	74.4	3.5	1.7 – 6.9
Family history of CHD				
(i) No	86.4	75.6		
(ii) Yes	13.6	24.4	2.1	0.9 – 4.7
Smoking habit				
(i) No	66.7	53.7		
(ii) Yes	33.3	46.3	1.7	0.9 – 3.3
Hypertension or Hypertension History				
(i) No	75.3	62.2		
(ii) Yes	24.7	37.8	1.9	0.9 – 3.7
Diabetes or Diabetes history				
(i) No	53.1	46.3		
(ii) Yes	46.9	53.7	1.3	0.7 – 2.4
Body Mass Index				
(i) Nonobese	59.3	62.2		
(ii) Obese	40.7	37.8	0.9	0.5 – 1.7

TABLE 2: Description of age and blood pressure in CHD and non-CHD groups.

Variables	N	Mean	SD	Min – Max
Age				
(i) CHD group	82	56.5	9.5	37 – 83
(ii) Non-CHD group	81	51.5	11.3	21 – 76
Systolic blood pressure (SBP)				
(i) CHD group	82	126.2	21.0	100 – 200
(ii) Non-CHD group	81	115.2	20.4	90 – 155
Diastolic blood pressure (DBP)				
(i) CHD group	82	85.6	15.5	60 – 120
(ii) Non-CHD group	81	80.5	14.0	60 – 110

TABLE 3: Full model of dyslipidemia with CHD.

Variables	B	p value	OR	CI 95%
Dyslipidemia	1.000	0.049	2.7	1.0– 7.4
Hypertension	-0.29	0.194	0.5	0.2– 1.4
Age ≥ 60 years	0.621	0.128	1.9	0.8– 4.1
Male	1.275	0.002	3.6	1.6– 8.2
Having family history of CHD	0.505	0.295	1.7	0.6– 4.3
Smoker	-0.037	0.926	1.0	0.4– 2.1
Diabetes	0.081	0.824	1.1	0.5– 2.2
Obese	-0.243	0.512	0.8	0.4– 1.6
Dyslipidemia*Hypertension	1.877	0.038	6.5	1.1 – 38.6

TABLE 4: Final model of dyslipidemia connection with CHD.

Variables	B	p value	OR	CI 95%
Dyslipidemia	0.924	0.047	2.5	1.0 – 6.3
Hypertension	0.545	0.150	1.7	0.8 – 3.6
Age ≥ 60 years	-0.642	0.227	0.5	0.2 – 1.5
Dyslipidemia*Hypertension	1.973	0.022	7.2	1.3 – 38.7

TABLE 5: Relationship of dyslipidemia with coronary heart disease according to hypertension.

	p value	OR	CI 95%
Dyslipidemia in nonhypertension group	0.047	2.5	1.0– 6.3
Dyslipidemia in hypertension group	<0.001	18.1	4.3 – 75.6

CHD. Dyslipidemia is investigated as a predictor of CHD; it has a role in the process of atherogenesis [11, 12].

The results showed that the relationship of dyslipidemia and the incidence of CHD was different according to hypertension status. At the same age, respondents with hypertension or history of hypertension and dyslipidemia had 18 times higher to develop CHD than nondyslipidemic respondents, whereas in nonhypertensive patients, respondents with dyslipidemia were 2.5 times higher to develop CHD compared to nondyslipidemic respondents. This study is in line with previous studies that stated that dyslipidemia interacts with high blood pressure (hypertension) in causing CHD [13, 14].

The risk of CHD in patients with dyslipidemia will increase if dyslipidemia is accompanied by one or more other CHD risk factors [15]. Various studies showed that other factors that can cause cardiovascular disease as hypertension. Dyslipidemia and hypertension are established risk factors of prime importance in cardiovascular disease [16]. If these two factors (dyslipidemia and hypertension) are present together, this will accelerate the process of atherosclerosis, thus increasing the risk of CHD. In Indonesia, people with hypertension are estimated at 15 million, but only 4% are controlled hypertension. Controlled hypertension means they suffer from hypertension and know that they are suffering from hypertension [17].

Cholesterol is a risk factor that can be changed from hypertension, so the higher the total cholesterol level, the higher the likelihood of hypertension [18]. The constriction and the rigidity of the blood vessel walls resulting from the buildup of cholesterol in the blood vessels that can cause increased blood pressure will have an impact on the increased risk of CHD. High cholesterol levels in the blood cause cholesterol deposits on blood vessel walls or the so-called plaque cholesterol. The precipitation of calcium ions in plaque cholesterol causes the soft plaque to become hard and rigid. This causes the blood vessel wall to become stiff and not elastic. In addition, in the presence of a hardened plaque cholesterol, this causes the inner walls of blood vessels to become narrow and not slippery, so that blood supply to the organ becomes reduced. If hardening occurs in the arteries that supply blood to the heart (coronary artery), then it causes CHD [19].

In addition, this study is also in line with the theory that the increase in fat levels is associated with the process of atherosclerosis. Dyslipidemia is an important risk factor for the initiation and progression of atherosclerosis and is strongly associated with cardiovascular events [20]. Pathophysiology of CHD originated from the formation of atherosclerosis [21]. Atherosclerosis is the formation of plaque in the walls of the large arteries, thus narrowing the lumen of the vessels that cause the blood flow disruption and decreases the elasticity of the blood vessels. Various studies have been conducted suspecting that the early lesions of atherosclerosis form a layer of fat. Dyslipidemia is a lipid metabolic disorder characterized by increased or decreased lipid fraction in plasma. The major lipid fraction disorders are increased total cholesterol, LDL cholesterol, triglycerides, and decreased HDL cholesterol levels. All lipid fractions have an important role in the process of atherosclerosis and are closely related to one another.

High triglyceride levels and high LDL cholesterol and low HDL cholesterol levels are associated with atherosclerosis, which is one of the risk factors for CHD. The results of Iskandar's study (2017) showed that there was a correlation between triglyceride cholesterol level and CHD occurrence, where the value of OR 1.99 (95% CI 0.97-1.00) was obtained, meaning that patients with high triglyceride levels had odds for CHD 1.99 times greater than patients who have normal triglyceride levels [22]. This is also consistent with Bao et al.'s study which found that total cholesterol, LDL cholesterol, HDL, and triglyceride levels were on average higher in patients with CHD than in the non-CHD group.

Increased total cholesterol levels in the blood leads to cholesterol deposits in the walls of blood vessels. In addition, the increase in total cholesterol also causes disruption to endothelial function by increasing the production of oxygen free radicals. This radical deactivates the production of nitric oxide, a major endothelial-relaxing factor. So if there is an increase in total cholesterol levels and increased levels of triglycerides in a long time, the endothelial permeability becomes increased which causes lipoproteins accumulation in it. Exposure of free radicals in endothelial cell endothelial cells causes LDL oxidation.

Previous research conducted in Korea showed that there was a strong relationship between LDL cholesterol and increased risk of CHD [23]. Low Density Lipoprotein (LDL) is the primary lipid in the process of formation of atherosclerosis. Based on the Guidelines for the Management of Dyslipidemia issued by the Indonesian Heart Association (IHA) in 2013, there is strong evidence of a link between LDL cholesterol and CHD occurrence based on clinical outcome studies, so that LDL cholesterol is the primary target in the management of dyslipidemia. The process of atherosclerosis begins with endothelial damage or dysfunction in artery walls. The possible cause of this endothelial damage can be caused by increased LDL levels. When LDL levels are high, then cholesterol elevated by LDL may precipitate in the subendothelial layer; therefore, LDL is atherogenic, that is the material that can cause atherosclerosis. When the endothelium presents a lesion, oxidized LDL causes various inflammatory reactions, which eventually attract monocytes

and neutrophils into the lesion area and increase the size of atheromatous plaque. It will also get worse if followed by a decrease in HDL levels.

Previous research conducted in Cameroon, Central Africa, showed that decreased HDL levels are the most common lipid lesions in causing CHD [21]. High Density Lipoprotein (HDL) is a lipid that acts as a protective factor. HDL plays an important role in reverse cholesterol traction (RCT), a process whereby excess cholesterol in peripheral tissue is returned to the liver for excretion. This process is often referred to as the main mechanism of HDL that is to protect the body from the risk of atherosclerosis and can even decrease the plaque regression. If there is a decrease in HDL levels, then the body protective against atherosclerosis will be reduced; consequently, the inner wall of blood vessels becomes narrow, not slippery, and not elastic, so that the blood supply to the organ becomes reduced. If the process continues on coronary arteries, it will cause coronary heart disease [24].

The results of this study indicated that there was no significant relationship between age and the incidence of CHD, but age was confounder of dyslipidemia relationship with CHD events. The results of this study differ from previous studies which stated that age is significantly associated with the incidence of CHD [25].

## 5. Conclusion

In the CHD group, the percentage of respondents with dyslipidemia was 50%, while in the non-CHD group, the percentage of respondents with dyslipidemia was 17.3%. The relationship of dyslipidemia with CHD differed according to hypertensive status of respondents. Once controlled for age, in hypertensive respondents or having a history of hypertension, respondents with dyslipidemia are 18 times higher to develop CHD than nondyslipidemic respondents. Whereas in respondents who are not hypertensive or have no history of hypertension, respondents with dyslipidemia are 2.5 times higher to develop CHD than nondyslipidemic respondents.

## Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

## Additional Points

**Recommendation.** Health workers should prioritize preventive programs by improving communication, information, and education to the public regarding lifestyles, especially those related to positive changes in behavior to control lipid profile and blood pressure. Changing lifestyles can be done by dieting healthy foods such as reducing saturated fat intake, increasing fiber intake, and reducing carbohydrate intake to control lipid profile and blood pressure and regular medical checkup in Posbindu Noncommunicable Diseases or Health Service Facilities.

## Conflicts of Interest

The authors declare no conflicts of interest.

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## Research Article

# Dietary Oxidized Linoleic Acid Modulates Plasma Lipids beyond Triglycerides Metabolism

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**Introduction.** Triglyceride (TG) is an independent risk factor for coronary heart disease. Previous work has shown that short-term supplementations of mouse chow with oxidized linoleic acid (OxLA) significantly reduce the level of plasma triglycerides. **Study Objective.** This study aims to determine the effects of longer-term supplementation of mouse chow with various concentrations of oxidized linoleic acid (OxLA) on plasma triglycerides. **Study Design.** The study consisted of forty C57BL/6 wildtype mice divided into four groups ( $n = 10$ ). Two groups were kept as controls. One control group (P) was fed plain chow and the second control group (C) was fed chow supplemented with linoleic acid. The other two experimental groups (A) and (B) were fed oxidized linoleic acid supplemented chow in the following doses: 9 mg/day of oxidized linoleic acid and 18 mg/day of oxidized linoleic acid/mouse. **Results and Conclusion.** Mice that were on a diet supplemented with the higher dose of oxidized linoleic acid showed a 39% decrease in hepatic PPAR- $\alpha$  and a significant decrease in the plasma HDL levels compared to the mice that were fed diets of plain and linoleic acid supplemented chow. Interestingly, the longer-term consumption of oxidized linoleic acid may predispose to atheropathogenesis.

## 1. Introduction

Triglycerides plasma levels are major risk factor for cardiovascular diseases. It is a complex polygenic trait that is modulated by a number of major pathways in the intestine, liver, adipose, and plasma. Although triglycerides are not directly linked to the pathogenesis of atherosclerosis, they greatly influence plasma lipoproteins, especially the low-density and very low-density lipoproteins (LDL) which are known risk factors for cardiovascular disease (CVD).

Linoleic acid is the most abundant polyunsaturated fatty acid (PUFA) in many lipid rich diets. Like other polyunsaturated fatty acids (PUFA), linoleic acid is a ligand of the peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) which is a regulating component of plasma triglyceride levels. We have previously shown that oxidized linoleic acid significantly lowers plasma triglyceride (TG) levels as compared to animals fed oleic acid. The changes were associated with increased APOA5 and acetyl-CoA oxidase genes expression in the mice

that were fed a diet supplemented with 9 mg/mouse/day of OxLA. Two apolipoproteins (Apo), Apo A5 and Apo CIII, were of particular interest due to their role in the level of triglycerides. These proteins modulated triglycerides by interchangeably binding to VLDL particles [1, 2].

PPAR- $\alpha$  induces the expression of proteins involved in the uptake, transport, and metabolism of fatty acids which results in decreasing the synthesis of triglyceride. In human ApoA5 is a direct target of PPAR- $\alpha$  which is consistent with the triglyceride-lowering role proposed for ApoA5 [3]. However PPAR- $\alpha$  link to ApoA5 in mice is not well understood. ApoA5 induces a clearance of VLDL from the plasma through electrostatic interactions with heparin sulfate proteoglycan (HSPG). This interaction aid colocalizes the VLDL to HSPG attached lipoprotein lipase (LPL), allowing for greater lipolysis [1, 4–6].

ApoCIII, like ApoA5, localized on VLDL particles but has the opposite effect as it promotes increases of plasma triglycerides. Within the liver, ApoCIII acts by utilizing

the available triglyceride substrates to increase synthesis of VLDL-TG. The increased synthesis of VLDL particles results in greater plasma TG levels. ApoCIII prevented lipolysis leading to increased plasma TG [6]. As a result, these two antagonistic proteins are considered key in triglyceride modulation [1, 4, 5].

Lipases are other proteins of interest in TG metabolism. They are key enzymes that break down lipids and lipoproteins; lipoprotein lipase (LPL) is antagonized by two angiopoietin-like proteins ANGPTL3/4. The proteins cause the LPL dimers to dissociate, leading to a loss of activity and lipolysis. ANGPTL3 is promoted when its gene is bound by the Liver X Receptor (LXR). Hepatic ANGPTL4 is activated by ligands of PPAR- $\alpha$ , which includes PUFA [3, 7].

By investigating the hepatic gene, protein expressions, and plasma protein levels, the study determines the range of modulators for plasma triglycerides, which is known to control biosynthesis and metabolism of TG and widely affects the plasma lipoproteins. The goal is to elucidate a mechanism by which oxidized linoleic acid modulates TG and hepatic lipoproteins metabolism.

## 2. Experimental Design

Normal C57BL/6 male mice were cared for in an animal facility with 12-hour light/dark cycles and all the protocols pertaining to this study were approved by the University of Massachusetts, Lowell, Institutional Animal Care and the Use Committee (IACUC.) The body masses of the mice were monitored over the course of 10 weeks. Water was provided ad libitum, while set amounts of diet were measured and supplied to each study group weekly.

## 3. Diets

13-Hydroperoxyoctadecadienoic acid (13-HPODE) was prepared as previously described [2]. Two prepared formulas of 13-HPODE were then shipped to Harlan Laboratories, Indianapolis, Indiana, US, for preparation of the experimental mouse chow formulas. The specialized diets were kept at 2°C until used. Four different diet formulas were provided to the groups of mice: a Standard Chow as plain control (P group), a chow supplemented with linoleic acid 9 mg/mouse/day, linoleic control (C group), oxidized linoleic acid, 9 mg/mouse/day (A group), and oxidized linoleic acid 18 mg/mouse/day diet (B group). Mice were fed the dietary formulas or kept on plain chow for two months.

## 4. Materials and Methods

Mice were euthanized at the end of the study. Whole blood was obtained from each mouse by heart puncture and placed in heparin tubes. The containers were spun in a cold centrifuge at 3000 rpm. The samples were later aliquoted and stored at -80°C. The plasma samples were analyzed for LDL, High-Density Lipoprotein (HDL), glucose, and total cholesterol using reagents and standards from Medica Corporation, Bedford, MA 01730. ApoA5, ApoCIII, ANGPTL3,

ANGPTL4, and hepatic lipase (HL) in plasma were analyzed using commercial ELISA kits.

*4.1. mRNA Extraction and Analysis of ApoA5, ApoCIII, PPAR-Alpha, and SREBP1 Genes.* Prior to collection of organs, chilled 1x phosphate buffered saline (PBS) was perfused through the heart, after which the liver (100 mg samples) and adipose were collected in homogenization tubes. The organs were then flash frozen in liquid nitrogen (LN2) and stored at -80°C. Single aliquots of liver and adipose were stored in 1 mL of Trizol for later RNA extraction. The RNA was extracted from liver samples with the Trizol reagent and then aliquoted. RNA was quantified using the Qubit fluorimeter (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Quality was assessed on a 1% agarose gel using ethidium bromide as a probe and detected on a UVP imager (UVP Biosystems, Upland, CA, USA). cDNA was prepared using iScript RT mix, which was diluted to 1:50 for gene expression analysis. Aliquots of 8  $\mu$ L were run with a Polymerase Chain Reaction (PCR) Master mix containing EvaGreen SsoFast Supermix. ApoA5, ApoCIII, PPAR $\alpha$ , and SREBP1 were run against GAPDH for control comparison.

*4.2. Protein Extraction and Analysis of ApoCIII, ANGPTL3, and ANGPTL4.* Hepatic protein was extracted through homogenization in 1 mL precipitation cocktail-10 mL radio-immunoprecipitation assay (RIPA) buffer with a complete mini ultra-protease inhibitor tablet. Homogenized samples were incubated on ice for 30 minutes and centrifuged for 15 minutes at 3000 rpm. The protein containing supernatant was removed and aliquoted. Concentrations were determined using the bicinchoninic acid (BCA) assay. All chemicals and materials used for the Western Blotting and gene expression were obtained from Bio-Rad Laboratories, Inc., Hercules, CA, USA.

For the western blot assay, 16  $\mu$ L of liver protein was loaded per lane on polyacrylamide 4–15% gels and ran with Western C Protein Plus Standards Ladder. The protein was then transferred onto 0.2  $\mu$ m PVDF membrane for blotting. A blocking buffer was prepared by dissolving nonfat dried milk (NFDM) in 1x Tris Buffered Saline (TBS) with .1% v/v Tween 20 (T). The NFDM-TBST buffer was used to block membranes and dilute antibodies. ApoCIII, ANGPTL3, ANGPTL4, and proprotein convertase subtilisin/kexin type 9 (PCSK9) antibodies were assayed at 1:500. Their incubation was followed with a secondary goat anti-rabbit-HRP (1:10000) which was coincubated with an anti-ladder-HRP (1:10000). B-Actin, the reference protein, is HRP primary tagged (1:25000). All proteins were visualized on a UVP Biosystems Imager with Dura West ECL signaling reagent.

## 5. Results

*5.1. Plasma Lipids, Glucose, and ELISA Measurements.* The plasma was analyzed for lipids and glucose. Overall, the mice that were fed diets supplemented with fatty acids showed increasing plasma levels of glucose and lipids (Figures

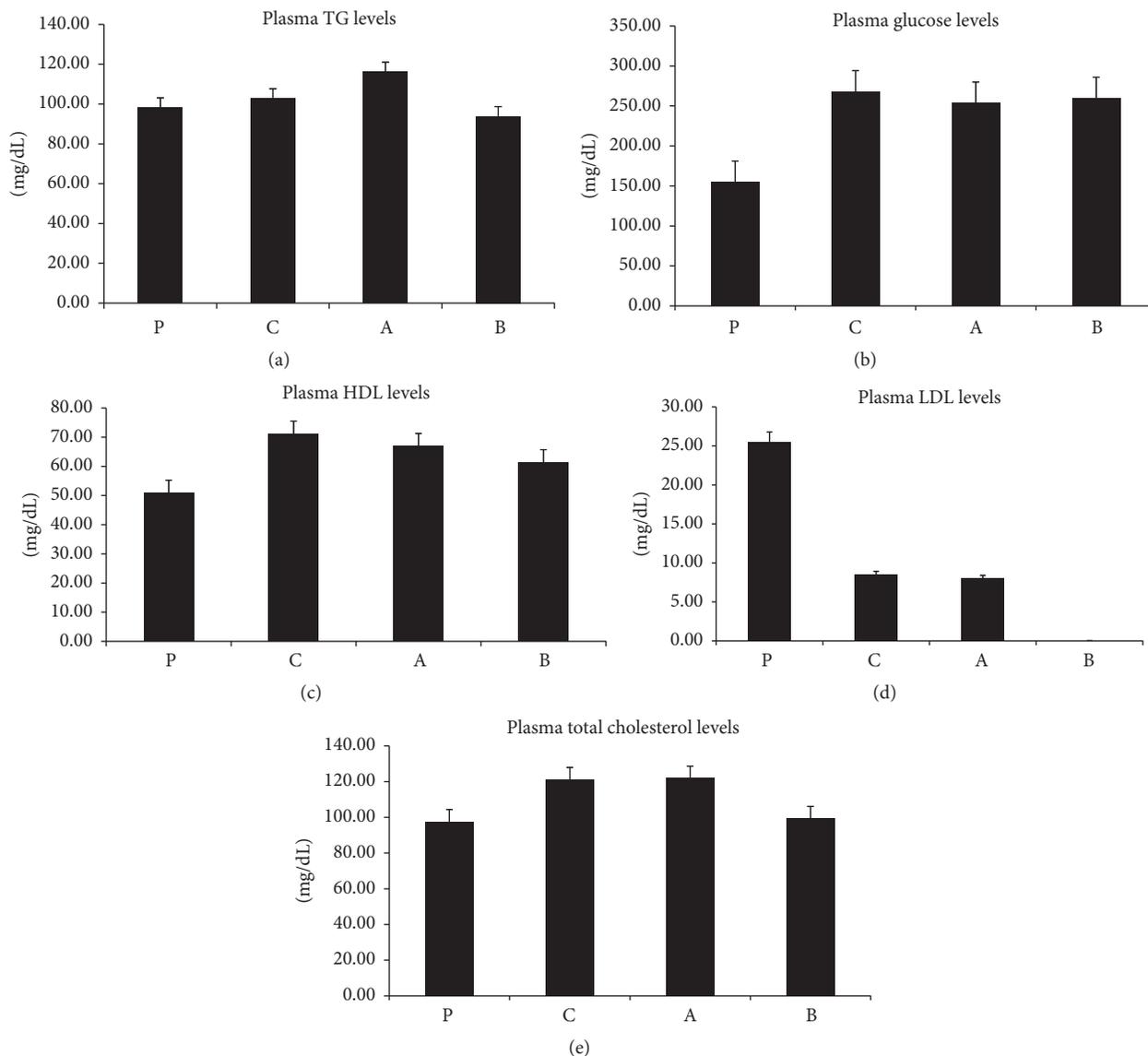


FIGURE 1: (a) The difference between the plasma levels of triglyceride in the 4 groups was not significant. (b) The difference between the plasma levels of glucose in plain control (Standard Chow) and control (chow supplemented with linoleic acid) is significant ( $P < 0.01$ ); glucose also significantly ( $P < 0.01$ ) decreased in higher concentration of OxLA compared to the plain chow. However the low oxidized LA has shown significantly ( $P < 0.01$ ) increased glucose compared to the plain group. (c) Plasma HDL levels increased among all the treated groups; they were however more significant ( $P < 0.05$ ) between the plain and control group and the LA and higher OxLA group ( $P < 0.01$ ). (d) Higher OxLA plasma LDL levels were significantly ( $P < 0.01$ ) reduced compared to the LA control. Plasma LDL was also greatly reduced in low OxLA compared to the plan and control groups. However the difference was not significant. (e) Total cholesterol plasma levels increased in LA and the low OxLA groups compared to the plain control and higher OxLA groups; however the difference was not significant.

1(a)–1(c) and 1(e)), with the exception of LDL (Figure 1(d)), which was decreased.

There were no substantial significant changes shown within the triglyceride measurements between the groups (Figure 1(a)) but a change in the weight gained by mice during the 10-week study was noted (data not included).

All groups that were fed a diet supplemented with fatty acids showed higher concentrations of total cholesterol (Figure 1(e)), glucose (Figure 1(b)), and HDL (Figure 1(c)) within their plasma.

Significantly greater blood glucose levels (Figure 1(b)) were seen in all three experimental groups. While the total

cholesterol (Figure 1(e)) showed an overall increase, only the groups that were fed a diet supplemented with a lower concentration of the oxidized fatty acid showed much higher levels. HDL (Figure 1(c)) levels considerably increased in the linoleic acid fed control group compared to the plain control or high oxidized fed mice. The LDL (Figure 1(d)) levels were low in all groups and they were significant only when the high oxidized fed mice are compared to the linoleic control fed group.

**5.1.1. ELISA.** Linoleic acid or oxidized linoleic acid supplementation has led to a decrease in plasma ApoCIII

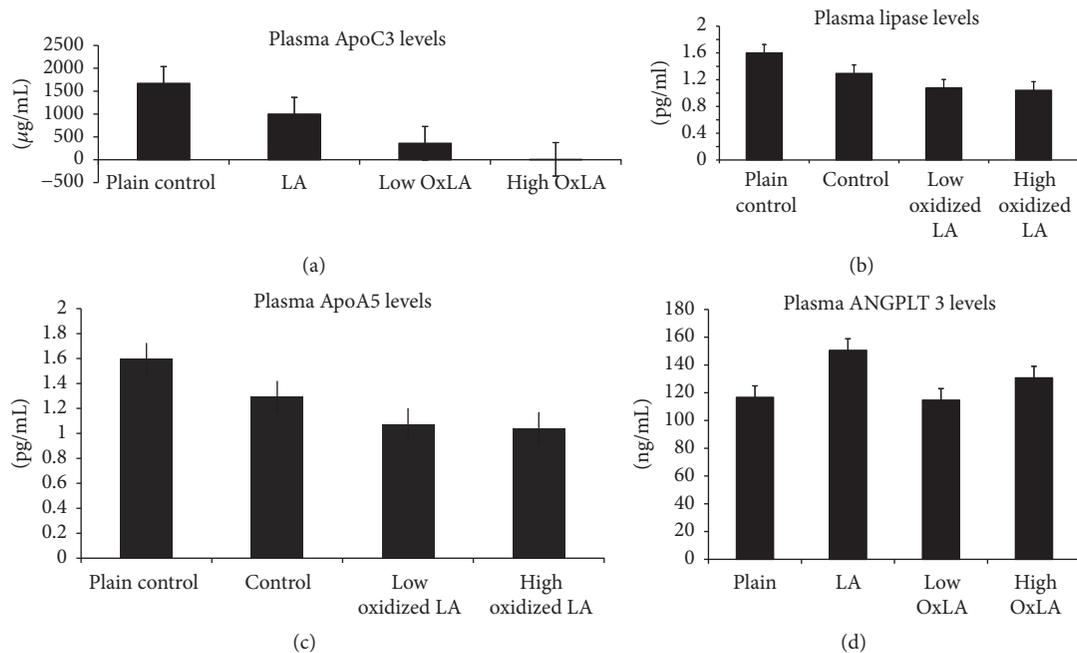


FIGURE 2: Plasma ApoC3 significantly decreased in a dose dependent manner in low OxLA ( $P < 0.05$ ) compared to plain group. The levels were also significantly reduced ( $P < 0.05$ ) between low OxLA and LA. Plasma ApoC3 levels were also significantly reduced ( $P < 0.01$ ) in higher OxLA group compared to the LA. (b) Oxidized linoleic acid supplementation led to dose dependent significant ( $P < 0.05$ ) decreases in plasma hepatic lipase when compared to the plain fed group of mice. The decreases in the hepatic lipase levels in the LA control group compared to the plain mice were not significant. (c) Plasma ApoA5 levels decreased but nonsignificantly in the treated groups compared to the plain control. (d) ANGPTL3 concentration decreased among the OxLA fed groups compared to the LA control linoleic acid group; interestingly the experimental groups had either similar or slightly elevated ANGPTL3 concentration compared to the plain groups.

(Figure 2(a)) levels. The drop was significant for both of the oxidized groups compared to the linoleic acid control group. The greatest drop was in group (B) which had the highest concentration of oxidized linoleic acid, demonstrating a dose dependent response.

**Plasma Hepatic Lipase (HL).** Plasma HL decreased within experimental groups (Figure 2(b)). The drop was significant for the OxLA groups compared to the plain groups; these differences were dose dependent.

**Plasma ApoA5 and ANGPTL3.** The group of mice that were fed the linoleic acid and 18 mg/mouse/day oxidized linoleic acid supplemented chows showed increased plasma levels of ApoA5 (Figure 2(c)) and ANGPTL3 (Figure 2(d)). The difference was not significant. The ApoA5 levels were elevated in the high oxidized linoleic acid group. However, ANGPTL3 had the highest concentration in the control linoleic acid group.

**5.2. Gene Expression.** ApoA5 gene was significantly upregulated (Figure 3(a)) in the linoleic acid control group. This aligns with the result for the plasma APOA5 measured by ELISA. The increase in the expression of ApoCIII noted in the linoleic acid control group was not significant (Figure 3(b)). SREBP gene expression was slightly downregulated in the linoleic acid control group. However, it was upregulated

(Figure 3(c)) in the oxidized groups. The upregulation was significant for the mice that were fed high OxLA diet.

PPAR- $\alpha$  expression peaked slightly for the linoleic control group and dropped for both oxidized linoleic acid groups (Figure 3(d)).

**5.3. Western Blot.** ApoCIII (Figure 4(a)) shows greater expression for the ApoCIII protein for all groups except the B group, which appears unchanged. The C group showed the highest protein expression followed by the P group and A group with the least upregulation.

ANGPTL3 (Figure 4(b)) shows intense protein expression for the P and C groups. The A group showed significant underexpression and the B group showed lesser expression.

ANGPTL4 (Figure 4(c)) showed significant ANGPTL4 overexpression on the linoleic acid control group. There was a slight increase in expression for the plain group. The high oxidized linoleic acid group showed no change and the low oxidized linoleic acid group showed differential expression between samples.

## 6. Discussion

Linoleic acid (LA) is an essential fatty acid that is required for physiological and developmental functions of mammals, particularly humans. Like all polyunsaturated fatty acids (PUFAs), LA is susceptible to oxidation that results in

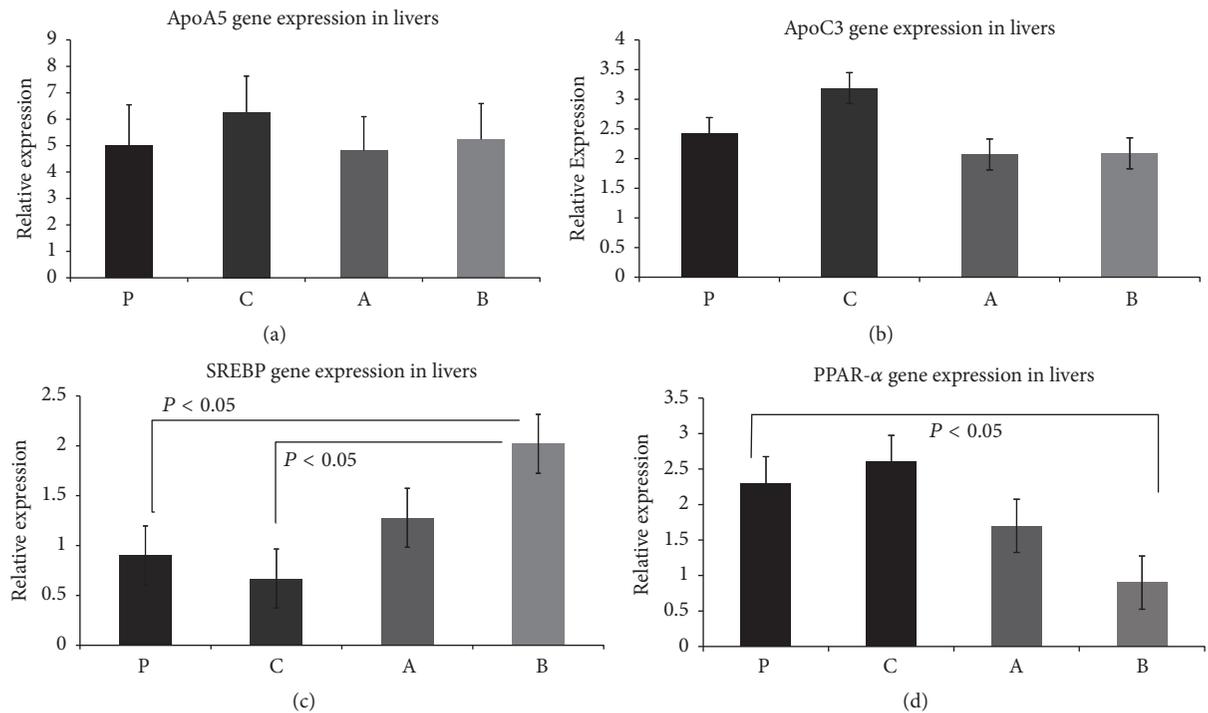


FIGURE 3: (a) ApoA5 is significantly upregulated in the linoleic acid control group (C group). P group (Standard Chow), C group (chow supplemented with linoleic acid 9 mg/mouse/day), A group (chow supplemented with oxidized linoleic acid 9 mg/mouse/day), and B group (chow supplemented with linoleic acid 18 mg/mouse/day). (b) ApoC3 is nonsignificantly upregulated in the linoleic acid control group (C group). P group (Standard Chow), C group, A group (chow supplemented with oxidized linoleic acid 9 mg/mouse/day), and B group (chow supplemented with linoleic acid 18 mg/mouse/day). (c) SREBP gene expression shows significant upregulation in the mice group fed a high concentration of oxidized linoleic acid. P group (Standard Chow), C group (chow supplemented with linoleic acid 9 mg/mouse/day), A group (chow supplemented with oxidized linoleic acid 9 mg/mouse/day), and B group (chow supplemented with linoleic acid 18 mg/mouse/day). (d) Slight peak in the PPAR- $\alpha$  gene expression in the linoleic acid control group (C group) and decreased expression in both oxidized linoleic acid concentration groups (A and B groups): P group (Standard Chow), C group (chow supplemented with linoleic acid 9 mg/mouse/day), A group (chow supplemented with oxidized linoleic acid 9 mg/mouse/day), and B group (chow supplemented with linoleic acid 18 mg/mouse/day).

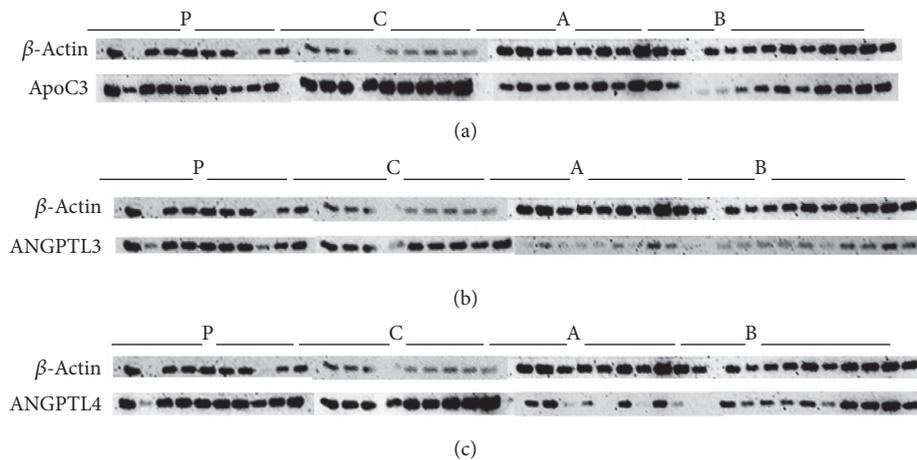


FIGURE 4: (a) Increased expression of ApoC3 over the contrast B-actin in all groups with more intense expression in group C while the B group seems unchanged. P group (Standard Chow), C group (chow supplemented with linoleic acid 9 mg/mouse/day), A group (chow supplemented with oxidized linoleic acid 9 mg/mouse/day), and B group (chow supplemented with linoleic acid 18 mg/mouse/day). (b) ANGPTL3 protein expression is increased in groups P and C, while it is underexpressed in groups A and B, with group showing much less expression. P group (Standard Chow), C group (chow supplemented with linoleic acid 9 mg/mouse/day), A group (chow supplemented with oxidized linoleic acid 9 mg/mouse/day), and B group (chow supplemented with linoleic acid 18 mg/mouse/day). (c) ANGPTL4 protein expression is increased intensely in group C, as well as the P group, which also shows a slight increase. Group A shows no change in expression levels while the B group displays much less expression between the dose dependent groups (A and B). P group (Standard Chow), C group (chow supplemented with linoleic acid 9 mg/mouse/day), A group (chow supplemented with oxidized linoleic acid 9 mg/mouse/day), and B group (chow supplemented with linoleic acid 18 mg/mouse/day).

several active metabolites that have biological relevance. 13-Hydroxyoctadecadienoic acid (13-HODE) (a common name for 13(S)-hydroxy-9Z, 11E-octadecadienoic acid (13(S)-HODE)) and 9-hydroxyoctadecadienoic acid (9-hydroxy-10(E),12(Z)-octadecadienoic acid or 9-HOPDE) are the most studied metabolites of LA. In the current study we used 9-HOPDE derivative to reassess our previous findings that showed significant reduction on TG after over two weeks of 9-HOPDE dietary intake. The present results of our study showed differential responses for how oxidized linoleic acid affects triglyceride metabolism in C57BL/6 mice. It appears that the prolonged dietary intake of the oxidized linoleic acid has a different effect than what we previously reported [2] on the short intake acute effect on TG and lipoprotein metabolism. This may suggest that extended dietary intake of oxidized fatty acids results in mixed favorable and unfavorable liver and plasma responses. We have seen some genes and plasma lipoproteins levels changes that are dose dependent for intake of oxidized linoleic. However, it is not evident that the intake of dietary OxLA resulted in significant metabolic alterations in TG and lipoprotein.

The plasma triglyceride levels were the highest for the group that had less oxidized linoleic acid in their diet which in part incorporates our previous findings. Interestingly, plasma triglyceride levels are comparable to the gain in body weight over the 10-week period (data not shown). ApoA5 is an important lipid modulating protein that acts on TG and VLDL particles. ApoA5 itself is a protein that is not highly expressed. It is described to stabilize lipid droplets in the liver and protects LPL through an electrostatic mechanism in the bloodstream. The relative abundance of mRNA (Figure 3(a)) for ApoA5 in the liver was low, although it did have a significantly higher concentration in the linoleic acid control group. There was a lower manifestation in the low oxidized group. In comparison, the plasma level showed no significant differences for ApoA5 (Figure 2(c)). However, it demonstrated greater concentration in the bloodstream for the high oxidized and control groups compared to the low oxidized group. ApoCIII is a hepatic and plasma protein that acts to block LPL activity and causes an increase in TG levels in the blood stream. Gene expression (Figure 3(b)) data showed a greater, though insignificant, abundance of mRNA for ApoCIII in the livers of the control linoleic acid group, while there was a lower abundance seen in the mice fed oxidized linoleic acid. The western blot (Figure 4(a)) data generally agreed with the gene expression. The highest amount of active hepatic protein was found in samples from the linoleic control group followed by the plain control group. The low oxidized group showed higher expression than the higher oxidized group. Plasma (Figure 2(a)) ApoCIII concentration was lower in the groups fed fatty acids diets. However, it was more significantly lower in those fed oxidized diets. This could possibly be due to cellular degrading of ApoCIII during or after translation, or the protein may have a loss of function in these mice. Plasma glucose (Figure 1(b)), HDL (Figure 1(c)), and total cholesterol (Figure 1(e)) had some differential changes across mice samples. The glucose increased in all the experimental groups, while the plasma total cholesterol was higher in the linoleic acid control and lower oxidized linoleic

acids fed groups compared to the mice on plain chow and high oxidized linoleic acids fed groups. SREBP is known to modulate the regulation of ANGPTL3, LDLR, and PCSK9, all of which affect the plasma lipids profile [8, 9]. SREBP may have induced more expression of the LDL receptors leading to the much lower LDL concentrations (Figure 1(d)) [9].

SREBP is equally produced in the intestine and liver. It also has a secondary function in increasing triglyceride rich lipoprotein production within the intestine. By promoting the activity of MTP, PCSK9 increases lipidation of ApoB that can lead to greater plasma lipid concentrations [9]. SREBP increases activities of ANGPTL3 and SREBP also with ANGPTL4 antagonized LPL and HL which affect their activity [6, 7]. The ANGPTL family of proteins causes dissociation of many lipases. The dissociation causes a loss of activity and decreased clearance of plasma triglycerides, which is one of the reasons why VLDL particles have less lipolysis thus leading to a diminished clearance. ANGPTL3 and ANGPTL4 are essential for LPL regulation [10]. ANGPTL3 (Figure 4(b)) expression levels were regulated for P and C groups, while group A has shown significant reduction and group B slight reduction. ANGPTL3 was upregulated in group C, though not significant, in the plasma (Figure 2(d)). Although ANGPTL3 did not have any significant changes, it may have acted in preventing further clearance of triglycerides [11].

ANGPTL4 has shown significant upregulation in the linoleic control group as shown in the western blot (Figure 4(c)).

*Conclusion.* This study demonstrates the ambiguity of the prolonged dietary oxidized fatty acids intake. The rationale for the differences between the short and extended period intake of the oxidized linoleic fatty acid and the conflicting outcomes compared to our previous study is not very clear. However, apparently the long term intake of oxidized linoleic acid may have unfavorable effects on lipoprotein metabolism. The mechanisms of actions vary greatly between the experimental formulas and controls. These findings strongly point towards the proatherogenic roles of the oxidized fatty acids. Future studies using LDLr  $-/-$  mouse models may be necessary to establish possible linkages to the pathogenesis of atherosclerosis [12, 13].

## Disclosure

Part of this work was published as an abstract in the Journal of Clinical Lipidology, 2017.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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## Review Article

# Industrial *Trans* Fatty Acid and Serum Cholesterol: The Allowable Dietary Level

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*Trans* fatty acid (TFA) from partially hydrogenated oil is regarded as the worst dietary fatty acid per gram due to its role in coronary heart disease. TFA consumption is decreasing worldwide, but some but not all observational studies indicate that TFA intake has little relevance to serum cholesterol levels in populations with low TFA intake (<1% *E* [percentage of total energy intake], <approximately 2 g/day). Few intervention trials examined the effect of TFAs on blood cholesterol at relatively low levels (<2% *E*); no definite evidence is available on the tolerable upper level of the intake. A series of our intervention studies in Japanese suggested that an industrial TFA intake at <1% *E* does not influence the serum cholesterol level. To establish allowable level, we must consider not only the dietary level of TFAs, but also the composition of dietary fats simultaneously consumed, that is, saturated and unsaturated fatty acids. These fatty acids strengthen or counteract the adverse effect of TFAs on serum cholesterol levels. In this review we describe the complex situation of the cardiovascular effects of industrial TFAs. The relationship between dietary industrial TFAs and concentration of plasma cholesterol should be evaluated from the viewpoint of dietary patterns rather than TFAs alone.

## 1. *Trans* Fatty Acid in Foods

*Trans* fatty acid (TFA) is defined as unsaturated fatty acid with at least one nonconjugated double bond in the *trans* configuration. There are several food sources of TFAs. TFA from partially hydrogenated vegetable oil is the major source of dietary industrial TFAs [1], and this type of TFA is regarded as contributing to cardiovascular events. There is a trend toward decreasing consumption of this type of TFA. The second major source of TFA is from ruminant fat, and in some cases ruminant fat is a major contributor of TFAs due to the reduction of the intake of industrial TFAs. The impact of ruminant fat TFAs on human health has not been conclusive, regarding both health benefits [2] and harmful effects [3] depending on the reports. A small amount of industrial TFA is also present in edible oils formed during the deodorization process at high temperature [4]. The physiological effects of TFA contained in edible oils are not well established [5, 6].

Since the TFA in partially hydrogenated oils (PHOs) is composed mainly of a number of positional isomers of the

octadecenoic acids, it is important to clarify which TFA isomer(s) is responsible for health hazards. Chatgialiloglu et al. [7] stressed the importance of lipid geometrical isomerism to the biological functions of fatty acids. Ferreri et al. [8] also summarized the significant role of isomerism of fatty acids in membrane functions. In most of the *in vitro* studies available, elaidic acid (9*t*-18:1) was examined as a representative TFA in PHO [9]. Elaidic acid is the major component of TFA in PHO, but in many cases it is not always the largest part, usually <30% of the total TFAs. It is plausible that different isomers exert different biological functions, if any. When the principal TFA(s) is revealed, more effective ways to lower the TFA contents of foods can be expected, contributing to human health.

TFA in humans is attributed not only to dietary origin but also to that endogenously formed through the production of free radicals during metabolism [7]. However, it is likely that most of the TFAs in humans are attributable to dietary origin, although the biological activities may differ between

TABLE 1: The *trans* fatty acid contents of major foods distributed in Japan.

	Avg.	Max. g/100 g	Min.	<i>n</i>
Shortening	13.58	31.21	1.15	10
Margarine and fat spread	7.00	13.49	0.36	34
Creams	3.02	12.47	0.01	10
Butters	1.95	2.21	1.71	13
Biscuits	1.80	7.28	0.04	29
Vegetable oils	1.40	2.78	0.00	22
Animal fats	1.34	2.70	0.64	4
Mayonnaise	1.24	1.65	0.49	9
Cheeses	0.83	1.46	0.48	27
Cakes, buns and pastries	0.71	2.17	0.26	12
Beef	0.52	1.45	0.01	70
Ice creams	0.24	0.60	0.01	14
Japanese buns	0.20	0.34	0.15	4
Breads	0.16	0.27	0.05	5

Reference [19].

these two sources of TFAs. Endogenously formed TFAs were detected in breast cancer tissue specimens [10] and erythrocyte and lymphocyte membranes of children with dermatological diseases [11].

There are several reports concerning the industrial and ruminant TFA content of the foods consumed in various countries [12–17], and Craig-Schmidt and Rong summarized the worldwide consumption of TFAs [18]. In general, the TFA contents of Japanese foods are comparable to those of the corresponding foods in the countries. The amounts of TFA consumed differ among countries, and Japan is probably one of the countries that consumes the least TFA. An example of the industrial and ruminant TFA contents of foods marketed in Japan is shown in Table 1 [19]. The contents of TFA in currently available foods containing partially hydrogenated oils may be somewhat lower than the values shown in this Table, reflecting manufacturers' efforts to reduce TFA contents after the information in the Table was published. However, it should be noted that the industrial TFA content differs widely even in the same foods. Nevertheless, TFA intakes from various foods among Japanese are relatively lower than those in the US and EU, as shown in Table 2 [19]. In the national data, the average intakes of industrial TFA and ruminant TFA in Japanese were estimated to be 0.403 g/day (0.19% *E*) and 0.262 g/day (0.12% *E*), and the 99th percentiles of these values were 1.778 g/day (0.76% *E*) and 1.465 g/day (0.66% *E*) [20].

The measurement of the TFA contents in erythrocytes or plasma should be useful to understand the dietary intake of TFA for estimating the allowable dietary level of TFA as a biomarker [21].

Although the industrial TFA content of vegetable cooking oils without partial hydrogenation is relatively low, vegetable oils are the highest source of dietary TFA among other foods, followed by milk. It is therefore important to determine how much TFAs people in Japan are consuming from each type

of food, rather than only the TFA content of the food. The US Food and Drug Administration (FDA) issued a ban in 2015 (applied from June 2018) regarding the use of partially hydrogenated oils [22], and this resulted in a decrease in TFA intake in the US. However, it is impossible to construct healthy diets that are completely free from TFAs, as milk and meat contain TFAs. In light of this situation, it is important to precisely identify the effects of low levels of TFA intake on serum cholesterol levels.

## 2. *Trans* Fatty Acid and Serum Cholesterol

In 1990, Mensink and Katan [23] reported that the consumption of a meal containing TFAs equivalent to 10.9% *E* (percentage of total energy intake) increased the serum LDL-cholesterol and decreased HDL-cholesterol concentrations in healthy subjects. Thereafter, a number of intervention studies have been conducted, and they revealed that a TFA intake above 4%–6% *E* resulted in elevated serum LDL-cholesterol concentrations [24]. Several epidemiological studies provided evidence that the consumption of excess TFAs from industrial sources increases the risk of cardiovascular disease (CVD) [25–27]. Though the influence of excessive industrial TFA intake on both blood lipid levels and the risk of cardiovascular disease has been well established [28], definitive evidence regarding the tolerable upper level of TFA intake does not exist [29].

In the human body, TFAs are metabolized in the same way that *cis* fatty acids are metabolized [30]. TFAs appear to affect serum cholesterol levels through multiple mechanisms including the hepatic production, secretion, and catabolism of circulating lipoproteins [31, 32]. The addition of TFAs increased the secretions of cholesterol [33] and apolipoprotein B-100 [34] by human hepatoma HepG2 cells in vitro. TFA intake increases the plasma activity of cholesterol ester transfer protein (CETP), which is responsible for the transfer

TABLE 2: The *trans* fatty acid (TFA) intake from various foods in Japanese.

	Food intake g/day	TFA intake mg/day
Vegetable oils	8.2	114.4
Milk	101.6	92.2
Margarine and fat spread	1.2	84.0
Beef	15.0	78.2
Breads	33.5	54.6
Cakes, buns and pastries	7.4	52.3
Mayonnaise	3.3	40.8
Others of dairy products	8.2	39.5
Biscuits	1.8	32.3
Others of confectioneries	5.3	26.0
Butters	1.1	21.5
Cheeses	2.3	19.0
Japanese buns	6.4	13.1
Fermented milk and lactic acid bacteria beverages	23.1	9.9
Animal fats	0.1	1.4
Others	—	20.8
Total	—	700.0

Reference [19]; TFA intake was calculated from the mean intake and TFA content of each food group. The mean intake of each food group was calculated using the data of National Health and Nutrition Survey carried out for all ages, in a total of 8,762 men and women. To obtain the mean TFA contents of 19 food groups, 386 foods of TFA were determined by gas chromatography.

of cholesterol esters from high-density lipoprotein (HDL) to low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) [35]. These metabolic alterations may at least in part explain the increase of LDL-cholesterol and the decrease of HDL-cholesterol by dietary TFAs.

The World Health Organization (WHO) recommended that TFA intake should be <1% *E* in order to prevent noncommunicable diseases [36]. This level was inferred from the probable safety zone in multivariable regression analyses between TFA intake and the ratio of LDL-/HDL-cholesterol observed in intervention trials [37]. However, the experimental evidence is limited [24]. In fact, the available data concerning the influence of low-level TFA intake (i.e., around 1% *E*) on serum cholesterol levels are insufficient [38].

Since there is a possibility that TFAs and SFAs may be associated with the development of nonalcoholic fatty liver disease (NAFLD) [39], this disease should also be considered as a workable marker for the allowable level of dietary TFAs. There are no definite data available regarding the effect of a low level of TFA on the induction of NAFLD. The current evidence is qualitative. The metabolism of TFA in the hepatocytes may be essentially the same as that of saturated or unsaturated fatty acids, though there are some differences in the oxidation rate. For example, TFA (elaidic acid), compared to oleic acid, was reported to be a better substrate for mitochondrial and peroxisomal oxidation, but a poorer substrate for cellular and very-low-density lipoprotein triacylglycerol synthesis [40].

Although the serum cholesterol level is a good biomarker for CVD risk, attention should also be paid to the biomarkers of systemic inflammation and endothelial dysfunction to confirm the effect of TFA.

### 3. Observation Studies on *Trans* Fatty Acid

Mozaffarian et al. [41] described the relationships between TFA intake and serum lipid levels. In their study, the concentrations of serum LDL- and HDL-cholesterol were measured in 823 generally healthy women living in the US, and the subjects' TFA intake was assessed with the use of a semiquantitative food-frequency questionnaire. The analysis revealed that the subjects' TFA intake was inversely associated with their HDL-cholesterol levels, positively associated with the ratio of LDL- to HDL-cholesterol (Table 3), and not associated with the LDL-cholesterol level. van de Vijver et al. [42] investigated the association between TFA intake and serum lipids in volunteers from eight European countries. They studied 327 male and 299 female apparently healthy volunteers, and TFA intake was assessed using a dietary history. The study results indicated that TFA intake was not associated with the LDL- or HDL-cholesterol levels or the ratio of LDL- to HDL-cholesterol.

The mean TFA intake in the Mozaffarian et al. study was 2.7 g/day (1.3% *E*) and that in the van de Vijver et al. study was 2.2 g/day (0.91% *E*). Thus, the TFA intakes in these studies were almost equal, but there was a clear-cut difference in the association between TFA intake and cholesterol response. These observations suggest the existence of a threshold level causing the different effects of TFAs, but the influence of a difference in the composition of dietary fat should not be excluded.

We investigated the relationship between TFA intake and the serum cholesterol levels in 133 young Japanese women [43]. Their TFA intake was assessed with a self-reported written dietary record and a photographic record with a scale

TABLE 3: Serum LDL- and HDL-cholesterol levels per quintile of *trans* fatty acid (TFA) intake.

	Quintiles of TFA					<i>p</i> for trend
	Q1	Q2	Q3	Q4	Q5	
Mozaffarian et al. [41] <sup>1</sup>						
TFA intake, g/d	1.8	2.3	2.7	3.1	3.9	—
LDL-C, mg/dL	118	115	123	118	122	—
HDL-C, mg/dL	70	66	63	63	63	<0.001
LDL:HDL ratio	1.8	1.9	2.1	2.1	2.1	<0.01
van de Vijver et al. [42] <sup>2</sup>						
TFA intake, g/d	0.7	1.4	1.9	2.6	4.4	—
LDL-C, mg/dL	147	154	150	143	143	0.62
HDL-C, mg/dL	58	58	56	58	58	0.27
LDL:HDL ratio	2.72	2.8	2.9	2.7	2.7	0.58

<sup>1</sup>Mean fatty acid intake: saturated; 20 g/day (9.9% *E*), n-6; 10 g/day (5.0% *E*), n-3; 1.2 g/day (0.6% *E*), P/S; 0.56. <sup>2</sup>Mean fatty acid intake: saturated; 30.4 g/day (12.5% *E*), monounsaturated; 29.3 g/day (12.2% *E*), polyunsaturated; 11.5 g/day (4.7% *E*), P/S; 0.38.

card, and the TFA intake was calculated by dietitians using commercially available nutrient calculation software and the data from the Basal Report of Evaluation of TFAs in Food [19]. In this context, the amounts of TFA consumed in the study were more accurate than those in the preceding trials. Our findings revealed a significant correlation between total fat and TFA intakes, whereas TFA intake was not correlated with the total, LDL-, or HDL-cholesterol levels (Figures 1(a) and 1(b)). However, there was a significant correlation between the subjects' saturated fatty acid (SFA) intake and serum LDL-cholesterol levels (Figure 1(c)). The mean intakes of TFA and SFAs were 0.36% *E* and 8.3% *E*, respectively.

These results suggest that the amounts of TFA consumed by young Japanese women may in general not adversely affect their serum cholesterol levels. In light of the relatively low intake of TFA, it appears that more attention should be paid to the intake of SFAs rather than that of TFAs.

Very recently, Yang et al. [44] studied the association between plasma TFA and serum lipid levels before and after the US FDA enacted food-labeling regulations in 2006, and they observed a 54% reduction in plasma TFAs in US adult men and women from 1999-2000 to 2009-2010. Despite the significant reductions, the subjects' plasma TFA concentrations were significantly and consistently associated with serum cholesterol levels. Yang et al. speculated that there does not appear to be a threshold under which the association between the plasma TFA concentration and lipid profiles might become undetectable. The correlation between plasma TFAs and TFA intake is weaker ( $r = 0.30$ ) compared to that between TFAs in erythrocytes and TFA intake ( $r = 0.43$ ) [21], and the content of plasma TFAs may be affected by the serum triacylglycerol concentration. Because subjects with hypertriglyceridemia often have hypercholesterolemia too, considerable attention must be paid to the interpretation of a causal relation between plasma TFA and serum cholesterol levels. The triacylglycerol level in the highest-TFA-quintile group in the Yang et al. study was more than twice that in the lowest-TFA-quintile group (198 mg/dL versus 85.5 mg/dL in 1999-2000, 175 mg/dL versus 74.2 mg/dL in 2009-2010). Because the TFA intake was not described in the Yang et al.

study, a direct comparison of our study with their observational study may not be appropriate. In addition, in their study, serum lipid levels were investigated in adult men and women, whereas only female subjects participated in our study.

#### 4. Intervention Studies on *Trans* Fatty Acid

A number of intervention studies [31] have demonstrated that industrial TFA at dietary levels above 4% *E*–6% *E* increases blood LDL-cholesterol and reduces HDL-cholesterol. These observations suggest that industrial TFA is more likely to elevate the risk of CHD compared to dietary SFAs, which increase both LDL- and HDL-cholesterol. It was estimated that the intake of TFAs in several European and Asian countries is no more than 2% *E* on average [42, 43, 45], a level that is much lower than the amounts examined in several intervention studies. It is possible that the subgroups in these countries may be consuming higher amounts of industrial TFA, as not all the products are free from TFA even at the present time.

In the US, the intake of industrially produced TFAs decreased substantially after the introduction of the 2003 Nutrition Labeling rule, and the current mean intakes of industrial TFA are estimated to be around 1 g/day, or approx. 0.5% *E* (based on a 2000 kcal daily intake) [46]. However, the number of intervention studies examining the effect on blood cholesterol levels of comparatively low TFA (<2% *E*) is limited [24, 38]. Our summary of six intervention studies assessing the effect of low levels of industrial TFAs is provided as Table 4 [47–52].

In one such study [47], there were no differences in the serum LDL- or HDL-cholesterol levels of moderately hypercholesterolemic subjects who consumed a margarine-containing diet with 3.3% *E* of TFAs and those who consumed a control diet containing 0.55% *E* of TFA over a 5-week period. However, the LDL-cholesterol levels increased after the intake of a diet that included butter (containing 1.3% *E* of TFA) compared to the control diet. Because the content of SFAs in the butter- and margarine-containing diets differed

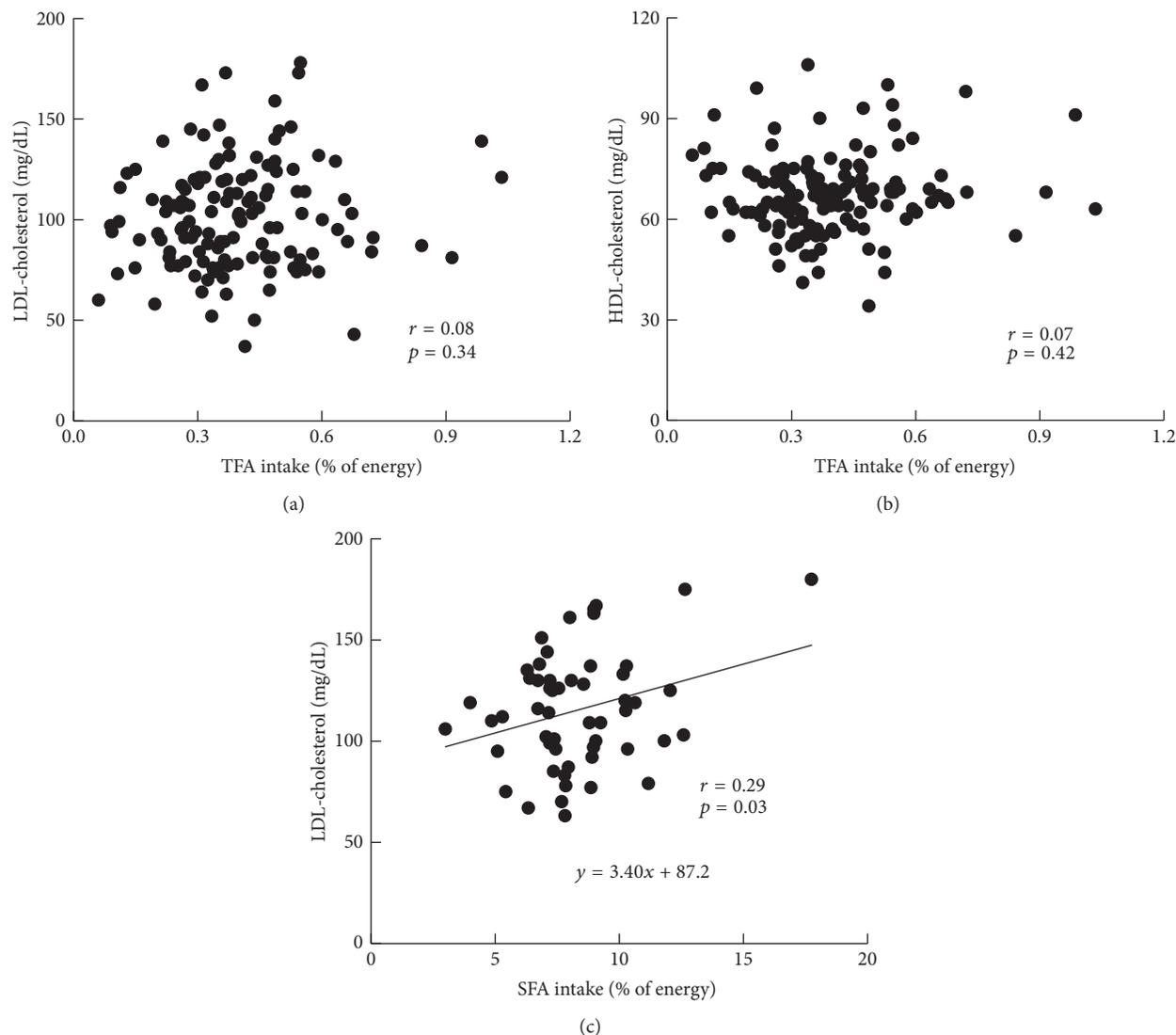


FIGURE 1: Relationships between the *trans* fatty acid (TFA) intake to LDL-cholesterol (a) and HDL-cholesterol (b), and the relationship between saturated fatty acid (SFA) intake to LDL-cholesterol (c) in 133 young Japanese women [43].

markedly (62% and 25%, resp.), it is difficult to attribute the observed change to TFA alone.

In another study [48], healthy subjects living in the US given a margarine meal containing 1.5% *E* TFAs for 5 weeks had significantly lower LDL-cholesterol levels compared to the values observed after a daily butter meal containing 0.5% *E* from TFAs. Again, there was a detectable difference in the contents of SFAs, and the margarine- and butter-containing diets contained 9% *E* and 16% SFAs. It is plausible that the increase in the LDL-cholesterol due to the butter-containing diet can therefore be attributed more to SFAs than to TFAs. SFAs might have a greater impact than TFAs if the content of TFAs is low in the daily diets on the basis of *E*% of intake.

In order to assess the effect of supplementation with 0.6% *E* or 1% *E* industrial TFAs, we carried out three intervention trials. We conducted a randomized, double-blind crossover trial with two treatment periods of 4 weeks each to assess

the effects of 0.6% *E* industrial TFA supplementation on serum cholesterol levels in 12 healthy young Japanese subjects ( $22.8 \pm 3.0$  years old) [49]. A 12-week washout period was set between each experimental period. The subjects consumed one cookie containing rapeseed oil (control) or partially hydrogenated rapeseed oil (TFA) every day throughout the treatment periods. The control and TFA cookies contained 0.04 g (0.02% *E*) and 1.13 g (0.6% *E*) of TFAs, respectively. Thus, the difference in dietary fatty acids other than TFAs was negligible in both groups. After the subjects' consumption of the control versus TFA diets, there were no significant between-group differences in the serum concentrations of total, LDL- or HDL-cholesterol. The number of subjects in this study, a total of 12, was too small to draw a conclusion. Larger-scale studies are required.

Under the same protocol [50], we conducted a randomized, double-blind parallel trial to assess the effects of 0.6% *E*

TABLE 4: Summary of six intervention trials that assessed the effect of low-level intakes of *trans* fatty acid (TFA) on serum cholesterol level.

Author (year)	Study design	Baseline demographics	TFA intake, E%	SFA intake, E%	Weeks	Serum cholesterol level
Lichtenstein et al. (1999) [47]	R, CR, CF	<i>n</i> = 36 Healthy, 63 y	Control: 0.55 TFA: 3.30 Butter: 1.25	Control: 7.3 TFA: 8.4 Butter: 16.7	5	Significant LDL-C and Total-C:HDL-C; Control < Butter No significance LDL-C and Total-C:HDL-C; Control versus TFA HDL; Control versus Butter; Control versus TFA
Denke et al. (2000) [48]	CR, CF	<i>n</i> = 226 Healthy adult, 41 y Healthy children, 12 y	Butter: 0.9 TFA: 1.5	Butter: 16 TFA: 9	5	Significant LDL-C, Total-C; TFA < Butter No significance HDL-C
Mensink (2008) [52]	CR, CF, DB, R	<i>n</i> = 44 Healthy, 41 y	TFA free: 0.2 Low TFA: 0.7	TFA free: 6.2 Low TFA: 2.3	3	Significant LDL-C, HDL-C, Total-C/HDL-C, and Total-C; Low TFA < TFA free
Takeuchi et al. (2011) [49]	CR, DB, R	<i>n</i> = 12 Healthy young, 23 y	Control: 0.1 TFA: 0.8	Control: 4.0 TFA: 3.0	4	No significance LDL-C, HDL-C, LDL-C/HDL-C, Total-C
Takeuchi et al. (2013) [51]	DB, P, R	<i>n</i> = 65 Healthy young, 18 y	Control: 0.4 TFA: 1.47	Control: 8.7 TFA: 9.8	4	No significance LDL-C, HDL-C, Total-C
Takeuchi et al. (2015) [50]	DB, P, R	<i>n</i> = 51 Healthy adult, 45 y	Control: 0.39 TFA: 1.09	Control: 8.0 TFA: 8.3	4	No significance LDL-C, HDL-C, Total-C

CR: crossover; CF: controlled feeding; DB: double-blind; HDL-C: HDL-cholesterol; LDL-C: LDL-cholesterol; P: parallel; R: randomized; SFA: saturated fatty acid; TFA: *trans* fatty acid; TG: triacylglycerol; Total-C: total cholesterol; y: year.

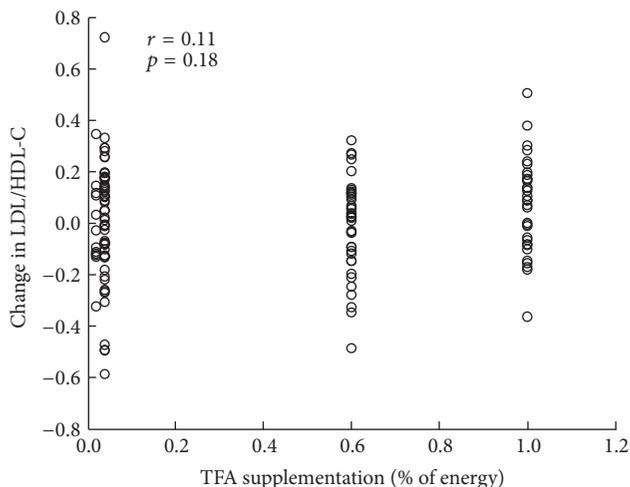


FIGURE 2: Meta-regression analysis of the change in the ratio of LDL-cholesterol/HDL-cholesterol (LDL/HDL-C) versus the supplementation level of *trans* fatty acids (TFAs) in three intervention trials [49–51].

industrial TFA supplementation on serum cholesterol levels in healthy adult Japanese women ( $44.6 \pm 4.2$  years old). Fifty-one volunteers consumed one cookie containing 0.6% *E* (the TFA diet group) or 0.04% *E* (the control diet group) of TFAs every day for 4 weeks. The volunteers also consumed approx. 0.4% *E* TFAs from their regular meals, and thus the mean TFA intakes of the control and TFA groups during the experimental period corresponded to 0.4% *E* and 1.1% *E*, respectively. Again, there were no significant differences in serum total, LDL- or HDL-cholesterol levels between the control and TFA groups. The results of this trial and our other trial described above [49, 50] indicate that dietary supplementation with 0.6% *E* industrial TFAs (a total TFA intake of approx. 1% *E*) would have little effect on serum cholesterol levels in young and adult healthy subjects.

In a series of trials [51], we addressed the effect of an additional 1% *E* industrial TFA intake on serum cholesterol levels. Sixty-five healthy young Japanese women consumed one cookie a day containing either 1% *E* or 0.04% *E* (control) of TFA for 4 weeks, in addition to their regular meals. The results again showed no significant differences in serum LDL- or HDL-cholesterol levels between the two groups. The results further supported that industrial TFAs at a dietary level of <1% *E* have little effect on serum cholesterol levels in healthy young women.

Indeed, our study protocol may not be appropriate to draw conclusions with respect to the number of participants and may not allow analyses at the subgroup level. However, we observed through three interventions that the plasma cholesterol levels did not change after industrial TFA intake in all participants. The background for this similarity is not apparent.

The results of our meta-regression analysis of changes in the ratio of LDL-/HDL-cholesterol versus the supplementation level of industrial TFAs in our three intervention studies are summarized in Figure 2. We found no significant correlation between the industrial TFA supplementation level and

changes in the ratio of LDL-/HDL-cholesterol. The results of these three intervention trials support the soundness of the 2003 WHO recommendation of <1% *E* of TFAs [36].

We have also studied the effects of 1% *E* industrial TFA supplementation on serum cholesterol levels in healthy adults with different obesity-related gene polymorphisms and observed little effect on serum cholesterol levels, regardless of genotype (here, the single nucleotide polymorphism) of fat mass- and obesity-associated gene or beta-3 adrenergic receptor (unpubl. data).

## 5. Linoleic Acid and *Trans* Fatty Acid

Mensink [52] compared the effects of a high-palmitic acid, *trans*-free semiliquid fat with those of a high-oleic acid, low-*trans* semiliquid fat on the serum lipids of healthy subjects. The results indicate that a high-oleic acid, low-*trans* fat has a more favorable impact on the serum lipoprotein profile than a *trans*-free fat high in palmitic acid. Mensink concluded that it is not possible to pinpoint a fat or oil as “good” or “bad” without considering its complete fatty acid composition.

In addition to SFAs, polyunsaturated fatty acids (PUFAs), in particular linoleic acid, favorably influence the blood cholesterol level, a well-known phenomenon commonly accepted in the dietary guidelines regarding the prevention of heart diseases. It has been pointed out that the cholesterol-raising effect of TFAs is attenuated by linoleic acid, as in the case of SFAs [53]. Hu et al. [54] also confirmed in a multivariable analysis of their observation study that the relative risk of cardiovascular disease was lowest when the intake of PUFAs was highest and that of TFAs was lowest. More recently, Hunter [24] proposed that the effect of TFAs on cholesterol levels can be counteracted by the addition of linoleic acid above the 6% *E* level.

Unfortunately, these indications have been almost ignored in the evaluation of the TFA-cholesterol relationship. Since the PUFA/SFA ratio (*P/S* ratio) of diets in Japanese is considerably high compared to that of people in Western countries, at 2 : 1 versus 1 : 1, it is probable that the expression of the cholesterol-raising effect of TFAs is being attenuated in Japanese. In any case, it is indeed important to consider the complex interaction of dietary fatty acids, not TFA alone but saturated and unsaturated fatty acids too.

## 6. Conclusion

Since the consumption of TFAs is currently decreasing in many countries, it is extremely important to clarify the influence that low dietary levels of TFAs exert on circulating cholesterol levels and cardiovascular diseases. The numbers of investigations of the effects of low-level TFAs on these parameters are insufficient. The results of the few existing studies indicate that TFA at <1% *E* has little adverse effect on the serum cholesterol level. A study conducted in Australia indicated that the relative impact of 0.59% *E* TFA exposure on CHD mortality is limited [55]. On the other hand, another epidemiological study suggested that there does not appear to be a threshold affecting serum lipid levels [44].

Regarding SFAs, although it is generally accepted that an excessive intake of SFAs adversely affects serum cholesterol

levels, this conclusion is controversial. The number of the countries that meet the Food and Agriculture Organization (FAO)/WHO recommendation of a mean intake of SFAs of <10% *E* is limited: only 11 of 40 countries reviewed [56], and it is thus necessary to reduce the industrial TFA intake without increasing the SFA intake. A decrease in total fat intake is generally accompanied by a lower intake of not only TFAs but also SFAs. This is the simplest correspondence, but it is also a troublesome approach. Hence, the determination of the tolerable upper level of industrial TFA intake based on reliable evidence is indispensable together with the removal of TFA from foods. In addition, when evaluating the impact of industrial TFAs on our health, it is most important to consider the total dietary pattern, not industrial TFA alone.

### Conflicts of Interest

The authors declare that there are no conflicts of interest regarding this study.

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## Review Article

# Do Omega-3/6 Fatty Acids Have a Therapeutic Role in Children and Young People with ADHD?

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*Background.* Attention deficit hyperactivity disorder (ADHD) is a debilitating behavioural disorder affecting daily ability to function, learn, and interact with peers. This publication assesses the role of omega-3/6 fatty acids in the treatment and management of ADHD. *Methods.* A systematic review of 16 randomised controlled trials was undertaken. Trials included a total of 1,514 children and young people with ADHD who were allocated to take an omega-3/6 intervention, or a placebo. *Results.* Of the studies identified, 13 reported favourable benefits on ADHD symptoms including improvements in hyperactivity, impulsivity, attention, visual learning, word reading, and working/short-term memory. Four studies used supplements containing a 9 : 3 : 1 ratio of eicosapentaenoic acid : docosahexaenoic acid : gamma linolenic acid which appeared effective at improving erythrocyte levels. Supplementation with this ratio of fatty acids also showed promise as an adjunctive therapy to traditional medications, lowering the dose and improving the compliance with medications such as methylphenidate. *Conclusion.* ADHD is a frequent and debilitating childhood condition. Given disparaging feelings towards psychostimulant medications, omega-3/6 fatty acids offer great promise as a suitable adjunctive therapy for ADHD.

## 1. Background

Attention deficit hyperactivity disorder (ADHD) is a common child-onset neurodevelopmental disorder occurring in children, adolescents, and adults, with an estimated prevalence of 5 to 7 per cent across cultures [1]. ADHD tends to be more common in boys than girls and is highly heritable, with pre- and perinatal factors also being implicated, although its definite cause remains unknown [2]. Although the rate of ADHD declines with age, at least half of children with the disorder will go on to have symptoms in adulthood [3]. The condition can impact heavily on mental health and education, lead to antisocial behaviour and personal dysfunction, and increase mortality risk [4]. Medications used to treat ADHD typically include methylphenidate (MPH; also, known as Ritalin), amphetamine, and atomoxetine which typically assume that there is a dopamine/norepinephrine deficit, although the aetiology of this condition is more complex [5]. Whilst MPH may ameliorate some comorbidities [6] it has been found to be ineffective in eliminating symptoms in 50 per cent of cases [7, 8]. Parents also appear to be

concerned about the long-term effects of their children using medications such as MPH [9].

Long-chain polyunsaturated fatty acids (LCPUFA) and particularly omega-3 fatty acids have been under the spotlight for decades. They are key regulators of brain neurotransmission, neurogenesis, and neuroinflammation, all having an important role in the prevention and treatment of psychological and behavioural dysfunction disorders [10]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are two fatty acids that are highly concentrated in the brain, exhibiting antioxidative, anti-inflammatory, and antiapoptotic effects, with these contributing to neuron protection [11].

The omega-6 fatty acid gamma linolenic acid (GLA) is also important in the generation of arachidonic acid (ARA) which is abundantly present in the brain [12, 13]. A recent meta-analysis found that combinations of omega-3 and omega-6 fatty acids (EPA and GLA) helped to improve symptoms of inattention in children with ADHD [14]. Brain lipids within cell membranes also act as signalling mediums, supporting neurotransmitter function with omega-3 fatty acids thought to play a key role in this which may help in the

prevention of anxiety disorders [15]. Laboratory research has also identified that omega-3 fatty acids may act in a similar way to “antipsychotics,” possibly by acting on brain receptors and helping to restore oxidative balance [16].

Omega-3 deficiencies have been found to alter dopaminergic and serotonergic systems, potentially modifying cerebral receptors in specific regions of the brain [17]. EPA and DHA are regarded as “essential fatty acids (EFAs)” that need to be obtained from food or supplement sources as they cannot be made in sufficient amounts by the human body [11]. The ratio of fatty acids (omega-6 : omega-3) which complete for the same enzyme pathways can also influence neurotransmission and prostaglandin formation, both of which are crucial in the maintenance of normal brain function [18, 19]. Furthermore, as the storage of the omega-3 fatty acids is limited, a continual exogenous supply is needed to obtain suitable levels [20].

A number of studies have measured LCPUFA status in individuals with ADHD. One study conducted on young adults (22.3 to 24.3 years) found the proportion of omega-3 fatty acids was significantly lower in the plasma phospholipids and red blood cells of ADHD participants compared with controls, whilst levels of saturated fatty acids were higher [21]. Another investigation found that whilst teenagers with ADHD consume similar amounts of omega-3 and omega-6 fatty acids to controls, their DHA status was significantly lower, indicating metabolic differences in fatty acid handling in those with ADHD [22]. Similarly, another trial showed that the proportions of saturated and polyunsaturated fatty acids were higher and lower, respectively, in paediatric patients with ADHD, compared with controls again indicating differences in lipid profiles [23]. Further meta-analytical evidence has concluded that children and young people with ADHD have elevated ratios of blood omega-6/3 indicating disturbances in fatty acid metabolism in these individuals [24].

Given that the human brain is nearly 60 per cent fat and the central role that EFAs have to play in the structure, synthesis, and functions of brain neurotransmitters [25], the present article evaluates evidence on whether LCPUFAs have a therapeutic role in the management of ADHD. Particular focus will be given on their potential effects in the management of ADHD along with their role as an adjunctive therapy.

## 2. Methods

**2.1. Approach.** The National Centre for Biotechnology Information (NCBI) search engine (PubMed) was used to extract relevant publications. English-language, human, randomised controlled trials (RCTs) published between 2001 and March 2017 were included. Data files were extracted from the NCBI collection depository and imported into Covidence software used to create systematic reviews.

**2.2. Exclusion/Inclusion Criteria.** Publications were excluded if they were not a RCT, did not use participants with ADHD, or were conducted on older adults with ADHD. For inclusion studies needed to be conducted on children or young people (up to 18 years of age), participants were considered to

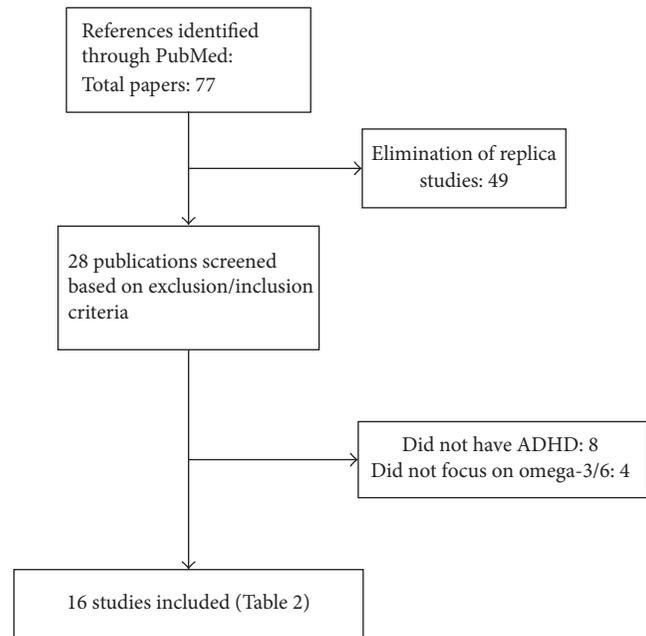


FIGURE 1: Algorithm of qualifying publications.

have ADHD at baseline and were taking an omega-3/6 supplement, including EPA, DHA, or GLA. Publications were further included if the full text was available or could be purchased.

The search terms “attention deficit hyperactivity disorder” or “ADHD” were combined with “long-chain n-3 fatty acids”, “omega-3/6 fatty acids”, “docosahexaenoic acid”, “eicosapentaenoic acid”, and “gamma linoleic acid”. Data extracted from each article included (1) author(s) and country of research, (2) subjects (gender, number of participants), (3) mean age, (4) study design and methods, (5) dose of supplement, and (6) main findings.

## 3. Results

The NCBI search identified 77 papers. After a further adjustment for replica papers, 28 articles remained for assessment. Of these, 12 papers were discarded after reviewing the abstracts and article content as they did not meet the inclusion criteria. This left 16 RCTs for general review. Figure 1 shows the algorithm of qualifying publications. Of these, one study was conducted in the United Kingdom, five in Europe, one in the United States, one in Mexico, two in Australasia, four in Asia, and two in the Middle East.

**3.1. Definitions.** As shown in Table 1, all of the publications identified included children or young people with ADHD at baseline. Most studies diagnosed ADHD according to the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV) criteria. Others used methods such as the Conners’ Parent Rating Scale (CPRS) and parent-reported learning difficulties [26–28]. Some studies focused more specifically on certain ADHD subtype. For example, Widenhorn-Müller et al. (2014) included the inattentive and

TABLE 1: Methods used to screen for ADHD.

Author	Definition used
Barragán et al. (2017)	ADHD of any subtype. Diagnosed according to the DSM-IV criteria and CGI-S scale.
Bos et al. (2015)	ADHD diagnosis confirmed by a trained researcher using the DISC-P.
Matsudaira et al. (2015)	ADHD diagnosis confirmed through a semi-structured interview based on the DSM-IV criteria.
Milte et al. (2015)	Diagnosis of ADHD or parent-rated symptoms >90th percentile on the CPRS and parent-reported learning difficulties.
Wu et al. (2015)	ADHD diagnosed according to DSM-IV and the Chinese version of CPRS. These rating scales about learning, attention, and behaviour were completed by the teachers and either parent(s) or guardians.
Widenhorn-Müller et al. (2014)	Met DSM-IV criteria for the ADHD combined subtype (hyperactive-inattentive) and the primarily inattentive or the hyperactive/impulsive subtype were included in the trial.
Manor et al. (2013)	Children were included if they had a score of at least 1.5 standard deviations above the normal for the patient's age and gender in the Teacher-Rated ADHD Rating Scale-IV School Version.
Hariri et al. (2012)	Conners' Abbreviated Questionnaires scores for hyperactivity were greater than 14.
Johnson et al. (2012)	Participants met DSM-IV criteria for a diagnosis of ADHD.
Milte et al. (2012)	Diagnosis of ADHD or parent-rated symptoms >90th percentile on the CPRS and parent-reported learning difficulties.
Perera et al. (2012)	All children in the program were clinically diagnosed using DSM-IV supported by positive scores in Swanson, Nolan, and Pelham version IV (SNAP) parent and teacher evaluation.
Gustafsson et al. (2010)	Clinical diagnosis of ADHD of combined type (fulfilling DSM-IV criteria A-E) with any neuropsychiatric comorbidity and who had been evaluated for pharmacological treatment.
Johnson et al. (2009)	Participants met DSM-IV criteria for a diagnosis of ADHD of any subtype, scoring at least 1.5 SD above the age norm for their diagnostic subtype using norms for the ADHD Rating Scale-IV-Parent Version.
Raz et al. (2009)	Parents were asked to present a formal ADHD diagnosis. The child performed a continuous performance test, while one of the parents filled in the essential fatty acids deficiency questionnaire and the DSM-IV questionnaire.
Hirayama et al. (2004)	Diagnosed or suspected as AD/HD according to DSM-IV and diagnostic interviews including behaviour observation by psychiatrists. In a strict sense, eight subjects might not be AD/HD according to the DSM-IV criteria, but two psychiatrists attending the summer camp strongly suspected them as AD/HD.
Voigt et al. (2001)	Previously been given a diagnosis of ADHD by a physician. Confirmatory diagnostic interview with a neurodevelopmental paediatrician to confirm responses to the telephone interview and to ensure that each met DSM-IV.

Key: ADHD, attention deficit hyperactivity disorder; CGI-S scale; Clinical Global Impressions-Severity scale; CPRS, Conners' Parent Rating Scale; DISC-P, Diagnostic Interview Schedule for Children-Parent Version; DSM-IV; Diagnostic and Statistical Manual of Mental Disorders.

hyperactive/impulsive subtypes within the trial whilst Voigt et al. (2001) studied those with oppositional defiant or conduct disorders. Other trials used adapted parental/researcher screening tools alongside the DSM-IV [29, 30].

**3.2. Omega Fatty Acids.** A total of 16 RCTs studied interrelationships between combinations of omega-3/6 fatty acids and ADHD symptoms (Table 2). Of these 13 reported beneficial effects, though the levels of effect appeared to depend on the dose of the intervention, ratio of the fatty acids, quality of the RCT, and ADHD subtype under investigation.

One of the most recent studies found that children (aged 6 to 12 years) receiving omega-3/6 fatty acids (Equazen) providing 558 mg EPA, 174 mg DHA, and 60 mg GLA in a 9:3:1 ratio over a period of 12 months did not need such a high dose of MPH to manage and reduce their ADHD symptoms (0.8 mg/kg/day versus 1.0 mg/kg/day). The completion rate was also higher in this group, whilst the

withdrawal rate and the incidence of adverse events were significantly lower. These findings indicate that omega-3/6 fatty acids may act as a useful adjunctive therapy to MPH, helping to improve tolerability, dosing, and adherence [31].

A 12-week RCT comprised of 76 male adolescents with ADHD using a similar dose of fatty acids found that supplementation improved blood levels of EPA, DHA, and total omega-3 fatty acids, though no effects on aggression, impulsivity, or anxiety were seen, possibly due to the smaller study sample size and shorter study length of this trial [32]. Two other trials have been undertaken using a similar 9:3:1 ratio of EPA, DHA, and GLA, respectively [33, 34]. This work was the first to trial omega-3/6 fatty acids finding that 1 in 8 patients benefited and experienced a reduction of more than 50% of ADHD symptoms, with strongest results seen amongst boys and those with ADHD inattentive subtype [34]. Later research by the same team of scientists found that omega-3/6 supplementation significantly improved the fatty

TABLE 2: Information extracted from trials looking at LC3PUFAs and ADHD.

Reference and country	Subjects M/F and sample size	Mean age	Study design and methods	Dose of supplement	Main findings
[31] Mexico	90 children (60 M, 30 F)	6–12 years Mean age 8.27 years	12-month trial (unblinded); MPH, omega-3/6 or a combination	Equazen: 558 mg EPA, 174 mg DHA, and 60 mg GLA (9 : 3 : 1 ratio)	Significantly better scores on ADHD. Adverse events were numerically less frequent with omega-3/6 or MPH + omega-3/6 than MPH alone.
[41] Netherlands	40 boys with ADHD and 39 matched, typically developing controls	Aged 8–14 years	16-week trial	10 g of margarine daily, enriched with either 650 mg of EPA/DHA or placebo	EPA/DHA supplementation improved parent-rated attention in both children with ADHD and typically developing children. Phospholipid DHA level at follow-up was higher for children receiving EPA/DHA supplements than placebo.
[32] United Kingdom	76 M adolescents with ADHD	12–16 years, mean = 13.7 years	12-week trial	Equazen: 558 mg EPA, 174 mg DHA, and 60 mg GLA (9 : 3 : 1 ratio)	In the treatment group, supplementation enhanced EPA, DHA, and total omega-3 fatty acid levels.
[26] Australia	90 Australian children with ADHD symptoms higher than the 90th percentile on the Conners' Rating Scales	7 to 12 years	4-month crossover study evaluating literacy and behaviour up to 12 months	Supplements rich in EPA, DHA, or LA	Increased erythrocyte EPA + DHA was associated with improved spelling ( $p < 0.001$ ) and attention ( $p < 0.001$ ), reduced oppositional behaviour ( $p < 0.003$ ), hyperactivity ( $p < 0.001$ ), cognitive problems ( $p < 0.001$ ), DSM-IV hyperactivity ( $p = 0.002$ ), and DSM-IV inattention ( $p < 0.001$ ).
[27] China	179 children with lower IQs or ADHD to receive	7 to 12 years	3-month trial: evaluated effects on visual acuity	Ordinary eggs or eggs rich in EPA and DHA	Both groups of children showed a significant improvement in visual acuity ( $p < 0.05$ ); however, visual acuity in the study group was significantly better than that of the control group ( $p = 0.013$ ).
[37] Germany	95 children diagnosed with ADHD according to DSM-IV criteria	6–12 years	16-week trial	Omega-3 fatty acid mix	Improved working memory correlated significantly with increased EPA, DHA, and decreased ARA.
[39] Israel	200 children diagnosed with ADHD	6–13 years	15-week trial followed by an open-label extension	300 mg PS-omega-3/day	Study results demonstrate that consumption of PS-omega-3 by children with ADHD, is safe and well tolerated, without any negative effect on body weight or growth.
[38] Malaysia	103 children	6–12 years	8-week trial	635 mg EPA, 195 mg DHA	Significant reduction in levels of CRP in the omega-3 group and significant increase in SOD and glutathione reductase. Significant improvement in ASQ-P score (measure of hyperactivity).
[33] Sweden	75 children and adolescents with DSM-IV ADHD	8–18 years	3-month trial. Omega-3/6 (Equazen) or placebo, followed by 3 months of open phase	Omega-3/6 (Equazen) or placebo Equazen: 558 mg EPA, 174 mg DHA, and 60 mg gamma linoleic acid (9 : 3 : 1 ratio)	Subjects with more than 25% reduction in ADHD symptoms were classified as responders. Compared to nonresponders, the 6-month responders had significantly greater n-3 increase at 3 months and decrease in n-6/n-3 ratio at 3 and 6 months ( $p < 0.05$ ).

TABLE 2: Continued.

Reference and country	Subjects M/F and sample size	Mean age	Study design and methods	Dose of supplement	Main findings
[28] Australia	90 Australian children with ADHD symptoms higher than the 90th percentile on the Conners' Rating Scales	7 to 12 years	4-month trial	Supplements rich in EPA, DHA, or safflower oil	Increased erythrocyte DHA was associated with improved word reading and lower parent ratings of oppositional behaviour. These effects were more evident in a subgroup of 17 children with learning difficulties.
[29] Sri Lanka	Children with ADHD $n = 48$ active group, $n = 46$ placebo	6–12 years	6-month trial	Capsule containing n3 and n6 (fish oil) and cold-pressed evening primrose oil	Statistically significant improvement was not found at 3 months of treatment between groups but was evident at 6 months of treatment ( $p < 0.05$ ) with inattention, impulsiveness, and cooperation with parents and teachers.
[40] Norway	92 children with ADHD	7–12 years	15-week RCT	0.5 g EPA versus placebo	EPA improved CTRS, inattention/cognitive subscale ( $p = 0.04$ ), but not Conners' total score.
[34] Sweden	75 children and adolescents with DSM-IV ADHD	8–18 years	3-month trial. Omega-3/6 (Equazen) or placebo, followed by 3 months of open phase	Equazen: 558 mg EPA, 174 mg DHA, and 60 mg GLA (9 : 3 : 1 ratio)	A subgroup of 26% responded with more than 25% reduction of ADHD symptoms and a drop of Clinical Global Impression scores to the near-normal range. After 6 months, 47% of all showed such improvement. Responders tended to have ADHD inattentive subtype and comorbid neurodevelopmental disorders.
[30] Israel	73 unmedicated children with a diagnosis of ADHD	7–13 years	7-week trial	480 mg LA, 120 mg ALA, placebo: 1000 mg of vitamin C	Both treatments ameliorated some of the symptoms, but no significant differences were found between the groups in any of the treatment effects.
[35] Japan	40 AD/HD (including eight AD/HD-suspected) children who were mostly without medication	6–12 years	2-month trial	Foods containing fish oil (fermented soybean milk, bread rolls, and steamed bread; 3.6 g DHA/week from these foods)	DHA-containing foods did not improve ADHD-related symptoms. Visual short-term memory and errors of commission (continuous performance) significantly improved in the control group compared with the changes over time in the DHA group.
[36] USA	63 children with ADHD, all receiving effective maintenance therapy with stimulant medication	6–12 years	4-month trial	345 mg DHA	No statistically significant improvement in any objective or subjective measure of ADHD symptoms.

Key. ADHD, attention deficit hyperactivity disorder; ALA, alpha-linolenic acid; ARA, arachidonic acid; CRP, C-reactive protein; CTRS, Connor Teacher Rating Scale; DHA, docosahexaenoic acid; DSM-IV; Diagnostic and Statistical Manual of Mental Disorders; EPA, eicosapentaenoic acid; F, female; GLA, gamma linoleic acid; LA, linoleic acid; M, male; MPH, methylphenidate; PS, phosphatidylserine; SOD, superoxide dismutase.

acid composition amongst study “responders,” that is, those with more than a 25 per cent reduction in ADHD symptoms [33].

Three studies used functional foods providing LC3PUFA. In a double-blind RCT, the ingestion of 10 g margarine daily providing 650 mg EPA/DHA improved parent-rated attention in children with ADHD after 16 weeks in 8–14-year-olds who continued with their usual medication. Another trial using “omega eggs” providing EPA and DHA found that daily consumption by 7–12-year-olds over 3 months significantly improved visual acuity and the red blood cell fatty acid profile of children with lower intelligent quotients or ADHD, indicating that the DHA content of ordinary eggs may not be sufficient [27]. Another work giving 6- to 12-year-olds ADHD DHA-enriched foods showed that ADHD symptoms did not improve though there were some significant improvements in short-term memory and errors of continuous performance [35].

Three studies concluded that there were limited associations between omega-3/6 fatty acid supplementation and ADHD outcomes. In one study, Conners’ Parent and Teacher Rating Scale was not regarded as being sensitive enough to detect small improvements in the behaviour of male adolescents [32]. Another work found that a supplement providing 480 mg of linoleic acid and 120 mg of  $\alpha$ -linolenic acid ameliorated some ADHD symptoms amongst 7–13-year-olds, although no significant differences were found, possibly because children were unmedicated [30]. Earlier work providing 6- to 12-year-olds with 345 mg DHA over 4 months did not find this to ameliorate ADHD, indicating that a longer trial period and inclusion of arachidonic acid may have been needed [36].

Remaining studies showed general benefits. An Australian study found that children with ADHD who had increased erythrocyte EPA + DHA levels had significantly improved spelling and attention and reduced oppositional behaviour, hyperactivity, and cognitive problems [28]. An omega-3 fatty acid mix taken over 16 weeks by German children aged 6 to 12 years also increased EPA + DHA erythrocyte levels and improved working memory but had no other effects on behaviour [37]. A short 8-week trial reported significant improvements in hyperactivity scores after supplementation with EPA + DHA [38]. In a trial where children had been taking MPH, supplementation with omega-3 and omega-6 fatty acids in the ratio of 1.6:1 led to significant improvements in inattention and impulsiveness, along with cooperation with parents and teachers after 6 months, indicating this was a safe and effective adjunctive therapy [29].

Other trials showed findings to be more prominent in certain subgroups. For example, a large trial of 200 children found that supplementation with phosphatidylserine-omega-3 reduced ADHD symptoms in a subgroup of hyperactive-impulsive, emotionally and behaviourally dysregulated ADHD children compared with the placebo [39]. Another work found that erythrocyte DHA levels increased after 4 months of supplementation with 4 capsules daily providing either (1) 108 mg DHA and 1109 mg EPA, (2) 1032 mg DHA and 264 mg EPA, or (3) 1467 mg of linoleic

acid [28]. The study also found that higher doses of DHA helped to improve the literacy and behaviour in children with ADHD, particularly in a subgroup with learning difficulties [28]. Norwegian work showed that 0.5 g EPA after 15 weeks improved symptoms in two ADHD subgroups: positional and less hyperactive/impulsive children [40].

#### 4. Discussion

The aetiology of ADHD is complex and multifactorial though diet, nutrition, and abnormalities in the metabolism of LCP-UFA are thought to have underlying roles [21, 22]. The present review has shown that omega-3 and omega-6 fatty acids have an important role to play in the management of ADHD. Previous work has shown that the tolerability of omega-3 fatty acids given to individuals with ADHD is high with only mild side effects reported such as incidental nose bleeds and gastrointestinal discomfort [42]. Severe side effects have not been documented and these minor complaints are regarded as being less severe than methylphenidate side effects [42].

Taken together, a growing body of clinically proven evidence suggests that dietary supplementation using omega-3/6 PUFAs may help to augment conventional ADHD treatments. Research carried out in Mexico at the National Health Institute with children prescribed with MPH and taking omega-3/6 fatty acids found that they required lower doses of the prescription medicine and experienced fewer medication-related side effects [31]. Similarly, other work has shown that omega-3/6 supplementation reduced behavioural and learning difficulties in children with ADHD that was refractory to MPH treatment alone [29]. Another RCT concluded that EPA was a safe complementary treatment option in omega-3 deficient ADHD children, with scope to benefit ADHD subgroups who are less responsive to stimulant treatments [40]. A recent review of 25 clinical trials has also concluded that two patients groups, in particular, could benefit from omega-3 fatty acids. The first is those with mild ADHD where omega-3 supplements could replace stimulant medications. The second is those with severe ADHD where omega-3 supplements could reduce the amount of stimulant medication being used, in turn, potentially reducing symptoms from the medications side effects [42].

These studies are further supported by evidence from meta-analytical studies. Evidence collated from ten trials comprised of 700 children has shown that omega-3 supplementation, with higher doses of EPA had modest effects in the treatment of ADHD, indicating potential roles in augmenting traditional pharmacological treatments, whilst providing an option for families who may decline other psychopharmacologic options [43]. An earlier meta-analysis also concluded that omega-3 fatty acids offer promise as a possible supplement to traditional therapies [44]. Interestingly, a series of interviews about treatment experiences showed that over half (52%) of parents expressed initial reluctance towards psychostimulants. Once psychostimulants were used by children and adolescents with ADHD, 73% concurrently used other treatments [45]. These findings indicate that parents are

concerned about their children using psychostimulants and are looking for accompanying treatment options.

In terms of study outcomes, most focused on ADHD symptoms. Whilst reduced hyperactivity and impulsivity were reported in most studies [26, 29, 31, 38, 40], other outcomes such as improved attention [41], visual acuity [27], improved word reading [28], and working/short-term memory were also observed [35, 37]. These findings indicate that LCPUFA supplementation has far-reaching effects, having additional benefits for learning. A recent 6-month 2-phase randomised trial with 154 children aged 9 and 10 years showed the omega-3/6 fatty acid supplementation improved reading ability in mainstream children and improved cognitive measures in children with attention problems, defined as those with ADHD symptom scores above the median [46].

With regards to dose a 9:3:1 ratio of EPA (558 mg) to DHA (174 mg) and GLA (60 mg) was used in four studies [31–34]. In the largest and longest studies, this was associated with improved hyperactivity and impulsivity subscores [31] and reduced ADHD symptoms [33], especially in the inattentive ADHD subtype and those with comorbid neurodevelopment disorders [34]. Other work using 635 mg EPA and 195 mg DHA also led to significant improvements in ADHD scores [38]. Studies using lower doses (345 mg DHA) tended not to yield significant findings in terms of ADHD symptoms [36]. Taken together, it appears that higher doses of fatty acids are needed to generate measurable effects. The ratio of omega-6 to omega-3 in studies and especially the ARA/DHA ratio may also have impacted on study outcomes, as this is regarded as being important for membrane fluidity [47].

Inconsistencies of lack of findings in some trials may have been attributed to interventions being too short. As erythrocytes survive in the body for 120 days, supplementation trials shorter than 12 weeks (84 days) may not be sufficient enough to detect changes in LCPUFA compositions [32]. Equally, as the turnover of fatty acids in the brain is thought to be rather low in 6- to 12-year-olds, longer periods of supplementation and/or higher doses may be needed to modify the fatty acid content of the central nervous system [36]. It should also be considered that some studies used different tools to assess ADHD symptoms, measures of attention, and scales of hyperactivity, some of which may be more sensitive than others. On a final note, it should be considered that the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5) is now out which includes new diagnostic groups such as “disruptive mood regulation” which have potential to be applied in future studies [48]. This could possibly increase prevalence rates of mental health disorders in future trials [48]. Continued research using the latest DSM-5 criteria, along with larger and longer interventions (more than 12 weeks), is now needed.

## 5. Conclusion

In conclusion, ADHD is a debilitating neurodevelopmental disorder that can impact heavily on children and young people's behaviour, mental health, education, and social/family lives. Whilst conventional medications have a role to play in the management of ADHD symptoms, new clinically

trials evidence indicates that omega-3/6 supplementation programmes can provide a promising adjunctive therapy, lowering the dose of psychopharmacologic medications needed and subsequently improving compliance with these. It also appears that parents are looking for complementary treatments for their children to use alongside traditional treatments.

## Abbreviations

ARA:	Arachidonic acid
ADHD:	Attention deficit hyperactivity disorder
CPRS:	Conners' Parent Rating Scale
CTRS:	Conners' Teacher Rating Scale
DHA:	Docosahexaenoic acid
DSM:	Diagnostic and Statistical Manual of Mental Disorders
EFA:	Essential fatty acids
EPA:	Eicosapentaenoic acid
GLA:	Gamma linoleic acid
LCPUFA:	Long-chain polyunsaturated fatty acids
MPH:	Methylphenidate
RCT:	Randomized controlled trial.

## Disclosure

The views expressed are those of the author alone and Equazen personnel had no role in writing the review.

## Conflicts of Interest

The author declares no conflicts of interest.

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## Clinical Study

# Erythrocyte Omega-3 Fatty Acid Content in Elite Athletes in Response to Omega-3 Supplementation: A Dose-Response Pilot Study

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**Introduction.** Supplementation of Omega-3 fatty acids (n-3FA) in athletes is related to the anti-inflammatory and/or antioxidant effect and consequently its action on all the processes of tissue restoration and adaptation to physical stress. **Objective.** Evaluate the Omega-3 Index (O3Ix) response, in red blood cells, to supplemental EPA + DHA intake in the form of high purity and stable composition gums (G), in elite summer athletes. **Method.** Twenty-four summer sport athletes of both sexes, pertaining to the Olympic Training Center in Spain, were randomized to two groups (2G = 760 or 3G = 1140 mg of n-3 FA in Omegafort OKids, Ferrer Intl.) for 4 months. Five athletes and four training staff volunteers were control group. **Results.** The O3Ix was lower than 8% in 93.1% of all the athletes. The supplementation worked in a dose-dependent manner: 144% for the 3G dose and 135% for the 2G, both  $p < 0.001$ , with a 3% significant decrease of Omega-6 FAs. No changes were observed for the control group. **Conclusions.** Supplementation with n-3FA increases the content of EPA DHA in the red blood cells at 4 months in a dose-dependent manner. Athletes with lower basal O3Ix were more prone to increment their levels. The study is registered with Protocol Registration and Results System (ClinicalTrials.gov) number NCT02610270.

## 1. Introduction

Docosahexaenoic acid (DHA C22: 6) and eicosapentaenoic acid (EPA C20: 5) are the most important polyunsaturated fatty acids (FAs) known as long-chain Omega-3 (n-3). Both are considered essential FA and are important components of the lipid bilayer of cell membranes. For its incorporation, they should be synthesized from essential fatty acid, alpha-linolenic acid (ALA), or taken directly preformed in the diet. At present, it is suggested that the administration of purified EPA and/or DHA can offer a wide range of beneficial effects [1], ranging from the plasticity, neuronal development, and functionality of the central nervous system [2] to the treatment and prevention of chronic diseases with an inflammatory component [3]. The indication of diet supplementation

in sport activities is due to the anti-inflammatory and/or antioxidant effect and consequently its action on all the processes of tissue restoration and adaptation to the physical stress and training, from the connective tissue to the neural development [4]. However, it has been shown that DHA is more effective than EPA in modulating specific markers of inflammation as well as blood lipids [5]. The epidemiological studies related to the impact of the supplementation on the physical activities are focused on supposed actions of the n-3 FA on muscle metabolism and tissue recovery [6], functional performance, and inflammation [7] and, with a very specified indications on sport, as exercise induced asthma [8], traumatic brain injury [9] or injury recovery, training adaptation, and sarcopenia [10]. Those studies report the use of different doses, concentration, and duration, and

they do not always reference the previous state of the amount of these FA in cell membranes or usual diet.

The use of biomarker-based approaches has made it possible to study and evaluate with criteria the Omega-3 Index (O3Ix), which is defined as the sum of EPA+DHA content in red blood cell (RBC) membranes and has been considered a risk factor for death from coronary heart disease and as a biomarker of n-3 FA status [11]. An O3Ix of  $\geq 8\%$  has been recommended for its cardiovascular protective effect [12] and has been postulated to be adequate also in the elite athlete [13]. Until now, Von Schacky et al.'s study [13] is the only one that targets elite athlete as reference values for O3Ix. Surprisingly, those subjects, with a diet geared toward better performance, demonstrate not only a low consumption of fish but also a low level O3Ix, far from the desired range over 8%. Well conducted studies have confirmed that dietary or supplemental intake of EPA + DHA is associated with higher levels of the O3Ix [14–16].

In the present study, the objective was to model the O3Ix response to supplemental EPA + DHA intake within attainable dietary ranges in athletes. We had the primary hypothesis that if adherence to treatment is adequate and diet is maintained, a change in the O3Ix would be observed depending on the dosage of the supplementation. This information will be important for making better EPA + DHA recommendations to achieve a target O3Ix for future studies on the evaluation of the effect of n-3 FAs in the different physical exercise activities.

## 2. Methods

This project has tried to follow the guidelines in the design, conduct, and reporting of studies of human health benefits of foods, summarized by Welch et al. [17].

**2.1. Participants.** Eligibility criteria were based on selecting healthy athletes of both sexes, from a specific age range and committed to the project. Athletes belonged to different summer sport federations and lived at the Olympic Training Center (OTC) of Sant Cugat del Vallés (Spain). Exclusion criteria included any type of inflammatory process or the use of anti-inflammatory medications, consumption of n-3 FA supplements and n-3 FA-supplemented foods in the past 3 months. planning to change dietary habits, or training schedule. In order to establish a control group (C), three athletes and four members of the technical staff met the same requirements for maintaining diet, weight, and daily activity level. All of them had their official sport preparticipation screening evaluation, signed informed consent, and went through a medical examination that included medical history, physical examination, anthropometric measurements, complete blood count, and standard chemistry panel to rule out the presence of any newly developed illness or inflammatory process. Diet analysis was conducted to evaluate, through a week registration, all the nutritional components of their diet. The study protocol was approved by the Ethical Committee of the Sports Council of the Generalitat de Catalunya 08/2014/CEIEGC. All procedures followed were

in accordance with the ethical standards of the Helsinki Declaration of 1975, revised in 2000.

**2.2. Intervention.** This was a randomized, parallel-group with control subjects study. Participants ( $n = 24$ ), 13 women (55%), were randomized to take two different dosages of n-3 FAs daily, as fish oil supplements in the form of gums (Omegafort OKids, Ferrer Intl.) for 4 months, the approximate time of lifespan of the RBC [17] and time that it takes the composition in FA of cell membranes to reach a new steady state [18]. To ensure even distribution among treatment groups, a computer randomization scheme was used, which is stratified by sex and age and balanced the size of the two blocks. Eligible participants were assigned to treatment group 2G, two gums daily, or 3G, three gums daily (i.e., 2G = 760 mg or 3G = 1140 mg of n-3 FA). All researchers and clinicians, except the Head Dept., and participants, were blinded to treatment assignment. Analysis of the fish oil gums verified that they contained 35,7% DHA, 27,7% EPA, 3,32% docosapentaenoic acid, 18,5% oleic acid, 3,1% vaccenic acid, 1,4% stearic acid, 1,2% palmitic acid, 1,7% arachidonic acid, and small amounts of other fatty acids (Table 1).

All participants were instructed to maintain their training schedule, diet and activity level, and their usual consumption of fatty fish as well as their no consumption of any supplementation during the study course. The participants were contacted monthly to ensure gummies intake compliance and to discuss any difficulties in following the treatment. Also, participants reported back to the Research Department after 8 weeks to return the gum boxes and to receive new supplies.

**2.3. Blood Sample Collection.** Blood samples collection was performed in the fasting state by venipuncture and harvested in  $K_2$ -EDTA-containing tubes before and after the intervention (12 hours without any intake with the exception of water, 48 hours without alcohol, and 12 hours without perform vigorous exercise). After each blood sample collection, a complete blood count and a general biochemistry profile were obtained. Whole blood was centrifuged at 1500  $\times g$  for 15 minutes at 4°C. Except for assays that required unfrozen specimens, samples were stored at  $-80^\circ C$  until they were analyzed.

**2.4. RBC Fatty Acid Analysis.** Erythrocytes were separated from the plasma by centrifugation (3000 rpm, 1500  $\times g$ , for 10 min) and washed with an equal volume of saline. These erythrocytes resuspended with saline were stored in a freshly 0.01% butylated hydroxyl toluene- (BHT-) treated Eppendorf vials at  $-80^\circ C$ . The fatty acids composition was determined using the method by Lepage and Roy [19]; erythrocyte's membranes were extracted from aliquots of 200  $\mu L$  of erythrocyte suspensions and the fatty acids converted to methyl esters by reaction with acetyl chloride for 60 min at 100°C. Methyl ester fatty acids (FAME) were separated and analyzed by gas chromatography performed on a Shimadzu GCMS-QP2010 Plus gas chromatograph/mass spectrometer (Shimadzu, Kyoto, Japan) and peaks were identified through mass spectra and by comparing with respect to a reference FAME mixture (GLC-744 Nu-Chek Prep. Inc., Elysian MN, USA) the elution

TABLE 1: Fatty acid composition of the diet supplement (gums).

Fatty acid	Composition* (%)	1 gum (mg)	2 gums (mg)	3 gums (mg)
<i>MUFA</i>				
Oleic (18:1, n-9)	18,5	111	222	334
Vaccenic acid (18:1, n-7)	3,1	19	37	56
Other MUFA	1,4	8	17	25
<i>SFA</i>				
Stearic acid (18:0)	1,4	8	17	25
Palmitic acid (16:0)	1,2	7	14	22
Other SFA	0,5	3	6	9
<i>PUFA n-6</i>				
Arachidonic acid (20:4, n-6)	1,7	10	20	30
Other n-6	1,9	12	23	35
<i>PUFA n-3</i>				
DHA (22:6, n-3)	35,7	214	428	643
EPA (20:5, n-3)	27,7	166	332	499
DPA (22:5, n-3)	3,3	20	40	60
Other n-3	3,5	21	42	63
Total DHA + EPA		380	761	1141

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; n-6, Omega-6; n-3, Omega-3; DHA, docosahexaenoic acid, EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid. \*To determine the quantitative fatty acid (FA) composition, FAs were analyzed by gas chromatography-mass spectrometry. The results express in molar % of total fatty acids.

pattern and relative retention times of FAME. The O3Ix was calculated as erythrocyte (EPA + DHA)/(total fatty acids)  $\times$  100% (percentage molar of total fatty acids) [11].

**2.5. Statistical Analysis.** Mean changes from baseline to 4 months were calculated and compared between groups using paired *t*-test. Differences among groups were tested by analysis of variance using a general linear model. All statistical tests were performed at a significance level of 0.05. Adjusted *p* < 0.05 was considered significant. Continuous data are reported as the mean  $\pm$  SD. For descriptive purposes, categorical data are presented as percentages. The statistical software program SPSS for Windows, version 13.0, was used for all data analysis.

### 3. Results

A total of 41 individuals were screened between May and June 2014; twenty-four of them met the inclusion criteria and were randomly assigned to any treatment group. Besides, seven subjects were selected as control subjects. Since the study is to determine the level of impregnation in the tissue not a placebo but a control that maintained the same diet was considered, all baseline measurements were completed during the selection process. One subject withdrew from the study between baseline and the final point due to an injury during training, and he voluntarily dropped out from the study. In addition, two subjects from the 2G and one from the 3G groups comment the lack of compliance at the first control and desired to abandon the study. Among study completers, compliance was presumably total in all groups. Anyway, real compliance was assessed by interrogation, by counting

returned capsules, and by analysis of red cell phospholipid fatty acid composition, which reflects dietary fatty acid composition. Those athletes, whose percentage of EPA in red cell membrane fatty acids differed,  $\geq 2$  Standard Deviations from the mean of the respective treatment group, were also considered noncompliant [20]. Noncompliant patients were excluded from the valid case analysis, but RBC analysis was performed confirming the absence of increase of EPA and consequently in O3Ix (Figure 1). Finally, from the 24 volunteers included in groups 2G and 3G, and once applied compliance criteria, the sample was reduced to nine and eleven subjects in both groups, respectively. Adherence to the therapy was 82% for 2G and 92% for 3G groups.

No significant differences were found between groups of participants with respect to baseline and diet characteristics. Nevertheless, the control group presented some differences related to age, height, and weight and slightly to the caloric intake, basically referred to as the technical staff. RBC FA content was similar between groups (Table 2). The mean O3Ix at study entry (mean  $\pm$  SD) was  $5.1 \pm 1.0\%$  with a range of 3.3% to 7.8%. On average, there were no gender differences in relation to O3Ix. Body weight, BMI, blood pressure, and heart rate did not change significantly during the study.

Distribution of O3Ix shows a Gauss distribution where a 93.1% of the athletes had values lower than 8% after EPA + DHA supplementation (Figure 2). The EPA + DHA supplementation increased the O3Ix in a dose-dependent manner (Table 2), affecting both EPA and DHA which resulted in a significant increase in O3Ix of 144% (116–157%) for the 3G dose and 135% (120–149%) for the 2G dose (*p* < 0.001). Participants who had lower basal O3Ix were more prone to increment their levels (Figure 1). Omega-3 FAs increase was

TABLE 2: Erythrocyte fatty acids profile of the participants as a function of the dose through the study.

Mean (SD)	2G (N:9)			3G (N:11)			Control (N:6)			Control versus 2G/3G	
	Basal	Post	$p \leq$	Basal	Post	$p \leq$	Basal	Post	$p \leq$	Basal	Post
SFA	44.7 (1.1)	45.6 (1.5)	NS	45.2 (0.7)	45.8 (0.8)	0.05	45.4 (0.9)	45.8 (0.8)	NS	NS	NS
MUFA	20.9 (1.6)	21.3 (1.2)	NS	20.0 (0.9)	21.0 (1.0)	0.05	21.0 (1.8)	21.9 (1.8)	NS	NS	NS
PUFA	34.4 (1.5)	33.6 (1.5)	NS	34.8 (0.9)	34.0 (1.2)	0.05	33.5 (1.4)	32.3 (1.4)	0.05	NS	0,05
n-6	27.9 (1.8)	25.2 (1.4)	0.01	28.6 (1.4)	25.4 (1.6)	0.001	27.5 (1.6)	26.7 (1.2)	NS	NS	0,05
n-3	6.4 (1.1)	8.4 (0.9)	0.001	6.3 (1.2)	8.6 (1.0)	0.001	6.1 (0.9)	5.6 (1.0)	NS	NS	0,001
n-6/n-3	4.5 (1.1)	3.0 (0.4)	0.01	4.7 (1.0)	3.0 (0.5)	0.001	4.6 (1.0)	4.9 (1.1)	NS	NS	0,01
O3Ix	5.0 (0.8)	6.8 (0.7)	0.001	4.9 (1,0)	7.0 (0,8)	0.001	4.8 (0,9)	4.4 (0,9)	NS	NS	0,001

Group 2G, two oil gums daily; 3G, three oil gums daily. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; n-6, Omega-6; n-3, Omega-3; and O3Ix, Omega-3 Index.

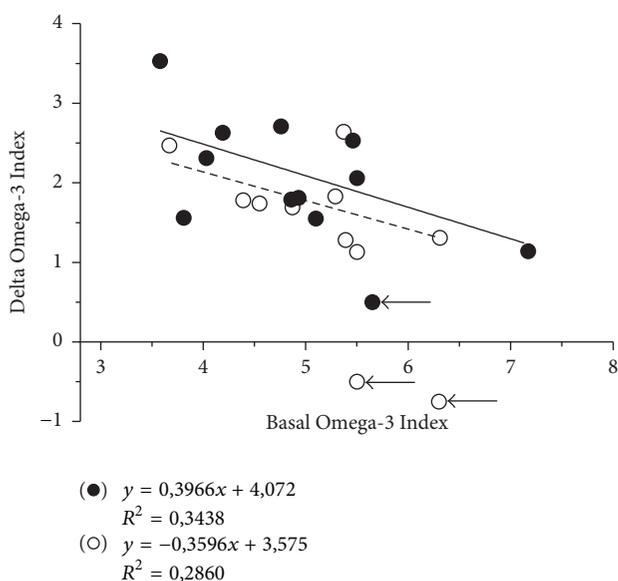


FIGURE 1: Changes on Omega-3 Index in athletes after 4 months of supplementation in function of the dose: (○) group 2G, two oil gums daily; (●) group 3G, three oil gums daily. The arrows show those subjects that were unable to comply with the treatment. These data were not added to the statistical evaluation; it is only to show the place in this figure. The line of tendency reflects the delta change of all subjects depending on the basal level and different doses.

accompanied by a significant decrease in total Omega-6 (n-6) FAs in both intervention groups from 28.0 to 25.2% (2G,  $p < 0.01$ ) and 28.6 to 25.4% (3G,  $p < 0.001$ ), respectively. No change in O3Ix was observed for the control group from baseline (93%). No significance changes were observed on the other FA except for MUFA, in the 3G group ( $20.0 \pm 0.9$  to  $21.0 \pm 1.0\%$ ).

#### 4. Discussion

The present study evaluated the effect of EPA + DHA supplementation in athletes on the O3Ix response. The required amount of Omega-3 intake is not clearly defined, although there are certain recommendations based on individualized dietary patterns by state and age [21]. The World

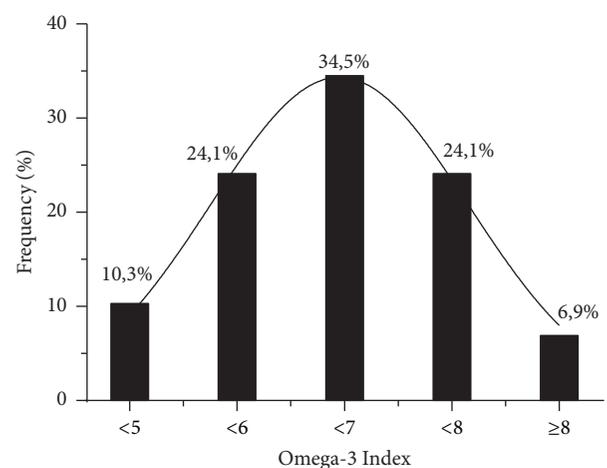


FIGURE 2: Distribution of the basal Omega-3 Index in the population of athletes of this study.

Health Organization establishes a need for consumption of 250–500 mg/day of EPA + DHA [22], while the International Society for the Study of Fatty Acids and Lipids [23] adjusts it to 500 mg/day. Certain effects as cardioprotective [24] or triglyceride decrease [25] at doses of 1 to 3–5 g/day of EPA + DHA are advised. In sport activities, similar dose also has been recommended [26]. However, the actual consumption of fatty fish does not become desirable for the different population groups and, as a result, the Omega-3 daily intake from the diet is insufficient. In Spain, the consumption of Omega-3 is 1.5 g/day with an average consumption of 0.2–2 g/day and it is estimated that Americans consume <100 mg/day of EPA + DHA [27]. While it may be considered satisfactory, the type of n-3 FAs consumed is basically ALA. As they have not the same biological effects and conversion of ALA to EPA and DHA is not carried out effectively, the EPA and DHA ingested are well below the recommendations of 0.25 to 0.5% of the daily energy, only reaching 0.05%. Moreover, the Omega-6/Omega-3 ratio in the same population is 15-16/1, well above the 4-5/1 that is considered suitable [28]. Therefore, the need for supplementation with n-3 FAs is real in all population age ranging from children to the elderly, and athletes are not out of this population.

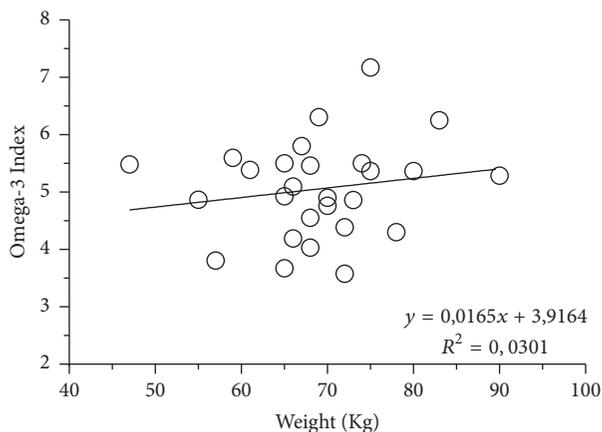


FIGURE 3: Relation of Omega-3 Index with body weight. There is a low correlation of the O3Ix distribution related to body weight in the population of this study.

We have found an average baseline O3Ix of 5.0% in the participants of the present study; this level is consistent with previous studies of adults' subjects reporting to be low habitual fish consumers [29] and in the same range of the athletes evaluated by Von Schacky et al. [13]. Our results suggest that athletes, with low fish intake who increased their dietary intake by 760 to 1140 mg/day of EPA + DHA, would experience an increase in O3Ix values of about 1.8% to 2.1% by mean, lower results than those observed when period of treatment is longer [30–32] over 3.5% and near to 5% increase (see Supplementary Material available online at <https://doi.org/10.1155/2017/1472719>). From these, it can be estimated that an average healthy adult with a low O3Ix (i.e., 5.0%) would require 1.5–2 g/day of EPA + DHA for 4 months or more to increase 2 index points and bring it to the desired 8%. Our different response could be related to the different ratio of EPA/DHA (44% EPA/56% DHA) as it has been argued by the different velocity of incorporation depending on that percentage [33]. That asseveration is not in agreement with Browning et al. study [30], with similar relation between DHA/EPA and fish oil content. Under this perspective, more concern has to be considered with the amount of Omega-3 offered and treatment duration, considering the different quantity and quality of products, which present different bioavailability [34].

Body weight does not explain the variability of O3Ix in response to EPA + DHA supplementation in our data (Figures 3 and 4). Flock et al. [15] demonstrated a greater tendency to respond to a given EPA + DHA intake in individuals with lower body weight, suggesting that, to achieve a target O3Ix, consumption recommendations of EPA + DHA should be made on the basis of body weight, in a similar way as it happens with current dietary protein requirements. This discrepancy with our data could be due to the fact that the weight of the athletes is usually adequate to their physical activity needs and their caloric and nutrient intake, in all cases under their daily requirements (except for PUFA) [35]. In Folk et al.'s study [15], it was estimated that the requirements to increase its O3Ix from 4.3% to 8% in individuals weighing

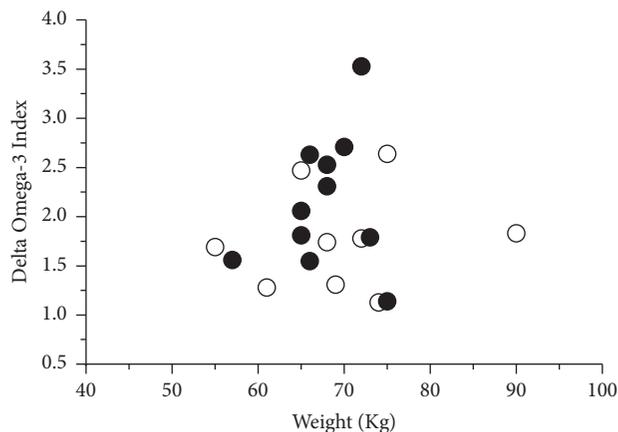


FIGURE 4: Delta change of the Omega-3 Index related to body weight and dosage: (○) group 2G, two oil gums daily; (●) group 3G, three oil gums daily. The changes on values of the O3Ix are more related to dosage than the previous body weight.

75 kg were about 1.2 g/day of EPA + DHA; 0.9 g/day if they weighed 55 kg; or 1.5 g/day for individuals weighing 95 kg. As it can be observed in Figures 3 and 4, that observation does not correlate similarly in the subjects of our intervention. It seems that this adjustment of doses is not needed in the athletes if their weight is the expected for performance and nourishment is adequate. Possibly in athletes a higher dose needs to be administrated to achieve the desired 8% in O3Ix.

We also found that the EPA + DHA incorporation into the membranes of RBC follows a dose-dependent increase in both groups assayed, which is potentially saturable. This suggests that EPA + DHA concentrations in the membrane of RBC could be regulated to some degree and it could reach a saturation point. This finding is consistent with previous observation that individuals, with higher content of EPA + DHA in their RBC membranes, incorporate, at a slower rate, additional EPA + DHA than those presenting a lower baseline level [36].

We did not find that women on average had a higher O3Ix than men at study entry. This relationship between sex and O3Ix seems not to be related only to the difference in body weight [37] as it appears when this factor was accounted for in the model by adjusting the dose per unit of body weight [38]. In the same study, the participants, that are more physically active, tended to experience greater elevation in O3Ix as dose increased, suggesting that something related to exercise may enhance incorporation of EPA + DHA in RBC membranes in individuals taking fish oil supplements.

With respect to the MUFA change in the 3G group, with lower levels from the beginning, it can be attributed to the 334 mg of MUFA ingested daily, but it was not statistically different. MUFA consumption can be beneficial when replacing carbohydrate and saturated fat in the diet but not when replacing PUFAs. Although MUFA showed to have positive impact on surrogate markers, the potential impact of MUFA intake alone on disease outcomes, such as CVD or diabetes, remains unclear. Therefore, the role of MUFAs on health and disease when consumed as an eating pattern (i.e.,

Mediterranean diet) should be more studied [39]. Maybe the explanation of the favorable properties lies in the oleic acid, the MUFA most abundant fatty acid found in food, or, more particularly, in its minor though highly bioactive molecules, the phenolic compounds, which have been associated with the prevention of the main chronic diseases [40].

The results presented here show that elite athletes, despite following a diet presumably healthy and suitable for their sporting activity, have an O3Ix well below that recommended and that, despite a strict follow-up of the DHA + EPA supplement in the diet during the study period, they do not reach the desired levels. Given the potential role of DHA and EPA in cardiovascular protection, preservation of the central nervous system, repair of the musculoskeletal system, and significant influence on cellular behavior and responsiveness to signals [41], it would be advisable to increase the intake of these Omega-3 FAs in the diet of these athletes. A study of its long-term benefits is guaranteed.

## 5. Strengths and Limitations

The strengths of this study were as follows: sample of subjects with elevated physical activity who were under a controlled and healthy diet, single blind study design that compared two doses of EPA + DHA with respect to a control group with a high adherence to the treatment, adequate duration of supplementation, 4 months, and the use of validated analytical methods to determine biomarker response to treatment.

Limitations include the scarce sample, the homogeneity of white, young, and healthy population, and the lack of background genetic data.

## 6. Conclusions and Further Research

It is confirmed that athletes even with a presumably healthy diet have low O3Ix. The marine-derived n-3 FA supplementation increases the RBC EPA + DHA content in a dose-time related manner. Future studies need to assess how EPA or DHA individually or different ratios of both affect O3Ix response and to clarify the potential correlation between changes in the O3Ix and its effect on prevalence and severity of the injury recovery.

### Disclosure

The funder had no role in the design and conduct of the study and in the collection, analysis, and interpretation of the data.

### Conflicts of Interest

The authors declare that they have no conflicts of interest.

### Authors' Contributions

Drobnic Franchek, Pons Victoria, and Banquells Montserrat carried out the design, selection of individuals, follow-up of the diets, statistic evaluation, and discussion of the results. Cordobilla Begoña, Rueda Félix, and Domingo Joan Carles carried out the methodology design of blood extraction,

Omega-3 blood analysis, and discussion of the results. Banquells Montserrat carried out the blood analysis samples and manipulation; Pons Victoria evaluated the daily diet of the athletes. Drobnic Franchek and Domingo Joan Carles wrote the article. All authors read and approved the final manuscript.

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