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# Metabolic effect of docosahexaenoic acid supplementation in different doses and formulations (ethyl- and glyceryl-) in hypercholesterolemic rats

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ABSTRACT

Docosahexaenoic acid (DHA) supplemented in pharmaceuticals and functional foods is commonly re-esterified as ethyl esters (DHA-EE) or alternatively as triacylglycerol (DHA-rTAG). The aim of this study was to investigate the modulation of plasmatic lipid profiles of two doses of DHA-EE and DHA-rTAG. During 8 weeks, Wistar rats were supplemented with high and low doses of DHA-EE and DHA-rTAG integrated in a hypercholesterolemic diet. DHA supplementation reduced total weight gain, adiposity index, HDL-cholesterol and glucose plasmatic concentration associated to the dose administered. T-CHO and TAG seem to be reduced more efficiently by high doses of DHA-rTAG, while adipose tissue distribution and reduction of FFA are improved with DHA supplementation, regardless of the dose and form of supplementation. Therefore, metabolic effects of DHA supplementation may be determined not only by the dosage administered, but also by the ester form in which this molecule is presented.



## 1. Introduction

A significant amount of epidemiological data and numerous interventional studies have demonstrated the positive relationship between fish consumption and the reduction of cardiovascular mortality and morbidity (Djoussé, Akinkuolie, Wu, Ding, & Gaziano, 2012; Eslick, Howe, Smith, Priest, & Bensoussan, 2009; Mozaffarian, Bryson, Lemaitre, Burke, &

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*Abbreviations*: LC-PUFA, long-chain polyunsaturated omega-3 fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; EFSA, European Food Safety Authority; EE, ethyl esters; TAG, triacylglycerols; rTAG, re-esterified TAG; hiEE, high dose DHA-EE; lowEE, low dose DHA-EE; hiTAG, high dose DHA-rTAG; lowTAG, low dose DHA-rTAG; T-CHO, total-cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; FFA, free fatty acids; TCA cycle, tricarboxylic acid cycle

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Siscovick, 2005). Long-chain polyunsaturated omega-3 fatty acids (LC-PUFA) have been identified as the main active cardioprotective ingredients in fish, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Supported by convincing scientific evidence and numerous intervention studies, the European Food Safety Authority (EFSA) recently published a positive opinion on the substantiation of health claims for functional foods containing EPA and/or DHA and the maintenance of several health related functions (EFSA Panel on Dietetic Products & Nutrition & Allergies (NDA), 2010). Daily intake recommendations for DHA and EPA were specified for each health claim, ranging from 250 mg/day to 2 g/day (EFSA Panel on Dietetic Products, Nutrition & Allergies, 2010). The American Heart Association recommends LC-PUFA intakes of 1–4 g according to the fasting triglyceride levels of the individual (Kris-Etherton, Harris, & Appel, 2003). The average consumption of fish products in many countries provides LC-PUFA lower than this amount (SACN, 2004; Health Council of The Netherlands, 2006). The gap between actual fish derived LC-PUFA intake and the daily-recommended intake is often filled with LC-PUFA supplements or functional foods enriched in LC-PUFA (Barrow, Nolan, & Holub, 2009; Kralovec et al., 2009; Reglero et al., 2008). With this aim, LC-PUFA are obtained from marine sources and usually re-esterified as ethyl esters (EE), which is the form of prescription drug in US and most EU countries (Schuchardt et al., 2011a). It differs from the naturally occurring form of DHA, such as fish oil, which is composed of triacylglycerols (TAG). Various studies suggest better bioavailability of DHA when re-esterified into TAG (rTAG) rather than EE (De Schrijver, Vermeulen, & Backx, 1991; Dyerberg, Madsen, Møller, Aardestrup, & Schmidt, 2010; Neubronner et al., 2011; Schuchardt et al., 2011a, 2011b), which may cause an effect on DHA biological efficiency (Schuchardt et al., 2011a). Despite the large number of investigations and institutional daily intake recommendations, there is controversy about the role of LC-PUFA in cardiovascular disease prevention. A recently published meta-analysis (Rizos, Nitzani, Bika, Kostapanos, & Elisaf, 2012) concluded that LC-PUFA supplementation is not associated with a reduction in cardiovascular, which contrast previous publications reporting links between LC-PUFA intake and reduction of cardiovascular diseases or associated risk factors (Bernstein, Ding, Willett, & Rimm, 2012; Eslick et al., 2009; Marik & Varon, 2009; Wei & Jacobson, 2011) . Also, while there is a consensus about the beneficial effect of LC-PUFA in plasma TAG concentrations (Kris-Etherton et al., 2003), there are discrepancies about its role in cholesterol regulation (EFSA Panel on Dietetic Products, Nutrition & Allergies, 2010). Many extrinsic (consumer age and gender, health status or environmental interferences) and intrinsic (dose, formulation, molecular form or dietary interferences) factors may alter the clinical outcome of LC-PUFA making difficult the comparisons among studies. In this context, animal models are a valuable tool for preclinical comparison of the effect of a limited number of factors in controlled conditions. Since the metabolism of glycerol and ethanol follows different routes and it may modify the bioavailability of the LC-PUFA transported, it is hypothesized that the metabolic effect of LC-PUFA would differ when it is supplemented in form of EE or TAG. The aim of this study was to investigate the efficiency of DHA-EE and DHA-rTAG as the main dietary source of lipids in the management of plasma lipid profiles in a hypercholesterolemic animal model.

## 2. Materials and methods

### 2.1. Highly concentrated DHA functional oils

The functional oils used in this study were provided by Nutra Omega Biotecnologica Oleica S.L. (NOBO, A Coruña, Spain). Refined fish oil (12EPA/18DHA) was chemically esterified by ethanolysis to produce EE and subsequently PUFA esters were concentrated in a process that included urea fractionation and molecular distillation, to reach >94% LC-PUFA in Ethyl Ester form. The DHA-EE with high DHA content was enzymatically transesterified with commercial lipases to produce DHA-rTAG containing 82.6% DHA, 6.5% docosapentaenoic acid ($n-3$ clupanodonic acid), 5.3% EPA and 5.6% of a mixture of essential $n-6$ and $n-9$ fatty acids, as analyzed by gas chromatography. The functional oils were produced and bottled in the absence of oxygen and were stored and transported at 4 °C in the absence of light.

### 2.2. Animals and diets

Male Wistar rats (200–225 g body weight) were obtained from an accredited supplier (Charles River Laboratories España, S.A. Barcelona, Spain), housed with free access to food and water, and maintained under a normal light–dark cycle in the Experimental Surgery Department of La Paz University Hospital (registration number: 280790001941). After an adaptation period, animals were distributed among four experimental groups and a control group ($n = 8$ per group). For 8 weeks, the experimental groups were fed a purified, essentially fat-free diet (Harlan Laboratories Inc. Indianapolis, Indiana, USA) supplemented with high (10%) or low doses (4%) of DHA-EE or DHA-rTAG oils (groups hiEE, lowEE, hiTAG and lowTAG, respectively). The diet of all groups was supplemented with 2% sheep wool cholesterol (Sigma Aldrich Chemie GmbH, Steinheim, Germany) and 0.4% cholic acid (Sigma Aldrich Chemie GmbH, Steinheim, Germany) as described elsewhere (Lecumberri et al., 2007). Nutrient content and energy distribution of each diet is summarized in Table 1. Throughout the intervention, the health of individual animals was monitored through veterinary observation. Food intake was assessed daily while animal weight gain was controlled weekly. The study protocol was approved by the Institutional Animal Ethics Committee of La Paz University Hospital, and procedures were performed in accordance with the Spanish law for protection of animals for experimentation and other research purposes: RD 1201/2005.

### 2.3. Sample collection and tissue preparation

Before dietary modification (baseline) and after 4 weeks of intervention, blood aliquots were obtained from the tail vein of fasted animal to monitor plasma lipid profiles (total-cholesterol [T-CHO], HDL-cholesterol [HDL-C], LDL-cholesterol [LDL-C] and TAG) and glucose. After 8 weeks on the diet, fasted animals were subjected to complete exsanguination

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