University of Chester

Department of Clinical Sciences and Nutrition

MSc, Diploma, Certificate

In

Exercise & Nutrition Science

Module Title: Research Project

Module Code: XN7523

Module Tutor: Prof. Stephen Fallows

Effect of n-3 Fatty Acid Supplementation in Hyperlipidemic Patients Taking Statins on Lipid Profile, Including Small Dense LDL

Student: Gediz Dogay, 1624263

2016

Year of Intake

23 October 2017

Date submitted

Review Paper Word Count: 4430

Research Paper Word Count: 4505

DECLARATION OF OWN WORK

I, the undermentioned, hereby declare that the work done for this assignment is my own work, references that I utilised are properly indicated in the bibliography and this work has not been submitted previously in support of a degree qualification or other course.

Gediz Dogay

Acknowledgements

With special thanks to my supervisor Dr. Sohail Mushtaq for his support, guidance and encouragement throughout this research, as well as Dr. Ceri Nicholas for giving me the opportunity for this educational experience.

I particularly would like to thank my husband Melo for believing in me in this and for his unwavering support.

CONTENTS

LIST OF TABLES

Literature Review

Research Paper

LIST OF FIGURES

LIST OF ABBREVIATIONS

CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FA	Fatty acids
FFA	Free fatty acids
HDL-C	High-density lipoprotein cholesterol
HMG-CoA	5-hydroxy-3-methylglutaryl-coenzyme A reductase
HR	Hazard ratio
LDL-C	Low-density lipoprotein cholesterol
MI	Myocardial Infraction
MUFA	Mono unsaturated fatty acids
Non-HDL-C	non-High-density lipoprotein cholesterol
OM-3 FA	Omega-3 fatty acids
PUFA	Poly unsaturated fatty acids
sdLDL-C	Small&dense low-density lipoprotein cholesterol
SFA	Saturated fatty acids
TAG	Triacylglycerol
TC	Total cholesterol

LITERATURE REVIEW

EFFECTS OF HIGH DOSE OMEGA-3 SUPPLEMENTS IN BLOOD LIPIDS

AND LIPOPROTEINS, INCLUDING small & dense LOW DENSITY

LIPOPROTEIN CHOLETEROL

Abstract

Cardiovascular disease (CVD) has been, and still is, the number one cause of death across the world, with 1 of each 3 person dying due to CVD. One of the known causes of CVD is atherogenic dyslipidemia. Dyslipidemia can be treated pharmacologically with HMG-CoA reductase inhibitors (statins) and/or with dietary changes. Clinical guidelines emphasize the use of statins for people at risk, yet accompanied dietary interventions have also shown promising results. Omega-3 fatty acids (OM-3 FA), especially animal derived EPA and DHA are recognised as anti-atherogenic nutrients with several proven beneficial impacts on cardiovascular outcomes such as reduced CV mortality or reduced CV events such as stroke and MI. Studies that investigate the role of EPA and DHA in lipid management therapy point to significant reductions in blood TAG, non-HDL-C and VLDL-C. LDL-C might slightly increase with high-dose n-3 supplementation but this effect is neutralised when combined with statins. When added to statin therapy, there are further benefits of EPA and DHA in altering blood lipids including a shift from small&dense LDL-C particles to less atherogenic large&buoyant ones. More research is needed in the role of combination therapies in lipid management including LDL-C particle size and concentration.

Introduction

Cardiovascular disease is the major cause of deaths and loss of disability-adjusted life years in the world (World Health Organization, 2016). Prevention of CVD can be achieved with a cardio-protective lifestyle including a healthy diet. Nonetheless, for established CV risks such as dyslipidemia, more advanced clinical strategies are required. Management of blood lipids usually involve pharmacological agents as defined by clinical guidelines. Most clinical guidelines consider blood lipids as traditional risk factors and focus on low density lipoprotein cholesterol (LDL-C) and non-high density lipoprotein cholesterol (non-HDL-C) as primary treatment targets. HMG-

CoA reductase inhibitors (statins) are suggested by all clinical guidelines as the gold standard in management of atherogenic dyslipidemia in order to reduce elevated LDL-C and non-HDL-C levels. Even though not yet present as a treatment target in the clinical guidelines, a more recently discovered risk factor, small & dense LDL-C particles, are suggested by several studies as the most atherogenic lipoprotein particle (Hoogeveen et al., 2014).

Even though statins are the main therapeutic agent in treatment of dyslipidemia, evidence states that there is a considerable role of diet in development and management of lipid-related problems (Thompkinson, Bhavana & Kanika, 2014). Certain nutrients are known as atherogenic, meaning that they aggravate the atherogenic process, while certain nutrients are anti-atherogenic. The polyunsaturated OM-3 FA are among the most-investigated anti-atherogenic nutrients (Mozaffarian & Wu, 2011). Studies that analysed the relation between OM-3 intake and CV end points point to a negative correlation (Burr et al., 1989; GISSI, 1999; Stark, Park & Maines, 2000). A subset of these studies analyse the physiologic effects of OM-3 on CV blood markers including blood lipids such as TAG, HDL-C, LDL-C and non-HDL-C. Their results collectively highlight the beneficial effects of OM-3 on these parameters either when used alone or in combination with statins. Albeit limited, there are also studies that investigate the alterations in LDL-C particle size and concentration with OM-3 use (Contacos, Barter & Sullivan, 1993; Nordøy, Hansen, Brox & Svensson, 2001; Maki et al., 2008). This review aims at understanding blood lipids as separate CV risk factors and analyse the literature that studied the correlation between OM-3 intake and blood lipids.

Cardiovascular Disease

Definition and Prevalence

CVD is defined by the World Health Organisation (2014) as a family of conditions that have an impact on heart and blood vessels. According to the Joint British Societies (2014) most of the CVD are caused by a common pathology of "atherosclerosis", which is the condition when a person's arteries are blocked by fatty plaques. Atherosclerosis causes CVD within time, when the blood supply to an organ or tissues is completely blocked by narrowed arteries or ruptured plaques, resulting in limited amounts of blood and oxygen flow to that area (British Heart Foundation, 2013). Heart attacks and strokes are usually acute events that are mainly caused by such blockage, in turn.

In 1996, Murray and Lopez stated that cardiovascular disease is the major cause of mortality and loss of disability-adjusted life years in the world. Since then, 20 years have passed yet CVD still ranks first in the world in terms of death cause, which can be interpreted as that the number of people that die from CVD every year exceed that of other diseases. According to World Health Organization (2016), 17.5 million people died from CVD in 2012, which consisted 31% of the overall deaths globally and more than three quarters of them took place in low- and middle-income countries. The annual costs of the disease are estimated at approximately 863 billion USD and the burden is expected to increase as a consequence of the rapid increase in obesity and the ageing populations (Julius, 2017).

Lipids as Traditional CVD Risk Factors

Atherosclerosis is a degenerative disease of the inner wall of the vascular endothelium. In normal physiology, endothelial cells act as an interface between the blood flow and the vessel

wall, hence, change in their phenotype causes dysfunction of the vessel wall, which, in turn, leads to atherosclerosis (Douglas & Channon, 2010). One of the etiological causes of atherosclerosis is considered to be dyslipidaemia, a cluster of lipid abnormalities (Grooper & Smith, 2013), ever since the Framingham Study defined lipids as CVD risk factors in early 1950s (Kannel, Dawber, Friedman, Glennon & McNamara, 1964) and Keys formulated the cholesterol hypothesis in 1951. Elevated Triacylglycerol (TAG) levels and low-density lipoprotein cholesterol (LDL-C), usually accompanied with low levels of high-density lipoprotein cholesterol (HDL-C) are components of dyslipidaemia. Dyslipidaemia aggravates the atherosclerosis process by leading to lipid accumulation on the arterial wall (Garg, Aggarwal, Kumar & Sharma, 2015) and each of its components is known to have a certain role in atherosclerotic process.

Serum TAG were first observed as an essential CV risk factor in the PROCAM study in 1979 (Assmann & Schulte, 1996) which was further confirmed by Hokanson and Austin (1996), who showed in their meta-analysis that fasting TAG are an independent risk factor. Findings of the subsequent epidemiological and clinical studies that investigate the relationship between TAG and CVD risk suggest that serum levels of TAG may relate to the presence of CV risk (Assmann et al., 1998; Jeppesen et al., 1998; Miller et al., 2011; Boullart & Stalenhoef, 2012), however, caution should be applied when interpreting their results, given that plasma TAG are highly fluctuating in the post-prandial state (Mora, Rifai & Buring, 2008). Furthermore, elevated plasma TAG are usually accompanied with low HDL-C (Ginsberg et al., 2007), which may be a confounding factor in studies not adjusted for HDL-C. Nevertheless, TAG, as the substrate of LDL-C synthesis, are still deemed as a target in clinical management of dyslipidemia and each 1 mmol/l reduction in TAG levels is believed to reduce CV risk by 14% in men and 37% in women (Hokanson & Austin, 1996).

LDL-C, on the other hand, has consistently been found to be directly associated with CVD risk in epidemiological studies, human clinical trials and animal studies (Ridker, 2014, Jarcho & Keaney, 2016). Discovered almost 30 years ago by Brown and Goldstein (1996), it is now considered as the major atherogenic lipoprotein particle due to its direct role in disease progression: circulating LDL particles are internalised and then accumulate in the vascular intima and once they are oxidised, they convert into foam cells, starting the atherosclerotic process (Grooper & Smith, 2013). There is a consensus in the literature that elevated LDL- C is associated with increased CVD risk and therefore LDL-C is the primary target of atherosclerosis treatment guidelines (Ridker, 2014). Further discussed by Cannon et al. (2015) is whether 'lower is better', given that a recent major clinical trial named IMPROVE-IT (n=18,144) has revealed a statistically significant decrease in CV events between two study groups, one of which was treated with a lipid-lowering drug alone (median LDL-C= 1.8 mmol/l) and the other with two different lipid-lowering agents (median LDL-C= 1.4 mmol /l), (hazard ratio, 0.936; 95% CI, 0.89 to 0.99; p=0.016). Although the absolute risk difference between two groups is only 2% in this study, this value is statistically significant and hence clinically important. Furthermore, the Cholesterol Treatment Trialists' collaborators reported 23% reduction in the risk of major coronary events when LDL-C is decreased by 1.0 mmol /l in their meta-analysis of 90,000 subjects in 2005 (Baigent et al., 2005), and then further reported 40-50% reduction when LDL-C is decreased by 0.9-1.4 mmol/l in a more recent and larger meta-analysis in 2010 involving 170,000 subjects (Baigent et al., 2010).

Despite the ample evidence that LDL-C is a strong risk factor for CVD, not all CV patients have elevated LDL-C levels (Hirayama & Miida, 2012). This is explained by a great number of researchers with the fact that development and progression of atherosclerosis is also dependent

on the type of LDL lipoprotein complexes (Rizzo & Berneis, 2006; Packard, 2006; Berneis & Krauss, 2002; Packard, Caslake & Shepherd, 2000). Two decades ago, cross-sectional studies reported different LDL-C phenotypes in terms of size and density (Lamarche, Lemieux & Despres, 1999), phenotype B, also called small&dense LDL-C (sdLDL), being more atherogenic (Hirayama & Miida, 2012). The reason is they can easily penetrate into the arterial wall due to their small size, and their longer circulation time in the blood stream increase their oxidation capacity (Ivanova, Myasoedova, Melnichenko, Grechko & Orekhov, 2017). Past evidence has pointed to an association between sdLDL and CVD (Austin et al., 1988; Krauss, 2010; Berneis & Krauss, 2002; Rizzo & Berneis, 2006) followed by more recent studies strengthening that association, one of which is the Frahangam Offspring Study that suggested sdLDL might be a better indicator of CVD then LDL-C itself (Ai et al., 2010). Suita Study (Arai et al., 2013) also showed that sdLDL is useful in predicting CVD. Subsequently, in the large prospective ARIC study conducted on 11,419 subjects by Hoogeveen et al (2014), sdLDL predicted CVD risk even for subjects within the desired LDL-C levels. Most recently, a study performed in a Chinese population suggests that sdLDL correlates to CVD risk by means of carotid artery intima media thickness (Shen et al., 2015). Taken together, these findings collectively support the earlier decision by National Cholesterol Education Program (2002) which considers predominance of sdLDL as a CVD risk factor. However, there seems to be a lack of standardization in the method of measuring sdLDL in terms of particle size and concentration in these given studies, which is a shared limitation of the available evidence as different methods deliver different results. Furthermore, with significant variations in methodology, meta-analysis would be difficult to conduct.

Acting conversely to all, HDL-C is responsible for the removal of excess cholesterol from peripheral macrophage foam cells in atherosclerotic lesions. (Ganjali et al., 2017). This process is also called the reverse cholesterol transport, and has a protective role in the development of atherosclerosis. Indeed, epidemiological studies have shown that low HDL-C levels is strongly related to increased atherosclerotic CVD risk (Stone et al., 2013). However, this does not necessarily mean that a high HDL-C level prevents atherogenesis. Some of the clinical studies like ILLUMINATE (Barter et al., 2007), dal-OUTCOMES (Schwartz et al., 2012), AIM-HIGH (AIM-HIGH Investigators, 2011), and HPS2-THRIVE (HPS2-THRIVE Collaborative Group, 2013) linking the increased levels of HDL-C with CV outcome data has failed in showing a correlation. This may be caused by that serum HDL-C levels do not always reflect the cholesterol efflux capacity from cells (Khera et al., 2011). Furthermore, HDL-C is recently reported to be a "chameleon-like" protein which loses its protective features in oxidation (Fogelman, 2015), while, atherosclerosis is already known to be a chronic inflammatory state (Grooper & Smith, 2013). Supportive of this, there are individuals that develop atherosclerosis despite having a high serum HDL-C level, therefore, HDL-C is not anymore deemed as a primary target in treatment of atherosclerosis. (Toth et al., 2013).

In the recent years, assessment of non–high-density lipoprotein cholesterol (non-HDL-C) gained importance as a more relevant target of dyslipidemia therapy given the fact that it provides measurement of all atherogenic particles (Rana, Boekholdt, Kastelein & Shah, 2012) that a LDL-C measurement typically excludes. There is plenty evidence to support this approach, including a meta-analysis of 8 randomized controlled trials that enrolled 62,154 patients, non-HDL was found to be a better indicator of CVD risk compared to LDL-C (Boekholdt et al., 2012). Furthermore, in a study involving 302,430 people by The Emerging Risk Factor Collaboration

showed that LDL-C (HR: 1.38, 95% CI, 1.09 to 1.73) is less effective as a CVD risk indicator compared to non-HDL (HR: 1.42, 95% CI, 1.06 to 1.91) (Angelantonio et al., 2009). As a result of these and other supporting evidence (Cui et al., 2001; Ingelsson et al., 2007; Liu et al., 2006; Mora et al., 2009), National Lipid Association and National Institute for Health and Care Excellence (NICE) suggested non-HDL-C as a superior marker than others in predicting CVD risk (Jacobson et al., 2015).

Lipid Management Therapy

Worldwide, there are several guidelines on lipid management, four of which are more commonly used in clinical practice **(Table 1).**

Table 1 Lipid Management Guidelines Used in Clinical Practice

Organisation	Last Update
American Heart Association/American College of Cardiology (AHA/ACC)	2013
National Lipid Association (NLA)	2014
National Institute for Health and Care Excellence (NICE)	2014
European Society of Cardiology (ESC)	2016

All these guidelines aim to define certain risk groups and provide instructions for treatment of each risk group. There is ongoing debate about AHA/ACC 2013 guideline as all lipid goals have been removed in it (Martin et al., 2014; Lopez-Jimenez et al., 2014). NICE and NLA both suggest non-HDL as the primary clinical target of lipid management therapy with statins, while ESC states LDL-C should be the primary target. All of them jointly emphasise that the HMG-CoA reductase inhibitors (statins) use is the gold standard in lipid management therapy given that its clinical benefits have been well-established in preventing atherosclerosis and CVD (Miller & Martin, 2016).

Dietary Therapy in Lipid Management

Diet is the most common non-pharmacological therapy for primary and secondary prevention of CVD due to the strong evidence showing its relation to CV risk factors, including blood lipid profile (Rees et al., 2013). Many studies investigate the effect of dietary changes on blood lipids. Backed by A-level evidence, there is a general agreement that reduction in saturated fatty acids (SFA) (Mensink, Zock, Kester & Katan, 2003) and trans fat (Mozaffarian, Aro & Willett, 2009) intake result in a decrease in LDL-C and TAG, while limiting alcohol intake (Rimm, Williams, Fosher, Criqui & Stampfer, 1999) as well as reducing mono- and disaccharides (Bantle, Raatz, Thomas & Georgopoulos, 2000) result in a decrease in TAG. Increase in dietary fibre (Brown, Rosner, Willet & Sacks, 1999) and reduction in total carbohydrate (Nordmann et al., 2006) also reduce both LDL-C and TAG.

Fats are the most essential component of dietary therapy in lipid management. Mensink et al. (2003) have shown in their large meta-analysis of 60 controlled trials that when energy from SFA is increased by 1% (%E), LDL-C increase by 1.6 mg/dL (0.04 mmol/l) while it decreases equally when 1% E from SFA is replaced with monounsaturated fatty acids (MUFA). Polyunsaturated fatty acids (PUFA) have a greater impact in LDL-C reduction with a 2.0 mg/dL (0.05 mmol/l) reduction when 1% SFA is replaced with PUFA. Marine derived omega-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are particularly suggested as cardioprotective and anti-atherogenic nutrients as a result of the evidence that showed their several favourable effects on overall CV health, including their lipid lowering properties (Dessi et al., 2013), both of which are reviewed in the next chapter.

Role of omega-3 Fatty Acids in CVD Prevention and Lipid Management

Since the early studies in Greenland Eskimos, intake of EPA and DHA have been consistently shown to have a cardioprotective role (Massaro, Scoditti, Carluccio & Caterina, 2010). Several observational and interventional studies associate increased intake of EPA and DHA with reduced CV endpoints. One of the proposed mechanisms to justify such beneficial outcomes include lipid modifying effect of EPA and DHA, which is also investigated by a great number of trials.

Effects of omega-3 Fatty Acids on CV Outcomes

Among numerous studies in the area, two early randomized trials have clearly shown the favourable effects of EPA and DHA on CV outcomes. Diet and Reinfarction Trial (DART) assigned 2,033 men with recent myocardial infraction (MI) to fish oil capsules and has shown to decrease CV mortality by 29% over 2 years (Burr et al., 1989). It is followed by GISSI Prevenzione trial in 1990s, one of the milestones in the evidence that randomized 11,324 participants to 850 mg of EPA/DHA/day versus no intervention for 3.5 years (GISSI, 1999). The study showed that fish oil consumption was associated with a significant reduction in sudden deaths by 45% (RR 0.55; 95 % CI, 0.39–0.77; p = 0.0006) and CV mortality by 30% (RR 0.69; 95 % CI, 0.66–0.93; p = 0.006). Both DART and GISSI trials continued as the second phases, however DART 2 (n = 3,114) showed conflicting results (Burr et al., 2003) due to several limitations in the experimental design such as lack of blinding, eating advice to the control group and early interruption that make it difficult to interpret the findings. GISSI-Heart Failure (GISSI-HF), on the other hand, was a large (n = 6,975), randomized placebo-controlled trial that assessed the association between heart failure and EPA&DHA intake (Tavazzi et al., 2008). After 3.9 years follow up, a moderate 9% reduction in all-

cause mortality was observed in the intervention group (HR 0.91; 95.5 % CI, 0.833–0.998; p = 0.041).

After 2010s, further RCTs followed up on the initial positive findings of DART and GISSI trials, albeit with conflicting results. These studies are summarised in **Table 2**.

Table 2 Most recent randomized controlled trials of OM-3 FA and CVD risk

Name & Author	Size & Inclusion Criteria	Intervention	Duration (years)	Result
Alpha-Omega (Kromhout, Giltay & Geleijnse, 2010)	n=4,837 history of past MI	376 mg/day EPA&DHA vs. a combined control group receiving either placebo or ALA 1.9 g/day	3.7	Major CV events n =671 (HR 1.01; 95% CI, 0.87–1.17)
OMEGA (Rauch et al., 2010)	n=3,851 recent MI	840 mg/day EPA&DHA vs. placebo	1	Major CV events n =331 (HR 1.21; 95% CI, 0.96–1.52)
SU.FOL.OM3 (Galan et al., 2010)	n=2,501 history of acute coronary event	600 mg/day EPA&DHA vs. a combined control group receiving either placebo or B vitamins	4.7	Major CV events n =157 (HR 1.08; 95% CI, 0.79–1.47; p=0.64)
ORIGIN (Bosh et al., 2012)	n= 12,536 dysglycemia and evidence of CVD	1 g/day EPA&DHA and insulin glargine vs. 1 g/day olive oil placebo and standard glycemic care	6.2	Sudden Death n=574 (HR 0.98; 95% CI, 0.87–1.10; p=0.72).
Risk and Prevention (The Risk and Prevention Study Collaborative Group, 2013)	n=12,513 high CVD risk without previous MI	1 g/day EPA & DHA vs. olive oil placebo	5	Sudden Death or CV hospitalization n =733 (HR 0.98; 95 % CI, 0.88–1.08; p=0.64)

Notes: MI: Myocardial Infraction, ALA: Alpha Linoleic Acid, HR: Hazard ratio, CI: Confidence interval

Nevertheless, caution should be applied when interpreting these studies, as they have certain limitations. Alpha-Omega trial prescribed only 0.37g/day EPA&DHA, which is much less than the dose given in all other previous studies, and also compared the results not with placebo but with a combined control of either placebo or n-3 ALA. Omega and SU.FOL.OM3 trials were underpowered to determine effects of n-3 use in preventing CV mortality, with an achieved

power of 0.17 and 0.14 respectively (CV death: Omega n=57, SU.FOL.OM3 n=40). The Origin trial was designed for a dysglycemic population and used n-3 FA together with insulin therapy, thus possibly prevented measureable effects of n-3 supplementation. Finally, the authors of Risk and Prevention trial acknowledge that lower than expected rates of CV endpoints is a limitation of their study, possibly due to the Italian cohort already on a healthy dietary pattern, accompanied by intensive preventive care during the 5 year follow-up period.

Supportive of the early results from DART and GISSI trials, numerous meta-analysis of smaller scale RCTs collectively report a negative association between EPA&DHA intake and risk of CV mortality (Mozaffarian & Rimm, 2006; Mente , Koning, Shannon & Anand, 2009; Wang et al., 2006; Brouwer et al., 2009; Harris et al., 2009; He et al., 2004; Hooper et al., 2004; Hooper et al., 2006; Leon et al., 2008; Marik & Varon, 2009)

Besides these given studies, JELIS trial, another large (n=18,645) and high-quality intervention study on a Japanese population, has shown a significant 19% relative reduction in major coronary events between the groups (Yokoyama et al., 2007). Designed differently, all participants were on statin prescription and the study compared statin monotherapy to a combination of 1.8g/day EPA plus statin. Similar favourable effects of combination therapies will be discussed later on in this chapter.

Overall, the evidence shows that a higher intake of EPA and DHA results in a reduced risk of CVD. Proposed mechanisms to justify these beneficial outcomes include lipid modifying effects of EPA and DHA (Pirillo & Catapano, 2013).

Effects of omega-3 Fatty Acids on Lipids and Lipoproteins

Published guidelines reviewed in **Table 1** emphasize the use of statins as primary treatment strategy for dyslipidemia. However, evidenced shows that patients who cannot use statins, or those who need additional lipid-lowering therapy can use omega-3 FA, especially EPA and DHA, either alone or in combination with statins for management of dyslipidemia. Suggested mechanism of action of these FA involves inhibition of TAG synthesis in hepatic cells, reduced very LDL-C (VLDL-C) production as well as increased VLDL-C metabolism (Bradberry & Hilleman, 2013).

Major clinical trials that examined the effects of EPA and DHA on blood lipids have shown positive results **(Table 3).**

Table 3 Major clinical trials about the role of EPA&DHA monotherapy on blood lipids

Name & Author	Size & Inclusion Criteria	Research Design	Duration	Result
EVOLVE Kastelein et al., 2014	n=399 ≥18 years fasting TAG≥5.7 mmol/l and <22.5 mmol/l	4 weeks dietary r/i **1st intervention** 12 weeks 3 groups 1)OM-3 2 g/day (n=100) 2) OM-3 3 g/day (n=101) 3) OM-3 4 g/day (n=99) 4) control (placebo) (n=99) (34% of the subjects were using statin as prescribed)	12 weeks	TAG ↓ 2 g: −26%, p < 0.01 3 g: -25%, p < 0.01 4 g: −31%, p<0.001 Non-HDL-C ↓ 2 g: −8%, p < 0.05 3 g: -7%, p < 0.05 4 g: −10%, p<0.01 HDL-C no sig. change LDL-C ↑ 2 g: 19%, p < 0.01 4 g: 19%, p<0.001 VLDL-C ↓ 2 g: −27%, p < 0.01 3 g: -25%, p < 0.01 4 g: −31%, p<0.001

Name & Author	Size & Inclusion Criteria	Research Design	Duration	Result
MARINE Bays et al., 2011	n= 229 fasting TAG≥5.7 mmol/l and <22.5 mmol/l	6 weeks dietary r/i **1st intervention** 12 weeks 3 groups 1)EPA 2 g/day (n=76) 2) EPA 4 g/day (n=77) 4) control (placebo) (n=76)		TAG ↓ 2 g: −20%, p < 0.005 4 g: −33%, p<0.0001 Non-HDL-C ↓ 2 g: −8%, p < 0.05 4 g: −18%, p<0.0001 HDL-C & LDL-C no sig. change VLDL-C ↓ 2 g: −15%, p < 0.05 4 g: −29%, p<0.001 LDL-C particle concentration ↓

Notes: ↓ reduction, ↑ increase, r/i: run in,

Backed by ample evidence, EPA & DHA monotherapy is commonly used for treating hypertriglyceridemia (HTG). In presence of very high TAG levels (≥500 mg/Dl; 5.7mmol/l), EPA&DHA 2 g/day to 4/day is shown to reduce TAG by 25% to 45% depending on the baseline TAG levels (Weintraub, 2014; Harris et al., 1997). Similarly in EVOLVE, MARINE, ORIGIN and Risk and Prevention studies, the plasma TAG levels were significantly reduced **(Table 2 & Table 3)**. In a systematic review and meta-analysis of 21 randomized controlled trials [n=7803] of the effect of EPA&DHA supplementation on blood lipids, a net reduction of 0.3 mmol/l (−0.4, −0.2, P<0.0001) was reported in blood TAG (Balk et al., 2006). Similarly, a comprehensive meta-analysis of 47 studies that compare the effects of EPA&DHA on blood lipids with placebo showed that TAG is reduced by 0.07 mmol/l over an average treatment period of 24 weeks in 16,511 subjects with hyperlipidemia (Eslick, Howe, Smith, Priest & Bensoussan, 2009).

LDL-C, however, is usually reported to rise on EPA&DHA treatment, possibly due to increased production of LDL-C from VLDL-C (Pirillo & Catapano, 2013). Balk et al. (2006) report in

their systematic review a 0.2 mmol/l (+0.08, +0.2, P= 0.0006) increase in LDL-C levels. In EVOLVE trial, LDL-C increased 19% (p<0.001) with 2g/day and 4 g/day EPA&DHA intake.

HDL-C increased +13% (p = 0.014) with 4 g/day EPA&DHA in a 4-months study on subjects with TAG ≥5.7 mmol/l (Harris et al., 1997) and Balk et al. (2006) reported in their review a 0.02 mmol/l (+0.01, +0.03, P= 0.0003) statistically significant increase. In a 6-weeks study there was only a non-significant 5.9% (p = 0.057) increase (Pownall et al., 1999).

LDL-C and HDL-C did not significantly change in all studies. Eslick et al. (2009), for instance, observed no clinically significant benefit for HDL-C (+0.05 mmol/l, 95% CI: 0.00 to 0.36) and no increase in LDL-C (+0.03 mmol/l, 95% CI: 0.54 to 1.62). Similarly, the Risk and Prevention Study reports a slight increase in the HDL level in the intervention group and no significant difference in LDL-C, while EVOLVE reports no significant increase in HDL-C. MARINE trial also reports no significant changes in HDL-C and LDL-C. Such slight changes or no change in HDL-C and LDL-C could be due to the combined use of EPA and DHA in these studies, given that these two fatty acids show reverse characteristics in LDL-C and HDL-C alteration. A recent review which evaluated 22 studies that either compare EPA with DHA or investigate EPA or DHA alone suggests that EPA and DHA may have distinct effects on blood lipids (Jacobson, Glickstein, Rowe & Soni, 2012). DHA is reported to have a stronger role in increasing LDL-C with a net increase of 3.3% in head-to-head comparative studies. Same sub-group analysis shows that DHA is associated with a 6.8% net decrease in TAG and a net increase in HDL-C by 5.9%. Furthermore, in 70% of DHA-alone studies, LDL-C is observed to increase as opposed to EPA alone studies all of which showed a decrease in LDL-C. HDL-C, on the other hand, is observed to increase in 85% of DHA-alone studies and was unchanged or decreased in EPA-alone studies. Although these findings are

remarkable, further studies are required to have a clear understanding of the distinct mechanisms of EPA and DHA that affect HDL-C and LDL-C alteration.

Although a number of studies report an increase in LDL-C after fish oil use, TAG reduction is more significant than the increase in LDL-C in subjects with mild to moderate HTG (Pirillo & Catapano, 2013). More importantly, the rise in LDL-C due to EPA and DHA is caused by a shift from small&dense LDL-C to large & buoyant LDL phenotype, therefore reducing the risk of atherosclerotic CVD (Jacobson, 2008; Woodman et al., 2003; Calabresi, Donati, Pazzucconi, Sirtori & Franceschini, 2000). This is further confirmed by the MARINE trial (Bays et al., 2011) as well as a meta-analysis and systematic review of 24 RCTs (n=1533) which also shows that LDL-C increase 3% with a concomitant change in particle size (Hartweg, Farmer, Holman & Neil, 2009). In addition to the possible improvement in LDL-C phenotype, EPA&DHA monotherapy also resulted in significant decreases in non-HDL-C in both MARINE and EVOLVE trials.

Overall, EPA and DHA monotherapy may result in an improved lipid and lipoprotein profile as a result of their role in reducing TAG and non-HDL-C as well as shifting to a large & buoyant LDL-C from sdLDL-C. However, clinical trials have shown that EPA and DHA supplementation is more effective when combined with other lipid-lowering drugs - mostly statins (Pirillo & Catapano, 2013).

Combination Therapies: Statin and Omega-3

Although statins are considered as the first choice in lipid management therapy in all clinical guidelines (**Table 1**) in cases when elevated lipid levels are persistent to statin, combination therapy with EPA and DHA can be used in order to reduce TAG, non-HDL levels and

LDL-C particle size (Kastelein et al., 2014). Several clinical trials have shown that blood lipids of dyslipidemic patients respond favorable to EPA&DHA/statin combinations **(Table 4)**. Addition of EPA & DHA resulted in significant further reductions in TAG compared to statin therapy alone in most of the past combination trials (Contacos, Barter & Sullivan, 1993; Mataki et al., 1998; Durrington et al., 2001; Chan et al, 2002; JELIS; Davidson, et al., 2007; Maki et al., 2013, Chan et al., 2016). A range of 9% to 55% reduction in plasma TAG have been observed, in most cases with a reduction of 30% and above **(Table 4)**. HDL-C, on the other hand, is shown to increase with a combination therapy in some studies (Nordøy, Hansen, Brox & Svensson, 2001; Chan, 2002; Davidson, et al., 2007; Maki et al., 2008), whereas in others it has remained unchanged (Contacos, Barter & Sullivan, 1993; Nordøy et al., 1998; Mataki et al., 1998; Durrington et al., 2001; Chan et al., 2016). It is therefore difficult to reach a conclusion about the role of n-3 and statins in altering HDL-C out of the results of past trials. When endogenous cholesterol synthesis is inhibited, EPA&DHA supplementation may have a role in decreasing total cholesterol without increasing LDL-C cholesterol (Nordoy, 1998). Supportive of this, combination of EPA&DHA with statins counteracted the expected increase in LDL-C due to n-3 use (as seen in monotherapies in **Table 3)**, hence no significant increase in LDL-C was observed in any of the combination trials listed in **Table 4**. On the contrary, small&dense LDL-C particles significantly decreased with the combined use of EPA&DHA with statin in trials that had measured LDL-C particle size and concentration (Contacos, Barter & Sullivan, 1993; Nordøy, Hansen, Brox & Svensson, 2001; Maki et al., 2008). The decrease in VLDL-C and chylomicrons with EPA&DHA was resulted in a significant decrease in atherogenic particles, therefore 10-20% significant reductions in non-HDL-C in all of the combination trials **(Table 4)**.

The past evidence of combination trials is limited. Furthermore, most of the studies recruited a small number of participants (less than 100) with an average duration of 6-8 weeks. Exemptions of this are the JELIS, COMBOS and ESPIRIT studies, all showing promising results for further research in the area. JELIS, the largest combination trial conducted to date (n=18,645), showed not only improvement in certain blood lipids, but also a 19% reduction in major coronary events (p<0.001) attributed to EPA treatment. However, this study was not double-blind. Completed by Davidson et al. in 2008, COMBOS trial (n=254) is the first study that showed an improvement in all measured lipid parameters (non-HDL-C, TC, TAG, HDL-C, VLDL-C). Maki et al. (2008) extended Davidson et al.'s study and reported an improvement also in LDL-C particle size when EPA&DHA and statins are combined. However, Bays et al. (2010), Ballantyne et al. (2012), Maki et al. (2013) and Chan et al. (2016), who all tested the efficacy of EPA&DHA with statin and reported positive results, did not measure LDL-C particle size and concentration in their studies despite the earlier findings.

Table 4 Randomized controlled trials of combined therapy of OM3-FA and statin (continued to page 33)

Name & Author	Inclusion Criteria	Size	Research Design	Results
Contacos, Barter & Sullivan, 1993 Effect of pravastatin and omega-3 fatty acids on plasma lipids and lipoproteins in patients with combined hyperlipidemia	TC >5.5 mmol/l TAGs >5.7 mmol/l	32	Dietary r/i= 6 weeks **1st intervention**6 weeks 3 groups **1)** 3 g/day EPA&DHA 2:1 (n=10) **2)** 40 mg/day statin (n=10) **3)** placebo (n=10) **2nd intervention** 12 weeks (n=31) 3 g/day EPA&DHA 2:1 + 40 mg/day statin	**1st intervention** **In statin group:** TC ↓ 23% p<.001 LDL-C ↓ 30% p<.001 LDL-C particle size no change **In n-3 group:** TAG↓ 30% p<.05 LDL-C particle size ↑ p<.05 LDL-C ↑ 13% p>0.05 VLDL-C ↓ 37% p<.05 **2nd intervention** **In statin+comb group:** TAG↓ 33% p<.05 LDL-C ↑ 10% p <.01 LDL-C particle size no change **In n-3+comb group:** TC ↓ 18%; p<.01 LDL-C ↓24% p<.05

Name & Author	Inclusion Criteria	Size	Research Design	Results
				In placebo+comb group: TC ↓ 25% p<.001 TAG ↓33% p<.05 LDL-C ↓ 26% p ><.01
Nordøy et al., 1998 Effects of simvastatin and omega-3 fatty acids on plasma lipoproteins and lipid peroxidation in patients with combined hyperlipidaemia	TC >5.3 mmol/l TAG >2.0 mmol/l <15.0 mmol/l	41	Dietary r/i= 16 weeks **1st intervention** 5 weeks, 2 groups: **1)** statin 20mg/day (n=20) **2)** placebo (n=20) **2nd intervention** 5 weeks 1 group (n=41) statin 20mg/day **3rd intervention** 5 weeks, 2 groups: **1)** 4g/day EPA&DHA + 20 mg/day statin (n=22) **2)** placebo + 20 mg/day statin (n=20)	**In n-3 group:** TC ↓ 10% p=.052 TAG↓ 55% p<.001 LDL-C no change p=0.4 Non-HDL ↓ 10% p=not given

Name & Author	Inclusion Criteria	Size	Research Design	Results
Mataki et al., 1998 Effect of eicosapentaenoic acid in combination with HMG-CoA Reductase Inhibitor on lipid metabolism	N/A	N/A	No dietary r/i **1st intervention**12 weeks 1 group: 1.8g/day EPA+ 5-10 mg/day statin (n=N/A) **2nd intervention**12 weeks 1 groups: 5-10 mg/day statin (n=N/A)	**1st intervention** TC ↓ 20% p=N/A TAG↓ 40% p=N/A LDL-C & HDL-C no change p=N/A **2nd Intervention** TC ↓ 1% p>.05 TAG ↑ 50% p=N/A
Durrington et al., 2001 An omega-3 polyunsaturated fatty acid concentrate administered for one year decreased triglycerides in simvastatin treated patients with coronary heart disease and persisting hypertriglyceridemia.	TAGs >2.3 mmol/l on statin	59	No dietary r/i **1st intervention** 24 weeks, 2 groups: **1)** 4g/day EPA&DHA + 10-40 mg/day statin (n=30) **2)** placebo + 10-40 mg/day statin (n=29) **2nd intervention**= 24 weeks, 1 group(n=46) 4g/day EPA&DHA+ 10-40 mg/day statin	**1st intervention** In n-3 group: TAG ↓25% p<.0005 TC ↓10% p<.025 LDL-C & HDL-C no change Non-HDL ↓10% p=not given VLDL ↓ 40% p<.005 **2nd intervention** In n-3 group: TAG further ↓ 15% p<.0005

Name & Author	Inclusion Criteria	Size	Research Design	Results
				TC further ↓ 2% p<.005
				LDL-C ↓ 15% p<.025
				HDL-C no change
				Non-HDL further ↓2% p=not given
				In placebo group:
				TAG ↓25% p<.0005
				VLDL ↓25% p<.005
				HDL-C, LDL-C, TC, non-HDL no change
Nordøy, Hansen, Brox & Svensson, 2001 Effects of atorvastatin and omega-3 fatty acids on LDL subfractions and postprandial hyperlipidaemia in patients with combined hyperlipidaemia.	TC >5.3 mmol/l TAG >2.0 mmol/l TAG <15.0 mmol/l	42	Dietary r/i= 12 weeks **1st intervention** 5 weeks 1 group (n=42) 10 mg/d statin **2nd intervention** 5 weeks 2 groups **1)** 1.68 g/d EPA&DHA + statin (n=22) **2)** placebo + statin (n=20)	**1st intervention** TAG ↓25% p<.05 TC ↓30% p<.01 LDL-C ↓35% p<.01 HDL-C ↑ 10% p<.01 **2nd intervention** n-3 group: HDL ↑ 10% p=.03 s&d LDL↓ p<.05 postprandial TAG ↓ p < 0.01 LDL-C no change

Name & Author	Inclusion Criteria	Size	Research Design	Results
Chan, 2002 Factorial study of the effects of atorvastatin and fish oil on dyslipidaemia in visceral obesity.	Obese (BMI>29) LDL >2.6 mmol/l Non-HDL>3.4 mmol/l TAGs >1.2 mmol/l	52	Dietary r/i= 3 weeks **intervention**6 weeks 4 groups **1)** 40 mg/day statin + placebo (n=13) **2)** 4g/day EPA&DHA+ placebo (n=12) **3)** 4g/day EPA&DHA + 40 mg/day statin (n=11) **4)** placebo + placebo (n=13)	**n-3 effects:** TAG ↓ 0.38 ±0.11 mmol/L P= 0.002 HDL-C ↑ +0.07 ± 0.04 mmol/L P= 0.041 LDL-C no change
Yokoyama et al., 2007 (JELIS) Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis	TC >6.5 mmol/l LDL-C>4.5 mmol/l	18,645	r/i= 4-8 weeks **1st intervention** 5 years, 2 groups **1)** 1.8 g/day EPA + 10-40 mg/day statin (n=9326) **2)** 5-10 mg/day statin (n=9319)	Clinical benefit of combination therapy with omega-3 fatty acids and statin has been reported EPA treatment was associated with a 19% ↓ in major coronary events p<0.001 **n-3 group:** TAG ↓9% p<.05 TC ↓19% p<.001 LDL-C ↓25% p<.001

Name & Author	Inclusion Criteria	Size	Research Design	Results
				HDL-C ↑ 3%
				control group
				TAG ↓3% p<.05
				TC ↓19% p<.001
				LDL-C ↓25% p<.001
				HDL-C ↑ 4%
Davidson, et al., 2007 (COMBOS) Efficacy and tolerability of adding prescription omega-3 fatty acids 4 g/d to simvastatin 40 mg/d in hypertriglyceridemic patients: an 8-week, randomized, double-blind, placebo-controlled study.	TAG levels >2.3 and <5.7 mmol/l LDL >10% ATPIII	254	8 week r/I with 40mg/day statin **1st intervention** 8 weeks, 2 groups **1)** 4g/day EPA&DHA + 40 mg/day statin (n=123) **2)** 40 mg/day statin + placebo (n=133)	**n-3 group:** non-HDL-C ↓9% p<.001 TC ↓4.8 p=.001 TAG ↓30% p<.001 VLDL-C ↓28% p<.001 HDL-C ↑ 3.4% p<.001 LDL-C ↑ 0.7% p=.052 **control group** non-HDL-C ↓2.2% p<.001 TC ↓1.7 p=.001 TAG ↓6% p<.001 VLDL-C ↓7% p<.001 HDL-C ↑ 1.2% p<.001 LDL-C ↓ 2.8% p<.052

Name & Author	Inclusion Criteria	Size	Research Design	Results
Maki et al., 2008 Effects of Adding Prescription Omega-3 Acid Ethyl Esters to Simvastatin (20 mg/day) on Lipids and Lipoprotein Particles in Men and Women With Mixed Dyslipidaemia	TAG levels >2.3 and <6.7 mmol/l Non-HDL> NCEP	39	5 weeks r/i **1st intervention** 6 weeks 2 groups 1) 4g/day EPA&DHA + 20 mg/day statin (n=123) 2) 20 mg/day statin + placebo (n=133) **2nd intervention** 6 weeks 2 groups (cross-over) 1) 4g/day EPA&DHA + 20 mg/day statin (n=123) 2) 20 mg/day statin + placebo (n=133)	**n-3 group:** Non-HDL↓ 40% p<.001 VLDL-C ↓ 42% p<.05 TAG ↓44% p<.05 TC ↓31 p<.05 HDL-C ↑ 16% p<.05 LDL-C ↓37 p>.05 sLDL ↓ 2% p<.05 **control group** Non-HDL↓ 34% p<.001 VLDL-C ↓ 22% p<.05 TAG ↓29% p<.05 TC ↓26 p<.05 HDL-C ↑ 11% p<.05 LDL-C ↓40 p>.05 sLDL ↓1% p<.05
Bays et al., 2010 Effects of prescription omega-3-acid ethyl esters on non-high-density lipoprotein cholesterol when			n-3 vs statin	**n-3 group:** Non-HDL↓ 40% p<.001 VLDL-C ↓ 54% p<.001 TAG ↓45% p<.001

Name & Author	Inclusion Criteria	Size	Research Design	Results
coadministered with escalating doses of atorvastatin.				TC ↓31 p=.002 HDL-C ↑ 12% p=.007 LDL-C no sig. difference **control group** Non-HDL↓ 34% p<.001 VLDL-C ↓ 37% p<.001 TAG ↓27% p<.001 TC ↓27 p=.002 HDL-C ↑ 10% p=.007 LDL-C no sig. difference
Vecka et al., 2012 N-3 polyunsaturated fatty acids in the treatment of atherogenic dyslipidemia.	TAG levels >2.3 and <5.7 mmol/l	56	n-3 vs statin	TAG ↓28% p<.001 VLDL-C ↓ 27% p<.001 HDL-C ↑ 4% p<.01 LDL-C ↑9 p<.01
Ballantyne et al., 2012 (ANCHOR) Efficacy and safety of eicosapentaenoic acid ethyl ester (AMR101) therapy in statin treated patients with persistent high triglycerides (from the ANCHOR study).	TAG levels >2.3 and <5.7 mmol/l	702	6-week r/i **1st intervention** 12 weeks 2 groups 1)2 g/day EPA + statin	TAG ↓ 2 g: −10%, p = 0.0005 4 g: −21%, p<0.0001 Non-HDL-C ↓ 2 g: −6%, p = 0.0054 4 g: −15%, p<0.0001

Name & Author	Inclusion Criteria	Size	Research Design	Results
			2)4 g/day EPA + statin 3)placebo + statin	HDL-C ↓ 4 g: −5%, p<0.01 LDL-C ↓ 4 g: −6%, p=0.006 VLDL-C 2 g: −10%, p < 0.01 4 g: −25%, p<0.0001
Maki et al., 2013 (ESPRIT) A highly bioavailable omega-3 free fatty acid formulation improves the cardiovascular risk profile in high-risk, statin-treated patients with residual hypertriglyceridemia (the ESPRIT trial)	TAG levels >2.3 and <5.7 mmol/l	n=647	6-week r/i **1st intervention** 6weeks 3 groups 1) 2g/day EPA&DHA + statin (n=215) 2)4g/day EPA&DHA + statin (n=216) 2) statin + placebo (n=216)	TAG ↓ 2 g: −15%, p < 0.01 4 g: −21%, p<0.01 Non-HDL-C ↓ 2 g: −4%, p < 0.05 4 g: −7%, p<0.01 LDL-C ↑ 2 g: 4.6%, p < 0.05 VLDL-C ↓ 2 g: −14%, p < 0.01 4 g: −22%, p<0.001

Name & Author	Inclusion Criteria	Size	Research Design	Results
Chan et al., 2016 Effect of omega-3 fatty acid supplementation on arterial elasticity in patients with familial hypercholesterolaemia on statin therapy	(DLCN) score >8 (definite FH) BMI< 40 k/m2	22	4-week r/i **1st intervention** 8 weeks 2 groups 1) 4g/day EPA&DHA + statin (n=123) 2) statin + placebo (n=133) **2nd intervention** 8 weeks 2 groups (cross-over)	**n-3 group:** Non-HDL↓ 10% p=.09 TAG ↓20% p=.01 TC ↓9 p=.06 LDL-C ↓10 p=.2 HDL-C ↓ 6% p=.5

Notes: ↓ reduction, ↑ increase, r/i: run in, NCEP: National Cholesterol Education Programme, DLCN: Dutch Lipid Clinic Network, FH: Familiar hypercholesterolemia

Conclusion

CV benefits of omega-3 fatty acids are well-documented by several studies. Large clinical trials have shown reduced CV events and reduced CV risk with sustained intake of marine-derived OM-3 FA EPA&DHA. These fatty acids have been also demonstrated to beneficially alter the blood lipid profile in subjects with dyslipidemia – which is one of the known causes of atherosclerosis. Although clinical guidelines endorse statin use in management of lipid-related problems, accumulating clinical data suggest that marine-derived n-3 fatty acids EPA&DHA significantly reduce blood TAG and VLDL-C when used without another lipid lowering agent. An increase in LDL-C levels may be expected with EPA&DHA monotherapy, however, when combined these fatty acids are combined with statins, this effect is usually counter balanced. Studies that compare statin monotherapy with n-3 combination therapies collectively show added benefits on blood lipid profile and conclude that EPA&DHA enhance statin-mediated improvements. When EPA&DHA is administered in combination with statin, significant benefits are achieved among a range of lipid parameters beyond LDL-C primary target. Such achievements include a range of 30 to 55% reductions in TAG, as well as 10-20% reductions in non-HDL. More importantly, in the limited number of the clinical trials that have monitored the changes in size and concentration of LDL-C particles with OM-3 FA use, significant reductions in small and dense LDL-C particles, a possibly more important predictor of future cardiovascular risk than LDL-C alone, have been reported. The hypothesis that sustained high-dose EPA&DHA intake could alter LDL-C particle phenotype can be drawn on the findings of these studies, however, additional strongly-designed clinical trials are required to establish their role in clinical management of LDL-C particles.

References

Ai, M., Otokozawa, S., Asztalos, B., Ito, Y., Nakajima, K., White, C., Cupples, A., Wilson, P. & Schaefer, E. (2010). Small dense LDL cholesterol and coronary heart disease: results from the Framingham Offspring Study. *Clinical Chemistry*, 56(6), 967–976.

AIM-HIGH Investigators. (2011). The role of niacin in raising high-density lipoprotein cholesterol to reduce cardiovascular events in patients with atherosclerotic cardiovascular disease and optimally treated low-density lipoprotein cholesterol Rationale and study design. The Atherothrombosis Intervention in Metabolic syndrome with low HDL/high triglycerides: Impact on Global Health outcomes (AIMHIGH). *American Heart Journal*,161, 471–477 e2.

Angelantonio, E., Sarwar, N., Perry, P., Kaptoge, S., Ray, K. K., Thompson, A., Wood, A,…, Danesh, J. (2009). Major lipids, apolipoproteins, and risk of vascular disease. *JAMA,* 302, 1993–2000.

Arai, H., Kokubo, Y., Watanabe, M., Sawamura, T., Ito, Y., Minagawa, A., Okamura, T & Miyamato, Y. (2013). Small dense low-density lipoproteins cholesterol can predict incident cardiovascular disease in an urban Japanese cohort: the Suita study. *Journal of Atherosclerosis and Thrombosis*, 20(2), 195–203.

Assmann, G. & Schulte, H.. (1996). Hypertriglyceridemia and elevated lipoprotein (a) are risk factors for major coronary events in middle-aged men. *American Journal of Cardiology*, 77(14), 1179-1184.

Assmann, G., Schulte, H., Funke, H. & von Eckardstein, A. (1998). The emergence of triglycerides as a significant independent risk factor in coronary artery disease. *European Heart Journal*, 19 (Suppl M), M8-14.

Austin, M. A., Breslow, J., Hennekens, C., Buring, J., Willett, W. & Krauss, R. (1988). Low-density lipoprotein subclass patterns and risk of myocardial infarction. *JAMA*, 260(13), 1917-1921.

Baigent, C., Blackwell, R., Emberson, J. Holland, L. E., Reith, C., Bhala, N., Peto, R., …, Collins, R. (2010). Efficacy and safety of more intensive lowering of LDL cholesterol: a meta-analysis of data from 170,000 participants in 26 randomised trials. *Lancet*, 13, 376(9753), 1670-81.

Baigent, C., Keech, A., Kearney, P. M., Blackwell, R., Buck, G., Pollicino, C., Kirby, A.,…, Simes, R. (2005). Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet*, 366, 1267-78.

Balk, E. M., Lichtenstein, A. H., Chunga, M., Kupelnick, B., Chewa, P. & Laua, J. (2006). Effects of omega-3 fatty acids on serum markers of cardiovascular disease risk: A systematic review. *Atherosclerosis*, 189, 19-30.

Ballantyne, C. M., Bays, H. E., Kastelein, J. J., Stein, E., Isaacsohn, J. L., Braeckman, R. A. & Soni, P. N. (2012). Efficacy and safety of eicosapentaenoic acid ethyl ester (AMR101) therapy in statin treated patients with persistent high triglycerides (from the ANCHOR study). *American Journal of Cardiology*, 110, 984–92.

Bantle, J. P., Raatz, S. K., Thomas, W. & Georgopoulos, A. (2000). Effects of dietary fructose on plasma lipids in healthy subjects. *American Journal of Clinical Nutrition,* 72, 1128–1134.

Barter PJ, Caulfield M, Eriksson M, et al. (2007). Effects of torcetrapib in patients at high risk for coronary events. *New England Journal of Medicine*, 357, 2109–2122.

Barter, A.M. Gotto, J.C. LaRosa, J. Maroni, M. Szarek, S.M. Grundy, *et al.* (2007). Treating to new targets, HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. *New England Journal of Medicine*, 357 (13), 1301–1310.

Bays, E., McKenney, J., Maki, K. C., Doyle, R. T., Carter, R. N. & Stein, E. (2010). Effects of prescription omega-3-acid ethyl esters on non-high-density lipoprotein cholesterol when coadministered with escalating doses of atorvastatin. *Mayo Clinic Proceedings*, 85(2), 122-8.

Bays, H. E., Ballantyne, C. M., Kastelein, J. J., Isaacsohn, J. L., Braeckman, R. A. & Soni, P. N. (2011). Eicosapentaenoic acid ethyl ester (AMR101) therapy in patients with very high triglyceride levels (from the Multi-center, placebo-controlled, Randomized, double-blind, 12-week study with an open-label Extension [MARINE] trial). American Journal of Cardiology, 108, 682–90.

Berneis, K. & Krauss, R. M. (2002). Metabolic origins and clinical significance of LDL heterogeneity. *Journal of Lipid Research*, 43, 1363-79.

Boekholdt, S., Arsenault, B., Mora, S., Pedersen, T., LaRosa, J., Nestel, P., Simes, R.,…, Kastelein, P. (2012). Association of LDL Cholesterol, Non–HDL cholesterol, and apolipoprotein B levels with risk of cardiovascular events among patients treated with statins: A meta-analysis. *JAMA,* 307(12),1302-1309.

Bosch, J., Gerstein, H. C., Dagenais, G. R., Díaz, R., Dyal, L., Jung, H., Maggiono, A. P.,…., ORIGIN Trial Investigators. (2012). n-3 fatty acids and cardiovascular outcomes in patients with dysglycemia. *New England Journal of Medicine,* 367(4), 309–18.

Boullart AC & Stalenhoef AF. (2012), Serum triglycerides and risk of cardiovascular disease. *Biochim Biophys Acta*, 1821, 867e875.

Bradberry, J.C. & Hilleman, D. E. (2013). Overview of omega-3 fatty acid therapies. *PT*, 38, 681–691.

British Heart Foundation. (2013). Cardiovascular disease. www.bhf.org.uk/heart-health/conditions/cardiovascular-disease.aspx.

Brouwer, I. A., Raitt, M. H., Dullemeijer, C., Kraemer, D. F., Zock, P. L., Morris, C., Katan, M. B.,…, McAnulty, J. (2009). Effect of fish oil on ventricular tachyarrhythmia in three studies in patients with implantable cardioverter defibrillators. *European Heart Journal*, 30, 820–6.

Brown L, Rosner B, Willet W & Sacks SM. (1999). Cholesterol-lowering effects of dietary fiber: a meta-analysis. *American Journal of Clinical Nutrition*, 69, 30–42.

Brown, M. S. & Goldstein, J.L. (1996). 1985 Nobel laureates in medicine. *Journal of Investigative Medicine*, 44(2), 14-23.

Brunzell, J. D., Zambon, A. & Deeb, S. S. (2012). The effect of hepatic lipase on coronary artery disease in humans is influenced by the underlying lipoprotein phenotype. *Biochimica et Biophysica Acta*, 1821(3), 365–372.

Burr, M. L., Ashfield-Watt, P. A., Dunstan, F.D., Fehily, A. M., Breay, P., Ashton, T., Zotos, P. C., ... Elwood, P. C. (2003). Lack of benefit of dietary advice to men with angina: results of a controlled trial. *European Journal of Clinical Nutrition*, 57:193–200.

Burr, M. L., Fehily, A. M., Gilbert, J. F., Rogers, S., Holliday, R. M., Sweetnam, P. M., Elwood, P. C.& Deadman, N. M. (1989). Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet*, 2, 757– 61.

Calabresi, L., Donati, D., Pazzucconi, F., Sirtori, C.R. & Franceschini, G. (2000). Omacor in familial combined hyperlipidemia: effects on lipids and low density lipoprotein subclasses. *Atherosclerosis*, 148, 387–96.

Cannon, C. P., Blazing, M. A., Giugliano, R.P., McCagg, A., White, J. A., Theroux, P., Darius, H.,…, Califf, R. M. (2015). Ezetimibe added to statin therapy after acute coronary syndromes. *New England Journal of Medicine*, 372, 2387-97.

Chan, D. C., Pang, J., Barrett, P., Sullivan, D. R., Mori, T. A., Burnett, R., vanBockxmeer, F. & Watts, G. F. (2016). Effect of omega-3 fatty acid supplementation on arterial elasticity in patients with familial hypercholesterolaemia on statin therapy. *Nutrition, Metabolism and Cardiovascular Diseases*, 26(12), 1140-1145.

Chan, D. C., Watts, G. F., Mori, T. A., Barrett, P. H., Beilin, L. J. & Redgrave, G. (2002). Factorial study of the effects of atorvastatin and fish oil on dyslipidaemia in visceral obesity. *European Journal of Clinical Investigation*, 32(6):429-36.

Contacos, C., Barter, P. J. & Sullivan, D. R. (1993). Effect of Pravastatin and ω-3 Fatty Acids on Plasma Lipids and Lipoproteins in Patients with Combined Hyperlipidemia. *Arteriosclerosis and Thrombosis*, 13(12), 1755-62.

Cui, Y., Blumenthal, R., Flaws, J. A., Whiteman, M. K., Langenberg, P., Bachorik, P. S. & Bush, T. L. (2001). Non–high-density lipoprotein cholesterol level as a predictor of cardiovascular disease mortality. *Archives of Internal Medicine*, 161, 1413–1419.

Davidson, M. H., Stein, E. A., Bays, H. E., Maki, K. C., Doyle, R. T., Shalwitz, R. A., Ballantyne, C. M., Ginsberg, H. N.; COMBination of prescription Omega-3 with Simvastatin (COMBOS) Investigators. (2007). Efficacy and tolerability of adding prescription omega-3 fatty acids 4 g/d to simvastatin 40 mg/d in hypertriglyceridemic patients: an 8-week, randomized, double-blind, placebo-controlled study. Clinical Therapeutics, 29(7), 1354-67.

Douglas, G. & Channon K. (2010). The Pathogenesis of Atherosclerosis. *Medicine*, 38, 8-15.

Durrington, P. N., Bhatnagar, D., Mackness, M. I., Morgan, J., Julier, K., Khan, M. A & France, M. (2001). An omega-3 polyunsaturated fatty acid concentrate administered for one year decreased triglycerides in simvastatin treated patients with coronary heart disease and persisting hypertriglyceridaemia. *Heart*, 85(5), 544-8.

Eslick, G. D., Howe, P., Smith, C., Priest, R. & Bensoussan, A. (2009). Benefits of fish oil supplementation in hyperlipidemia: a systematic review and meta-analysis. *International Journal of Cardiology*, 136, 4–16.

Fogelman, A. (2015). Further evidence that high-density lipoprotein is a chameleon-like lipoprotein. *European Heart Journal*, 36(43):3017-9.

Galan, P., Kesse-Guyot, E., Czernichow, S., Briancon, S., Blacher, J. & Hercberg, S. (2010). Effects of B vitamins and omega 3 fatty acids on cardiovascular diseases: a randomised placebo controlled trial. *The BMJ*, 341, c6273.

Garg, R., Aggarwal, S., Kumar, R. & Sharma, G. (2015). Association of atherosclerosis with dyslipidemia and co-morbid conditions: A descriptive study. *Journal of Natural Science Biology and Medicine*, 6(1), 163–168.

Ginsberg, H. N., Bonds, D. E., Lovato, L. C., Crouse, J. R., Elam, M. B., Linz, P. E., O'connor, P. J., ..., ACCORD Study Group. (2007). Evolution of the lipid trial protocol of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial. *American Journal of Cardiology*, 99, 56-67.

GISSI, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. (1999). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. *Lancet,* 354, 447–55.

Gropper, S. & Smith, J. (2013). *Advanced Nutrition and Human Metabolism*. (6th ed.). Belmont, USA: Wadsworth

Harris, W. S., Ginsberg, H. N., Arunakul, N., Shachter, N. S., Windsor, S. L., Adams M, Berglund, L. & Osmundsen, K. (1997). Safety and efficacy of Omacor in severe hypertriglyceridemia. *Journal of Cardiovascular Risk, 4, 385–91.*

Harris, W. S., Mozaffarian, D., Lefevre, M., Toner, C. D., Colombo, J., Cunnane, S. C., Holden, M., Klurfeld, M., Morris, M. & Whelan, J. (2009). Towards establishing dietary reference intakes for eicosapentaenoic and docosahexaenoic acids. *Journal of Nutrition*, 139, 804S–19S.

Hartweg, J., Farmer, A. J., Holman, R. R., & Neil, A. (2009). Potential impact of omega-3 treatment on cardiovascular disease in type 2 diabetes. *Current Opinion in Lipidology*, 20(1), 30-38.

He, K., Song, Y., Daviglus, M. L., et al. (2004). Accumulated evidence on fish consumption and coronary heart disease mortality: a meta-analysis of cohort studies. *Circulation,* 109, 2705–11.

Hirayama, S. & T. Miida, T. (2012). Small dense LDL: an emerging risk factor for cardiovascular disease. *Clinica Chimica Acta*, 414, 215–224.

Hokanson, J. E. & Austin, M. A. (1996). Plasma triglyceride is a risk factor for cardiovascular disease independent of high density lipoprotein cholesterol: a meta analyses of population based prospective studies. *Journal of Cardiovascular Research*, 3, 213-19.

Hoogeveen, R., Gaubatz, J. W., Sun, W., Dodge, R. C., Crosby, J. R., Jiang, J., Couper, D.,…., Ballantyne, C. M. (2014). Small dense low-density lipoprotein-cholesterol concentrations predict risk for coronary heart disease: the Atherosclerosis Risk in Communities (ARIC) study. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34(5), 1069–1077.

Hooper, L., Thompson, R. L., Harrison, R. A., Summerbell, C. D., Moore, H., Worthington, H. V., Durrington, P. N., …., Ebrahim, S. B. (2004). Omega 3 fatty acids for prevention and treatment of cardiovascular disease. *Cochrane Database Systematic Reviews*, CD003177.

Hooper, L., Thompson, R. L., Harrison, R. A., Summerbell, C. D., Ness, A. R., Moore, H. J., Worthington, H. V., ... Smith, G. (2006). Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. *The BMJ,* 332, 752– 60.

HPS2-THRIVE Collaborative Group. (2013). HPS2-THRIVE randomized placebo-controlled trial in 25673 high-risk patients of ER niacin/laropiprant: trial design, pre-specified muscle and liver outcomes, and reasons for stopping study treatment. *European Heart Journal*, 34:1279–1291.

Ingelsson, E., Schaefer, E. J., Contois, J. H., McNamara, J., Sullivan, L., Keyes, M., Pencina, M. J., …., Vasan, R. S. (2007). Clinical utility of different lipid measures for prediction of coronary heart disease in men and women. *JAMA*, 298,776–85.

Ivanova, E., Myasoedova, V., Melnichenko, A., Grechko, A. & Orekhov, A. (2017). Small Dense Low-Density Lipoprotein as Biomarker for Atherosclerotic Diseases. *Oxidative Medicine and Cellular Longevity.* https://doi.org/10.1155/2017/1273042

Jacobson, A., Glickstein, S., Rowe, J., Paresh N. & Soni, P. (2012). Effects of eicosapentaenoic acid and docosahexaenoic acid on low-density lipoprotein cholesterol and other lipids: A review. *Journal of Clinical Lipidology*, 6, 5-18.

Jacobson, T. (2008). Role of n-3 fatty acids in the treatment of hypertriglyceridemia and cardiovascular disease. *American Journal of Clinical Nutrition*, 87, 1981S–90S.

Jacobson, T., Ito, M., Maki, K, Orringer, C., Bays, H., Jones, P., McKenney, J., …, Brown, V. (2015). National Lipid Association Recommendations for Patient-Centered Management of Dyslipidemia. *Journal of Clinical Lipidology*, 9, 129–169.

Jeppesen, J., Hein, H. O. & Suadicani, P. (1998). Triglyceride concentration and ischemic heart disease: an eight-year follow-up in the Copenhagen Male Study. *Circulation*, 97, 1029-36.

Joint British Societies. (2014). Joint British Societies' consensus recommendations for the prevention of cardiovascular disease.100:ii1–ii67.

Julius, U. (2017). History of Lipidology and Lipoprotein Apheresis. *Atherosclerosis*. doi: 10.1016/j.atherosclerosissup.2017.05.034

Kannel, W. B., Dawber, T. R., Friedman, G. D., Glennon, W. E. & McNamara, P. M. (1964). Risk factors in coronary heart disease. An evaluation of several serum lipids as predictors of coronary heart disease; The Framingham Study. *Annual Internal Medicine*, 61,888-899.

Kastelein, J., Maki, K., Susekov, A., Ezhov, M., Nordestgaard, B., Machielse, B., Kling, D. & Davidson, M. (2014). Omega-3 free fatty acids for the treatment of severe hypertriglyceridemia: The EpanoVa fOr Lowering Very high triglyceridEs (EVOLVE) trial. *Journal of Clinical Lipidology*, 8, 94-106.

Keys, A. (1951). Giant molecules and cholesterol in relation to atherosclerosis. *Bulletin of the Johns Hopkins Hospital*, 88(5), 473-483.

Khera, A., Cuchel, M., Llera-Moya, M., Rodrigues, A., Burke, M., Jafri, K., French, B.,....Rader, D. (2011). Cholesterol Efflux Capacity, High-Density Lipoprotein Function, and Atherosclerosis. *New England Journal of Medicine*, 364(2), 127–135.

Krauss, R. (2010). Lipoprotein subfractions and cardiovascular disease risk. *Current Opinion in Lipidology*, 21(4), 305-411.

Kromhout, D., Giltay, E. J. & Geleijnse, J. M. (2010). n-3 fatty acids and cardiovascular events after myocardial infarction. *New England Journal of Medicine*, 363, 2015–26.

Lamarche, B., Lemieux, I. & Despres, J. P. (1999). The small dense LDL phenotype and the risk of coronary heart disease: Epidemiology, pathophysiology and therapeutic aspects. *Diabetes & Metabolism*, 25, 199-211.

Leon, H., Shibata, M. C., Sivakumaran, S., Dorgan, M., Chatterley, T. & Tsuyuki, R. T. (2008). Effect of fish oil on arrhythmias and mortality: systematic review. *The BMJ*, 337, a2931.

Liu, J., Sempos, C. T., Donahue, R. P., Dorn, J., Trevisan, M., Grundy, S. M. (2006). Non-high-density lipoprotein and very-low-density lipoprotein cholesterol and their risk predictive values in coronary heart disease. *American Journal of Cardiology*, 98, 1363–1368.

Maki, K. C., McKenney, J. M., Reeves, M. S., Lubin, B. C. & Dicklin, M. R. (2008). Effects of adding prescription omega-3 acid ethyl esters to simvastatin (20 mg/day) on lipids and lipoprotein particles in men and women with mixed dyslipidemia. *American Journal of Cardiology*, 102(4), 429-33.

Maki, K. C., Orloff, D. G., Nicholls, S., Dunbar, R., Roth, E., Curcio, D., Johnson, J., Kling, D. & Davidson, M. H. (2013). A highly bioavailable omega-3 free fatty acid formulation improves the cardiovascular risk profile in high-risk, statin-treated patients with residual hypertriglyceridemia (the ESPRIT trial). *Clinical Therapeutics*, 35(9), 1400-11.

Marik, P.E. & Varon, J. (2009). Omega-3 dietary supplements and the risk of cardiovascular events: a systematic review. *Clinical Cardiology*, 32, 365–72.

Martin, S., Abd, T., Jones, S., Michos, E., Blumenthal, R. & Blaha, M. (2014). 2013 ACC/AHA Cholesterol Treatment Guideline: What Was Done Well and What Could Be Done Better. *Journal of the American College of Cardiology*, 63, 2674–8.

Massaro, M., Scoditti, E., Carluccio, M. A. & Caterina, R. (2010). Nutraceuticals and Prevention of Atherosclerosis: Focus on ω-3 Polyunsaturated Fatty Acids and Mediterranean Diet Polyphenols. *Cardiovascular Therapeutics*, 28, e13–e19.

Mataki, H., Watuji, K., Miwa, S., Matsushita, K., Miki, T., Go, M., Ishida, K.,…, Yokoyama, M. (1998). Effect of eicosapentaenoic acid in combination with HMG-CoA Reductase Inhibitor on lipid metabolism. *Atherosclerosis*, 134 (2), 129-134.

Mensink, R. P., Zock, P. L, Kester, A. & Katan, M. B. (2003). Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *American Journal of Clinical Nutrition*, 77, 1146–1155.

Mente, A., de Koning, L., Shannon, H. S., Anand, S. S. (2009). A systematic review of the evidence supporting a causal link between dietary factors and coronary heart disease. *Archives of Internal Medicine*, 169, 659–69.

Miller M, Stone NJ, Ballantyne C, Bittner V, Criqui MH, Ginsberg HN, Goldberg AC, Howard WJ, Jacobson MS, Kris-Etherton PM, Lennie TA, Levi M, Mazzone T & Pennathur S. (2011) Triglycerides and cardiovascular disease: a scientific statement from the American Heart Association. *Circulation*, 123, 2292e2333.

Miller, P. & Martin, S. (2016). Approach to Statin Use in 2016: an Update. *Current Atherosclerosis Report*, 18, 20.

Mora, S., Otvos, J. D. , Rifai, N., Rosenson, R. S., Buring, J. E., Ridker, P. M. (2009). Lipoprotein particle profiles by nuclear magnetic resonance compared with standard lipids and apolipoproteins in predicting incident cardiovascular disease in women. *Circulation*, 119, 931–939.

Mora, S., Rifai, N. & Buring, J. (2008). Fasting compared with nonfasting lipids and apolipoproteins for predicting incident cardiovascular events. *Circulation,* 118, 993-1001.

Mozaffarian, D. & Rimm, E. B. (2006). Fish intake, contaminants, and human health: evaluating the risks and the benefits. *JAMA*, 296, 1885–99.

Mozaffarian, D. & Wu, J. (2011). Omega-3 Fatty Acids and Cardiovascular Disease. *Journal of the American College of Cardiology*, 58(20), 2047-67.

Mozaffarian, D., Aro, A., Willett & W. C. (2009). Health effects of trans-fatty acids: experimental and observational evidence. *European Journal of Clinical Nutrition*, 63, S5–S21.

Murray, L. & Lopez, A. D. (1996). *The global burden of disease: a comprehensive assessment of mortality and disability from diseases, injuries, and risk factors in 1990 and projected to 2020*. Boston, US: Harvard University Press.

N.J. Stone, J.G. Robinson, A.H. Lichtenstein, C.N. Bairey Merz, C.B. Blum, R.H. Eckel, *et al.* (2013). ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circulation*, 129 (25 Suppl 2), S1–S45.

National Cholesterol Education Program. (2002). Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report. *Circulation*, 106(25), 3143–3421.

Nordmann, A., Briel, M., Keller, U., Yancy, W., Brehm, B. & Bucher, C. (2006) Effects of low-carbohydrate vs low-fat diets on weight loss and cardiovascular risk factors: a meta-analysis of randomized controlled trials. *Archives of Internal Medicine*, 166, 285–293.

Nordøy, A., Bonaa, K., Nilsen, H., Berge, R., Hansen J. & Ingebretsen, O. (1998). Effects of simvastatin and omega-3 fatty acids on plasma lipoproteins and lipid peroxidation in patients with combined hyperlipidaemia. *Journal of Internal Medicine*, 243(2):163-70.

Nordøy, A., Hansen, J., Brox, J. & Svensson, B. (2001). Effects of atorvastatin and omega-3 fatty acids on LDL subfractions and postprandial hyperlipemia in patients with combined hyperlipemia. *Nutrition, Metabolism and Cardiovascular Diseases*, 11(1), 7-16.

Packard, C. (2006). Small dense low-density lipoprotein and its role as an independent predictor of cardiovascular disease. *Current Opinion in Lipidology*, 17(4), 412–417.

Packard, C., Caslake, M. & Shepherd, J. (2000). The role of small, dense low density lipoprotein (LDL): a new look. *International Journal of Cardiology*, 74(1), S17-22.

Pirillo, A & Catapano, A. L. (2013). Omega-3 polyunsaturated fatty acids in the treatment of atherogenic dyslipidemia. *Atherosclerosis*, 14, 237-242.

Pownall, H. J., Brauchi, D., Kilinc, C., Osmundsen, K., Pao, Q., Payton-Ross, C., Gotto, A. M. & Ballantyne, C. M. (1999). Correlation of serum triglyceride and its reduction by omega–3 fatty acids with lipid transfer activity and the neutral lipid compositions of high-density and low-density lipoproteins. *Atherosclerosis,* 143, 285–97.

Rana, J., Boekholdt, S., Kastelein, P. & Shah, P. (2012). The Role of Non-HDL Cholesterol in risk stratification for coronary artery disease. *Current Atherosclerosis Report*, 14, 130–134.

Rauch, B., Schiele, R., Schneider, S., Diller, F., Victor, N., Gohlke, H., Gottwik, M.,..., OMEGA Study Group. (2010). OMEGA, a randomized, placebo-controlled trial to test the effect of highly purified omega-3 fatty acids on top of modern guideline-adjusted therapy after myocardial infarction. *Circulation,* 122, 2152–9.

Rees, K., Hartley, L., Flowers, N., Clarke, A., Hooper, L., Thorogood, M. & Stranges, S. (2013). Mediterranean dietary pattern for the primary prevention of cardiovascular disease. *The Cochrane Database of Systematic Reviews*. 8:CD009825.

Ridker, P. (2014). LDL cholesterol: controversies and future therapeutic directions. *Lancet*, 384, 607–17.

Rimm, E. B, Williams, P., Fosher, K., Criqui, M. & Stampfer, M. J. (1999). Moderate alcohol intake and lower risk of coronary heart disease: meta-analysis of effects on lipids and hemostatic factors. *The BMJ,* 319, 1523–1528.

Rizzo, M & Berneis, K. (2006). Low-density lipoprotein size and cardiovascular risk assessment. *Monthly Journal of the Association of Physicians*, 99(1), 1–14.

Rizzo, M. & Berneis, K. (2006). The clinical relevance of low-density-lipoproteins size modulation by statins. *Cardiovascular Drugs and Therapies*, 20, 205–217.

Schwartz, G. G., Olsson, A.G., Abt, M.(2012). Effects of dalcetrapib in patients with a recent acute coronary syndrome. *New England Journal of Medicine*, 367, 2089–2099.

Shen H, Xu L, Lu J, Hao T, Ma C, Yang H, Lu Z, Gu Y, Zhu T, Shen G. (2015). Correlation between small dense low-density lipoprotein cholesterol and carotid artery intima-media thickness in a healthy Chinese population. *Lipids in Health and Disease*, 29(14):137-142.

Stark, K. D., Park, E. J. & Maines, V. A. (2000). Effect of a fish-oil concentrate on serum lipids in postmenopausal women receiving hormone replacement therapy in a placebo-controlled, double blind trial. *American Journal of Clinical Nutrition*, 72, 389–394.

Tavazzi, L., Maggioni, A. P., Marchioli, R., Barlera, S., Franzosi, M. G., Latini, R., Lucci, D.,…, Gissi-HF Investigators. (2008). Effect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial): a randomised, double-blind, placebo-controlled trial. *Lancet,* 372, 1223–30.

The Risk and Prevention Study Collaborative Group. (2013). n-3 fatty acids in patients with multiple cardiovascular risk factors. *New England Journal of Medicine*, 368(19), 1800–8.

Thompkinson, D. K., Bhavana, V. & Kanika, P. (2014) Dietary approaches for management of cardio-vascular health- a review. *Journal of Food Science and Technology*. 51(10):2318–2330.

Toth, P., Barter, P., Rosenson, R., Boden, W., Chapman, J., Cuchel, M., D'Agostino, R.,…, Rader, D. (2013). High-density lipoproteins: A consensus statement from the National Lipid Association. *Journal of Clinical Lipidology*, 7, 484–525.

Wang, C., Harris, W. S., Chung, M., Lichtenstein, A. H., Balk, E. M., Kupelnick, B., Jordan, H. S. & Lau, J. (2006). n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review. *American Journal of Clinical Nutrition*, 84, 5–17.

Weintraub, H. S. (2014). Overview of prescription omega-3 fatty acid products for hypertriglyceridemia. *Postgraduate Medicine*, 126, 7–18.

Woodman, R. J., Mori, T. A., Burke, V., Puddey, I. B., Watts, G., Best, J. D. & Beilin, L. J. (2003). Docosahexaenoic acid but not eicosapentaenoic acid increases LDL particle size in treated hypertensive type 2 diabetic patients. *Diabetes Care*, 26, 253.

World Health Organization. (2016). Cardiovascular Diseases Fact Sheet 317. Retrieved from: www.who.int/mediacentre/factsheets/fs317/en/ (accessed 2 February 2017)

Yokoyama, M., Origasa, H., Matsuzaki, M., Matsuzawa, Y., Saito, Y., Ishikawa, Y., Oikawa, S.,…, Japan EPA lipid intervention study (JELIS) Investigators. (2007). Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet, 369, 1090–8.

RESEARCH PAPER

EFFECT OF N-3 FATTY ACID SUPPLEMENTATION IN HYPERLIPIDEMIC

PATIENTS TAKING STATINS ON LIPID PROFILE, INCLUDING SMALL DENSE

LDL: A RANDOMIZED CONTROLLED TRIAL

Journal Decision

This work would potentially be published in Atherosclerosis journal. Atherosclerosis brings together papers that concern "*arterial and vascular biology and disease, as well as their risk factors including: disturbances of lipid and lipoprotein metabolism, diabetes and hypertension, thrombosis, and inflammation*". According to the 2016 Web of Science citation reports, Atherosclerosis has an impact factor of 4.239 and ranks 37 in the field of Cardiac & Cardiovascular Systems. Having 9 similar clinical studies published in the past 5 years, Atherosclerosis seems to be a reasonable target for publishing this work.

Abstract

Background: Epidemiological and clinical evidence suggests that high-dose intake of long-chain OM-3 FA have a favourable role in altering blood TAG and non-HDL-C when combined with statins in hyperlipidemic patients. Their efficacy in altering LDL-C particle size and concentration is yet to be confirmed.

Aim: This study evaluated the effects of adding 4/day EPA+DHA to stable statin therapy on non-HDL-C, TAG, HDL-C, LDL-C and small&dense LDL-C particle concentration in a group of hyperlipidemic patients.

Methods: In this randomized, placebo-controlled, double-blind parallel group study, 44 subjects who were already on statin therapy for >8 weeks and had non-HDL-C levels above the National Lipid Association Recommendations were randomized into two groups. For 8 weeks, together with their prescribed atorvastatin, the intervention group received 4 g/day EPA+DHA (in ethyl ester form) while the control group received 4 g/day olive oil (placebo). Baseline measurements of non-HDL-C, TAG, TC, HDL-C, LDL-C, VLDL-C and LDL-C particle concentration were repeated at week 8. Differences in dietary intake was assessed with a weighted 3-day food diary at week 4. Primary outcome measures were the percent change in LDL-C III non-HDL-C and particle concentration from baseline.

Results: At the end of treatment, the median percent change in LDL-C III particle concentration was significantly greater with OM-3 FA plus atorvastatin compared with placebo plus atorvastatin (-67.5% vs -0%, respectively; P <0.001). OM-3 FA plus atorvastatin was associated with significant reductions in non-HDL-C (-9.5% vs 4.7%, P<0.01), TAG (-21.5% vs 6.2%, P <0.001) and VLDL-C (-36.9% vs 4.0%, P<0.001) and TC (-6.6% vs 2.1%, P<0.001). Between the groups, no significant

difference in percent change in LDL-C, HDL-C, as well as LDL-C I and LDL-C II particle concentration was observed.

Conclusion: In these adult, white patients with hyperlipidemia, P-OM3 plus atorvastatin improved LDL-C phenotype, non-HDL-C and other lipid and lipoprotein parameters to a greater extent than atorvastatin alone.

Introduction

Hyperlipidemia, is one of the known etiological causes of atherosclerosis (Julius, 2017). Epidemiological studies have shown that elevated serum levels of triglycerides (TAG) and low density lipoprotein cholesterol (LDL-C) as well as low levels of high density lipoprotein cholesterol (HDL-C) are independently and partially linked to the development of atherosclerosis (Garg, Aggarwal, Kumar, & Sharma, 2015). Although much focus has been on LDL-C as the primary therapeutic target of atherosclerosis, recent National Lipid Association guideline (Jacobson et al., 2015) suggest that non-high density lipoprotein cholesterol (non-HDL-C) might be a superior indicator of cardiovascular disease risk (CVD) than LDL-C is, based on immense clinical evidence (Boekholdt et al., 2012). More importantly, LDL-C phenotype is also suggested to be an important determinant of atherosclerotic process. Small and dense LDL-C particles have an increased capacity to penetrate into the arterial wall and are more readily oxidized compared to the large and buoyant ones (Ivanova, Myasoedova, Melnichenko, Grechko & Orekhov, 2017). Consistent with this, clinical studies collectively show a positive relation between serum concentration of small&dense LDL-C particles and CVD risk (Austin et al., 1988; Krauss, 2010; Berneis & Krauss, 2002; Rizzo & Berneis, 2006; Arai et al., 2013; Hoogeveen et al., 2014; Shen et al., 2015).

Although statins are the first drug of choice to alter blood lipids, accumulating evidence suggests that marine-derived omega-3 fatty acids (OM-3 FA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have a capacity to treat hyperlipidemia (Massaro, Scoditti, Carluccio & Caterina, 2010). High-dose EPA&DHA supplementation effectively decreased plasma triglycerides and non-HDL-C in several clinical studies. In two large clinical trials EVOLVE (n=399) (Kastelein et al., 2014) and MARINE (n=229) (Bays et al., 2011), 4g/day EPA&DHA supplementation alone resulted in 31 %($p<0.001$) and 33% ($p<0.0001$) reduction in serum TAG as well as 10% ($p<0.01$) and 18% ($p<0.0001$) reduction in non-HDL-C, respectively. LDL-C, however, is usually reported to rise on EPA&DHA treatment, possibly due to increased production of LDL-C from VLDL-C (Pirillo & Catapano, 2013). Balk et al. (2006) reported in their systematic review a 6 mg/dL (+3, +8, P= 0.0006) increase in LDL-C levels. In the EVOLVE trial, LDL-C increased 19% ($p<0.001$) with 4 g/day EPA&DHA intake. The increase in LDL-C with EPA&DHA monotherapy, however, is usually counter balanced when these fatty acids are combined with statins (Nordoy et al., 1998). Indeed, clinical trials that compare statin monotherapy with n-3 combination therapies collectively show added benefits on blood lipid profile and conclude that EPA&DHA enhance statin-mediated improvements, with a range of 30 to 55% reductions in TAG, as well as 10-20% reductions in non-HDL-C without concomitant increase in LDL-C (Davidson et al., 2007; Maki et al., 2013, Bays et al., 2010; Ballantyne et al, 2012). In COMBOS trial (n=254), 4g/day EPA+DHA supplementation combined with simvastatin resulted in 10% reduction in non-HDL-C ($p<.001$) with no accompanying increase in LDL-C (Davidson et al., 2007), while the same study design in ESPRIT trial (n=647) showed 7% decrease in non-HDL-C ($p<0.01$) with no significant change in LDL-C (Maki et al., 2013).

As opposed to the great number of clinical studies that investigate the role of OM-3 FA in altering blood lipids, their effect on LDL-C particle size and concentration has not been widely investigated. Nordøy, Hansen, Brox & Svensson (2001) reported a significant decrease in LDL-C particle size (p<.05) when 1.68 g/d EPA&DHA is combined with statin, despite the relatively low-dose of OM-3 FA supplements. Similar results are reported by Maki et al. (2008) with 4g/day EPA+DHA combination therapy with statin. Subsequent studies that tested the efficacy of EPA&DHA with statin and reported positive results did not measure LDL-C particle size and concentration in their studies despite these earlier findings (Bays et al., 2010; Vecka et al., 2012; Ballantyne et al., 2012; Maki et al., 2013; Chan et al., 2016).

In the present study, the efficacy of dietary supplementation with 4 g/day EPA+DHA to statin therapy for lowering non-HDL-C and small&dense LDL-C particle concentration in statin treated hyperlipidemic subjects was investigated. This study was approved by the University of Chester Faculty of Clinical Sciences and Medicine Research Ethics Committee (REF: 1288/17/GD/CSN) **(Appendix I: Ethical Approval)**.

Methods

Participants

Eligible participants were Caucasian men or women aged between 50 and 79 years who had been receiving a stable dose of atorvastatin for the control of LDL-C levels at least for 8 weeks before initial screening. Inclusion criteria was current combined hyperlipidemia with a non-HDL-C level above the National Lipid Association treatment goals (Jacobson et al., 2015) **(Table 5).**

Table 5 NLA Treatment goals for non–HDL-C, LDL-C in mg/dL

	Treatment Goal	
Risk category	Non-HDL-C	LDL-C
Low	130	100
Moderate	130	100
High	130	100
Very-high	100	70

Exclusion criteria included current use of OM-3 supplements, recent history of certain heart, kidney, liver, or cancer (patients that have had any type of heart surgery, patients that are diagnosed with any type of cancer and/or have had any kind of cancer therapy, patients that have had kidney failure, patients that have had liver failure) in the past 6 months, as well as ongoing pregnancy or lactation. All subjects were asked to give a signed informed consent **(Appendix II: Informed Consent)** before study-protocol was conducted.

Recruitment

100 patients who had been prescribed to atorvastatin for more than 8 weeks were invited for the initial screening, during which their serum LDL-C was measured. 38 of them did not meet the inclusion criteria, and 12 eligible subjects did not give consent to take part in the intervention. 44 subjects, out of the remaining 50, were randomly recruited for the study. The random sampling was done via www.randomizer.com.

Study Design

This was a randomized, double-blind, placebo controlled, parallel groups study. **Figure 1** summarizes the trial design. Before entry into the intervention phase of the study, 8-weeks of dietary lead in was done in accordance with NICE guidelines (2014) and subjects were instructed

to maintain this diet throughout the study. Adherence to dietary advice was measured by a 3-days weighted food diary at Week 4. Nutrient content of the diet was calculated with Nutritics software (Nutritics Ltd, Ireland) using UK: SACN 2015 food composition tables.
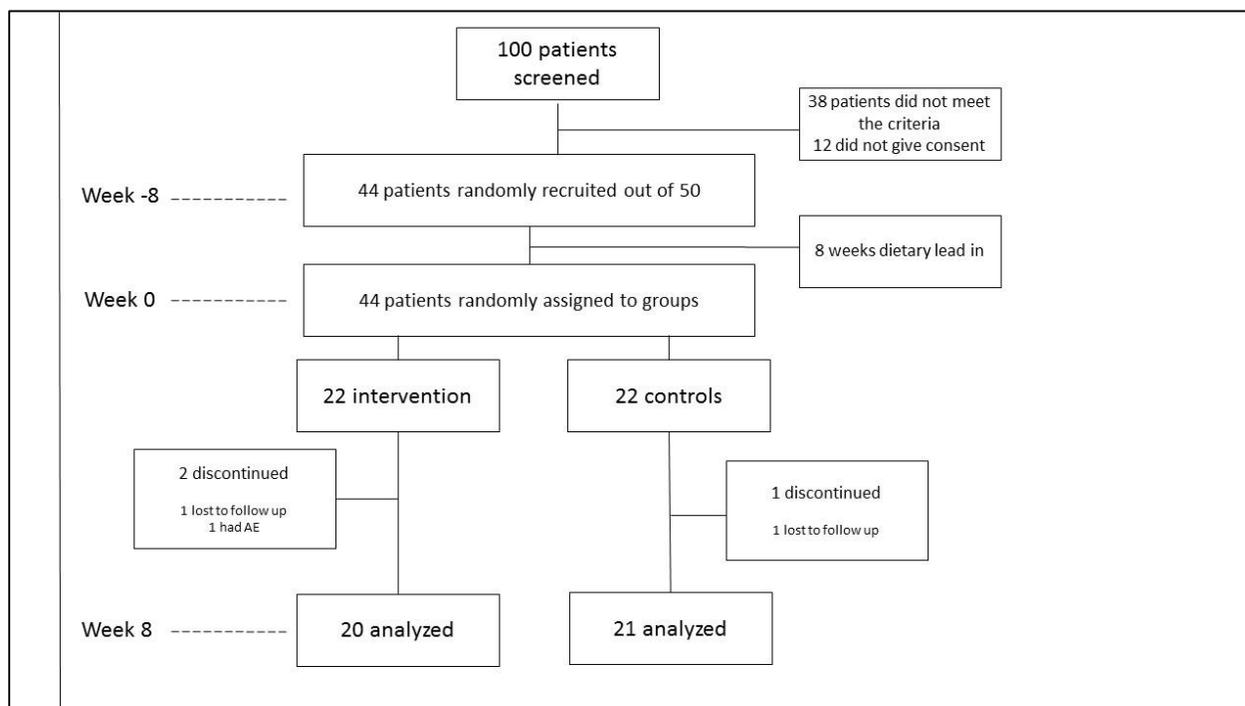
Figure 1 Study Flowchart

After dietary lead-in, baseline measurements of fasting blood Triglycerides (TAG), Total Cholesterol (TC), High-density Lipoprotein cholesterol (HDL-C), Low-density Lipoprotein cholesterol (LDL-C), and LDL-C sub groups (LDL-C I, LDL-C II, LDL-C III, LDL-C IV, LDL-C V) were done for all subjects at 2 visits separated by 1 week, and the means were used as baseline values. Non-High-density Lipoprotein cholesterol (non-HDL-C) was calculated by subtracting HDL-C from TC. After baseline measurements, all 44 subjects were randomized by www.randomizer.com in equal numbers to receive either EPA (%75) & DHA (%25) 4g/day ethyl esters (Peak EPA, Wiley's Finest™) or olive oil (placebo) 4g/day for 8 weeks in combination with the same dose of atorvastatin they have been prescribed to. The total EPA and DHA dose recommended by American Heart Association for lipid lowering is approximately 4 g/day (Miller et al., 2011), which is the common

therapeutic dose used in several major clinical trials including COMBOS, MARINE, ESPIRIT, EVOLVE, ANCHOR. For all subjects, atorvastatin dosage has been kept constant throughout the trial.

Both groups took 4 one-g capsules orally 4 times daily and compliance was measured by the number of capsules consumed relative to the number estimated to be consumed. OM-3 tablets included EPA&DHA ethyl esters (1000g), other OM-3 FA (60 mg), fish gelatin, glycerine, purified water and alpha tocopherol, while placebo tablets contained extra virgin olive oil (1000g0, bovine gelatin, glycerine, purified water.

Baseline measurements were repeated at the end of week 8. Study participants and investigators remained blinded to all laboratory results until the last subject completed the 8-week intervention period. During the treatment phase, subjects attended clinic visits at weeks 4 and 8.

Biochemical Measurements

Laboratory analyses were performed by Pax Clinic (Istanbul, Turkey) on the serum or plasma of 12 hours fasting blood samples. LDL-C, VLDL-C and HDL-C were measured with homogeneous enzymatic colorimetric assay of Roche Diagnostics (USA). TAG and TC was measured with enzymatic colorimetric assay of Roche Diagnostics (USA). LDL-C subgroups were analyzed with electrophoresis by Lipoprint (Quantimetrix, USA).

TC: Cholesterol esters were cleaved by cholesterol esterase to produce free cholesterol and FA. Cholesterol oxidase then catalysed the oxidation of cholesterol to cholest‑4‑en‑3‑one and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide effected the

oxidative coupling of phenol and 4‑aminophenazone to form a red quinone‑imine dye. The colour intensity of the dye formed was proportional to the cholesterol concentration. It was determined by measuring the increase in absorbance.

HDL: In the presence of magnesium ions, dextran sulfate selectively formed water‑soluble complexes with LDL-C, VLDL-C and chylomicrons which were resistant to PEG‑modified enzymes. The cholesterol concentration of HDL‑C was determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups. Cholesterol esters were broken down quantitatively into free cholesterol and FA by cholesterol esterase. In the presence of peroxidase, the hydrogen peroxide generated reacted with 4‑amino‑antipyrine and HSDA to form a purple‑blue dye. The colour intensity of this dye was directly proportional to the cholesterol concentration and was measured photometrically.

LDL: Cholesterol esters and free cholesterol in LDL were measured on the basis of a cholesterol enzymatic method using cholesterol esterase and cholesterol oxidase in the presence of surfactants which solubilize only LDL. The enzyme reactions to the lipoproteins other than LDL were inhibited by surfactants and a sugar compound. Cholesterol in HDL-C and VLDL-C was not determined. Cholesterol esters were broken down quantitatively into free cholesterol and FA by cholesterol esterase. In the presence of oxygen, cholesterol was oxidized by cholesterol oxidase to Δ4‑cholestenone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide generated reacted with 4‑aminoantipyrine and EMSE to form a red purple dye. The colour intensity of this dye was directly proportional to the cholesterol concentration and was measured photometrically.

LDL-C sub fractions: Lipophilic dye binded proportionally to the relative amount of cholesterol in each lipoprotein and the prestained lipoproteins subsequently underwent electrophoresis. During the first phase of electrophoresis, the lipoprotein particles were concentrated by the loading and stacking gels into a sharp narrow band. As the lipoprotein particles migrated through the separating gel matrix, they were resolved into lipoprotein bands according to their particle sizes from largest to smallest due to the sieving action of the gel.

Statistical Analysis

Power analysis was performed by using the G*Power software (v3.1.9) program to determine the sample size. At the baseline, a pilot study was performed with 10 people in each group. When the percentage change in non-HDL values of the groups was investigated, this was found to be 3.25±17.02 in the control group and -8.86±3.81 in OM-3 group. According to evaluation performed by using these data, effect size was calculated to be d=0.982 and each group should include 18 people to obtain 80% power at a level of $\alpha=0.05$. Sample size was assigned as 44 to allow for subject attrition and other potential causes of study withdrawal up to 20%. Demographic and baseline analysis were performed for all study participants, while efficacy analysis were performed only on subjects that successfully completed 8-weeks study protocol. The primary efficacy end point was non-HDL and LDL-C III particle concentration percentage change from baseline; secondary efficacy end points were changes in TAG, TC, LDL-C, VLDL-C and HDL-C.

Normal distribution of the sample was checked with Shapiro-Wilk's test given the fact that sample size is less than 100 (Coakes & Steed, 2013). Independent samples t-test was used for the intergroup comparisons of quantitative variables with normal distribution and Mann Whitney U

test was used for the intergroup comparisons of quantitative variables without normal distribution. Paired Samples t-test was used for the in-group comparisons of quantitative variables with normal distribution and Wilcoxon Signed Ranks test was used for the in-group comparisons of quantitative variables without normal distribution. Pearson's chi-square test was used for comparison of qualitative data. Levene's test was conducted to check homogeneity of variance between groups (Cohen & Holliday, 1996). P value was taken as 0.05 in determining significance.

Results

Subjects

Of the 44 subjects randomly assigned to the trial, 41 have completed it. In the treatment arm, 1 subject reported adverse effect (nausea) and 1 was lost to follow-up, while in the placebo group 1 subject was lost to follow-up.

The baseline demographics of the patients are listed in **Table 6**. The subjects were predominantly men (63%) with a mean age of 60.62±9.56 years and body mass index of 23.8±3.0 kg/m². No statistically significant difference was determined between groups regarding age, BMI values and gender ratios; as well as baseline TAG, TC, HDL-C, non-HDL-C, LDL-C, VLDL-C, LDL-C I, LDL-C II and LDL-C III values (p>0.05) **(Table 6 & Table 8).**

Table 6 Baseline Characteristics of the Participants

	Control (n=21)	OM-3 (n=20)	p
	Mean±SD	Mean±SD	
Age (years)	60.91±8.56	60.32±9.53	0.830

BMI (kg/m^2)		24.10±2.94	23.50±3.17	0.525
Gender, n (%)	Female	8 (36.4)	8 (36.4)	[b]0.999
	Male	14 (63.6)	14 (63.6)	

Diet

The analysis of total energy derived from dietary components shows no significant difference between the groups **(Table 7)**.

Table 7 Comparison of diets during the intervention periods as assessed by dietary records

Dietary Compounds	Control	OM-3	p
	Median (Q$_1$, Q$_3$)	Median (Q$_1$, Q$_3$)	
Total energy (kcal)	3222 (3090, 3420)	3274 (3105, 3420)	0.879
Total energy (MJ)	13.5 (12.9, 14.3)	13.7 (13.0, 14.3)	
CHO (%E)	51.25 (50.8, 52)	51.25 (50.9, 52)	0.629
Free Sugars (%E)	5.25 (5.1, 6.1)	5.25 (5.2, 5.3)	0.820
Protein (%E)	16.4 (16.3, 17.2)	16.4 (15.9, 17.1)	0.378
Fat (%E)	32.1 (31.4, 32.9)	32.1 (30.7, 32.7)	0.859
SFA (%E)	9.5 (8.9, 9.9)	9.5 (8.9, 9.8)	0.962
MUFA (%E)	10.9 (10.6, 11.3)	11.2 (10.6, 11.9)	0.364
PUFA (%E)	9.25 (8.8, 9.6)	9.25 (8.8, 10.8)	0.972
EPA (%E)	0.91 (0.89, 0.98)	0.91 (0.89, 0.97)	0.730
DHA (%E)	0.92 (0.91, 0.97)	0.92 (0.91, 0.97)	0.392

Values are **median (Q$_1$, Q$_3$),** Q$_1$: First quartile and Q$_3$: Third quartile. Values were derived from food composition tables.

Laboratory Measurements

Baseline and week 8 values as well as percentage change from baseline to the end of treatment for the primary and secondary endpoints are shown in **Table 8.**

Table 8 Outcome variables at baseline and the end of treatment in mmol/L and their percent change (%)

		Control (n=21)	OM-3 (n=20)	p
		Mean±SD	Mean±SD	
TAG	Baseline	1.61±0.85	1.62±0.60	0.924
	8 week	1.66±0.86	1.29±0.37	0.090
	% Difference	6.20±10.14*	-21.51±12.15***	<0.001***
TC	Baseline	4.97±0.81	4.91±0.72	0.820
	8 week	5.05±0.83	4.55±0.65	0.049*
	% Difference	2.06±2.97**	-6.55±3.59***	<0.001***
HDL-C	Baseline	1.40±0.47	1.34±0.32	0.677
	8 week	1.42±0.49	1.37±0.30	0.716
	% Difference	2.90±6.73*	1.09±6.97	0.403
Non-HDL-C	Baseline	3.55±0.60	3.47±0.59	0.999
	8 week	3.62±0.72	3.20±0.54	0.042*
	% Difference	4.65±21.00	-9.47±4.58***	0.007**
LDL-C	Baseline	2.61±0.65	2.66±0.61	0.831
	8 week	2.64±0.61	2.64±0.55	0.985
	% Difference	0.91±4.35	-0.08±5.20	0.510
VLDL-C	Baseline	0.93±0.25	0.90±0.29	0.684
	8 week	0.98±0.25	0.55±0.20	<0.001***
	% Difference	3.95±9.93	-36.88±11.75***	<0.001***
LDL-C I	Baseline	1.07±0.35	1.03±0.30	0.689
	8 week	1.08±0.36	1.03±0.24	0.647
	% Difference	1.41±10.21	3.89±15.73	0.551

		Control (n=21)	OM-3 (n=20)	p
		Mean±SD	Mean±SD	
LDL-C II	Baseline	0.56±0.20	0.63±0.26	0.381
	8 week	0.56±0.19	0.67±0.30	0.209
	% Difference	-0.74±16.24	1.55±17.27	0.664
LDL-C III,	Baseline	0.05 (0.02, 0.10)	0.09 (0.05, 0.15)	0.158
median (Q$_1$, Q$_3$)	8 week	0.05 (0.02, 0.10)	0.02 (0.00, 0.08)	0.090
	% Difference	0 (0, 0)	-67.5 (-100, -31.25)**	<0.001***

Q$_1$: First quartile Q$_3$: Third quartile   *p<0.05   **p<0.01   ***p<0.001

Primary endpoints

No statistically significant difference was determined between groups regarding post-intervention week **LDL-C III** concentration. The percent change in the control group was not found to be significant, however the percent change in OM-3 group at the 8th week from baseline was determined to be significant (p< 0.001). Also, the percent change in LDL3 value observed in OM-3 group was determined to be significantly different compared to the control group (p<0.001).

8th week **non-HDL-C** concentration was determined to be significantly lower in OM-3 group compared to the control group (p= 0.042). No significant change was determined in the control group, but the change in OM-3 group at the 8th week from baseline was determined to be significant (p<0.001). The percentage change in non-HDL value observed in OM-3 group was determined to be statistically significantly different compared to the control group (p= 0.007).

Secondary endpoints

The percentage change in **TAG** concentration of the control group (+6.20±10.14 %) and OM-3 group (-21.51±12.15 %) at the 8th week from baseline was determined to be statistically

significant (p= 0.012, p<0.001, respectively). However, the percent change observed in OM-3 group was also determined to be statistically significantly different compared to the control group (p<0.001).

TC at week 8 was determined to be significantly lower in OM-3 group when compared to the control group (p= 0.049). The percent change both in the control group (2.06±2.97) and OM-3 group (-6.55±3.59) at the 8th week from baseline was determined to be significant; respectively (p: 0.004), (p<0.001). However, the percentage change observed in OM-3 group was also determined to be significantly different compared to the control group (p<0.001).

No significant difference was determined between groups regarding 8th week **HDL-C** concentration. The percent change in the control group at the week 8 from baseline was determined to be significant (p=0.049). No significant change was determined in the OM-3 group. No significant difference was determined between groups regarding the percentage change observed in HDL values.

Week 8 **LDL-C**, **LDL I** and **LDL II** cholesterol concentrations show no significant difference between groups. The percent change of these values in the control and OM-3 groups at the 8th week from baseline was not found to be significant. No significant difference was determined between groups also regarding the percentage change observed in these values.

VLDL-C concentration was determined to be significantly lower in OM-3 group compared to the control group at week 8 (p<0.001). The percentage change in the control group was not found to be significant, but the percentage change in OM-3 group was determined to be

significant (p<0.001). The percentage change in VLDL value observed in OM-3 group was determined to be significantly different compared to the control group (p<0.001).
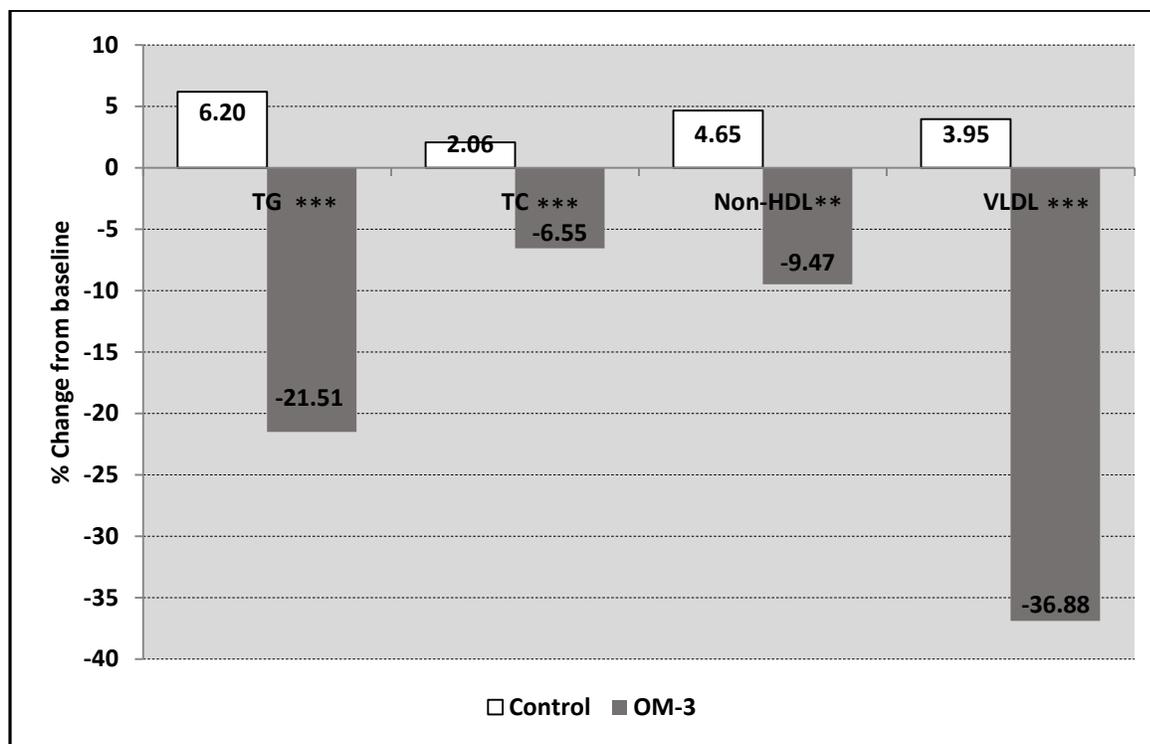
*p<0.05   **p<0.01   ***p<0.001

Figure 2 Mean percent change in TAG, TC, Non-HDL and VLDL from baseline to the end or treatment

Discussion

In this randomized, parallel-group, double-blind, placebo-controlled trial of men and women with hyperlipidemia on statins, 4-g/d EPA+DHA supplementation produced significant reductions in the primary endpoints non-HDL-C and LDL-C III particle concentration (-9.47±4.58, p<0.001 and -67.5 (-100, -31.25), p<0.01, respectively) compared with OO.

The role of marine OM-3 FA in altering LDL-C particle phenotype had not been much studied in the past. An early study by Contacos, Barter & Sullivan (1993) reported a significant increase in LDL stokes' diameter from 25.0 to 25.9 nm (P<.05), however LDL-C III particle

concentration was not measured in this study. In their study of combined therapy with 1.68 g/day EPA&DHA with atorvastatin (n=42), Nordøy, Hansen, Brox & Svensson (2001) reported a significant %40 (p<0.05) reduction in LDL-C III particle concentration in the treatment arm. However, not only this figure was not significantly different than the change in the control group, but also the serum concentration of large and buoyant LDL-C I and LDL-C II were also reduced in their study as opposed to this one, in which a non-significant increase in large and buoyant LDL-C particles were also observed, signalling to a shift within the phenotypes. Maki et al. (2008) found a significant increase in LDL-C particle size from 19.9 (19.2, 22.0) nm to 20.4 (19.3, 21.7) nm (p= 0.024) in their cross-over study (n=39) when 4/g EPA&DHA is combined with simvastatin. Different from Contacos, Barter & Sullivan (1993), Maki et al. (2008) also measured LDL-C III particle concentration and reported no significant change between control and intervention arms. It is of particular importance that this present study showed a difference in LDL-C III particle concentration, given the fact that LDL-C III and IV particle concentration is suggested to be a stronger predictor of CVD than LDL-C particle size is (Otvos et al., 2006; Cromwell & Otvos, 2004).

Previous studies have shown similar results regarding the effect of OM3-FA supplementation on non-HDL-C level. Davidson et al. (2007) found in COMBOS trial that dietary supplementation with 4g/day EPA&DHA during 40 mg/day simvastatin therapy resulted in a 9.1% reduction (p<0.001) in non-HDL-C compared to 2.2 % (p<.001) with corn oil. The same dose of OM-3 and statin caused 6.9% reduction (p<0.01) in ESPIRIT trial by Maki et al. (2013), compared to 0.9% (p>0.05) with olive oil. Similarly, Nordøy et al. (1998), Durrington et al. (2001) and Chan et al. (2016) reported approximately 10% significant reduction in non-HDL-C in their studies of combination therapy. On the other hand, higher reductions in non-HDL-C were also reported in

the past. Maki et al. (2008), for instance, found 40% reduction (p<0.001) in their study of a similar design with 39 subjects, however, the control group also showed a remarkably great and significant reduction (34%, p<0.001) in that study. Furthermore, in the ANCHOR trial, Ballantyne et al. (2012) also reported 15% reduction (p<0.0001) in non-HDL-C. Reason for this could be the use of EPA ethyl esters alone in this study, given that EPA might lead to greater average reductions in non-HDL-C than DHA (Jacobson, Glickstein, Rowe & Soni, 2012). However, little is known about these fatty acids' solo effects on individual lipids (Mori & Woodman, 2006) and available studies point out that they have complementary roles to each other (Schaefer et al., 2010), therefore their combined use is more widely preferred, as it was done in this study.

In the present study, the decrease in non-HDL-C cholesterol in the OM-3 group was achieved mainly due to lowering of VLDL concentrations (-36.88±11.75 %, p<0.0001), as well as other triglyceride-rich lipoproteins such as chylomicron remnants. The significant reduction in VLDL-C concentration is consistent with the previous evidence (Davidson et al., 2007; Maki et al., 2008; Bays et al., 2010; Ballantyne et al., 2012; Maki et al., 2013). In human physiology, increased amount of VLDL-C particles positively correlate with increased TAG levels (Dagnelie, Rietveld, Swart, Stijinen & van den Berg, 1994). These findings collectively confirm that marine OM-3 down regulate VLDL metabolism through decreasing TAG synthesis and increasing triglyceride clearance (Harris et al., 1997).

The significant 21.51±12.15 % (p<0.001) reduction in TAG in the present study is slightly smaller than the findings of some of the previous studies. Kastelein et al. (2014) reported a 31% reduction (p<0.001) and Bays et al. (2011) reported 33% (p<0.0001) reduction in serum TAG with 4g/day EPA&DHA monotherapy. Davidson et al. (2007) reported 28% reduction when the same

OM-3 dose is combined with statin. The relatively smaller reduction in TAG achieved in this study might be due to the difference in the baseline characteristics of the subjects when compared to the abovementioned studies. Baseline TAG of the intervention group (145.05±52.67 mg/dL, 1.62±0.60 mmol/l) was much less than EVOLVE and MARINE and trials (655 and 679 mg/dL, 7.40 and 7.67 mmol/l, median values, respectively) and the percent reduction in TAG levels highly depends on the baseline values (Weintraub, 2014; Harris et al., 1997). Supportive of this, the baseline TAG value of the OM-3 group in ESPIRIT trial (Maki et al., 2013) was 287± 82.8 mg/dL (3.25±0/94 mmol/l) and the achieved reduction in serum TAG was −20%, (p<0.01), similar to this study.

Reducing blood TAG concentration is important for two reasons. First of all, TAG, as the substrate of LDL-C synthesis, is deemed as a target in clinical management of dyslipidemia and each 1 mmol/l reduction in TAG levels is believed to reduce CV risk by 14% in men and 37% in women (Hokanson & Austin, 1996). More importantly, it has been long known that clinically significant reductions in TAG levels is typically accompanied by a shift in LDL particle size from smaller and denser particles (LDL-C III, IV) to larger and more buoyant particles (LDL-C I, II) (Krauss & Dreon, 1995). Serum TAG concentration is inversely related to the LDL-C particle size and it can been hypothesized that reducing TAG would result in an increase in LDL-C particle size (Watson et al., 1994). In this study, the significant decrease in serum concentration of LDL-C III (-67%, p<0.01) from 3.5 mg/dL to 1 mg/dL in the OM-3 group and the increase (albeit not significant) in LDL-C I from 39.86±11.69 mg/dL to 40.25±8.96 mg/dL and in LDL-C II from 24.45±10.22 mg/dL to 25.65±11.78 mg/dL is likely to be linked to the significant decrease in serum TAG.

In the control group, TC, TAG and HDL-C at week 8 had shown significant changes from baseline, although values are minor. One possible explanation why control group changes occurred in the present study is that late-onset effects of the dietary run-in and continued adherence to the healthy & balanced diet might have improved these variables.

The present study has certain limitations. First of all, small sample size and short intervention duration limits the conclusions that can be drawn. Although the lipid alterations achieved in this study are consistent with the findings of larger studies with longer duration, findings should still be confirmed in a larger population. Furthermore, the capsule load was high (4 capsules per day) and compliance was measured only by capsule count. Ideally, it needs to be confirmed by blood tests of EPA and DHA fatty acids. In this study, a supplemental form of OM-3 containing EPA & DHA in ethyl ester form was used. However, recent studies point out that EPA & DHA in free fatty acids (FFA) form may have 4-fold greater bioavailability than OM-3 ethyl esters (Davidson et al., 2012) given that their absorption does not involve pancreatic lipase (Maki et al., 2013). Particularly when taken on an empty stomach, FFA formulation would have provided great flexibility in the dosing schedule. However, this prescription OM-3 is not available in the Turkish market.

The use of OO as a control might had certain non-neutral effects on the outcome variables due to its high oleic acid content and the role of oleic acid in CVD prevention (Pineo & Anderson, 2008). However, 4 g/day is a too low dose to bias the result, especially when compared to the amount used in studies such as PREDIMED, which showed that 50 g/day use of OO showed improved CVD risk (Estruch et al., 2013). Several studies used OO as control given the lack of a true placebo.

Conclusion

There is a clinical need to effectively reduce small&dense LDL-C particle concentration for CVD prevention. Agents that lower blood TAG are usually successful in altering LDL-C particle phenotype. However, efficacy of OM-3 FA, which is a TAG lowering agent, in LDL-C particle altering has been much investigated. In this double-blind, randomized, controlled trial of combined use of OM-3 FA with atorvastatin in patients with hyperlipidemia, supplementation of EPA and DHA at 4 g/d dosage improved the overall lipid profile in 8 weeks, including significant lowering of LDL-C III and non-HDL-C concentrations. Further studies are required to confirm the results of this trial.

References

Arai, H., Kokubo, Y., Watanabe, M., Sawamura, T., Ito, Y., Minagawa, A., Okamura, T & Miyamato, Y. (2013). Small dense low-density lipoproteins cholesterol can predict incident cardiovascular disease in an urban Japanese cohort: the Suita study. *Journal of Atherosclerosis and Thrombosis*, 20(2), 195–203.

Austin, M. A., Breslow, J., Hennekens, C., Buring, J., Willett, W. & Krauss, R. (1988). Low-density lipoprotein subclass patterns and risk of myocardial infarction. *JAMA*, 260(13), 1917-1921.

Balk, E. M., Lichtenstein, A. H., Chunga, M., Kupelnick, B., Chewa, P. & Laua, J. (2006). Effects of omega-3 fatty acids on serum markers of cardiovascular disease risk: A systematic review. *Atherosclerosis*, 189, 19-30.

Ballantyne, C. M., Bays, H. E., Kastelein, J. J., Stein, E., Isaacsohn, J. L., Braeckman, R. A. & Soni, P. N. (2012). Efficacy and safety of eicosapentaenoic acid ethyl ester (AMR101) therapy in statin treated patients with persistent high triglycerides (from the ANCHOR study). *American Journal of Cardiology*, 110, 984–92.

Bays, E., McKenney, J., Maki, K. C., Doyle, R. T., Carter, R. N. & Stein, E. (2010). Effects of prescription omega-3-acid ethyl esters on non-high-density lipoprotein cholesterol when coadministered with escalating doses of atorvastatin. *Mayo Clinic Proceedings*, 85(2), 122-8.

Bays, H. E., Ballantyne, C. M., Kastelein, J. J., Isaacsohn, J. L., Braeckman, R. A. & Soni, P. N. (2011). Eicosapentaenoic acid ethyl ester (AMR101) therapy in patients with very high triglyceride levels (from the Multi-center, placebo-controlled, Randomized, double-blind, 12-week study with an open-label Extension [MARINE] trial). American Journal of Cardiology, 108, 682–90.

Berneis, K. & Krauss, R. (2002). Metabolic origins and clinical significance of LDL heterogeneity. *Journal of Lipid Research*, 43, 1363-1379.

Boekholdt, S., Arsenault, B., Mora, S., Pedersen, T., LaRosa, J., Nestel, P., Simes, R.,…, Kastelein, P. (2012). Association of LDL Cholesterol, Non–HDL cholesterol, and apolipoprotein B levels with risk of cardiovascular events among patients treated with statins: A meta-analysis. *JAMA,* 307(12),1302-1309.

Chan, D. C., Pang, J., Barrett, P., Sullivan, D. R., Mori, T. A., Burnett, R., vanBockxmeer, F. & Watts, G. F. (2016). Effect of omega-3 fatty acid supplementation on arterial elasticity in patients with familial hypercholesterolaemia on statin therapy. *Nutrition, Metabolism and Cardiovascular Diseases*, 26(12), 1140-1145.

Coakes, S. J. & Steed, L. G. (2013). *SPSS: version 20:0 for Windows: Analysis without anguish*. Singapore: Wiley.

Cohen, L., Holliday, L. (1996). *Practical Statistics for Students: An Introductory Text*. London: Sage

Contacos, C., Barter, P. J. & Sullivan, D. R. (1993). Effect of Pravastatin and ω-3 Fatty Acids on Plasma Lipids and Lipoproteins in Patients with Combined Hyperlipidemia. *Arteriosclerosis and Thrombosis*, 13(12), 1755-62.

Cromwell, W. C. & Otvos, J. D. (2004). Low-density lipoprotein particle number and risk for cardiovascular disease. *Current Atherosclerosis Report*, 6, 381–387.

Dagnelie, P. C., Rietveld, T., Swart, G. R., Stijinen, T. & van den Berg, J. W. (1994). Effect of dietary fish oil on blood levels of free fatty acids, ketone bodies, and triacylglycerol in humans. *Lipids*, 29, 41–45.

Davidson, M. H., Johnson, J., Rooney, M. W., Kyle, M., Kling, D. (2012). A novel omega-3 free fatty acid formulation has dramatically improved bioavailability during a low-fat diet compared with omega-3-acid ethyl esters: the ECLIPSE study. *Journal of Clinical Lipidology*, 6, 573–584.

Davidson, M. H., Stein, E. A., Bays, H. E., Maki, K. C., Doyle, R. T., Shalwitz, R. A., Ballantyne, C. M., Ginsberg, H. N.; COMBination of prescription Omega-3 with Simvastatin (COMBOS) Investigators. (2007). Efficacy and tolerability of adding prescription omega-3 fatty acids 4 g/d to simvastatin 40 mg/d in hypertriglyceridemic patients: an 8-week, randomized, double-blind, placebo-controlled study. Clinical Therapeutics, 29(7), 1354-67.

Durrington, P. N., Bhatnagar, D., Mackness, M. I., Morgan, J., Julier, K., Khan, M. A & France, M. (2001). An omega-3 polyunsaturated fatty acid concentrate administered for one year decreased triglycerides in simvastatin treated patients with coronary heart disease and persisting hypertriglyceridaemia. *Heart*, 85(5), 544-8.

Estruch, R., Ros, E., Salas-Salvadó, J., Covas, M. I., Corella, D., Arós, F., Gómez-Gracia, E.,…, PREDIMED Study Investigators. (2013). Primary prevention of cardiovascular disease with a Mediterranean diet. New England Journal of Medicine, 368(14), 1279-90.

Garg, R., Aggarwal, S., Kumar, R. & Sharma, G. (2015). Association of atherosclerosis with dyslipidemia and co-morbid conditions: A descriptive study. *Journal of Natural Science Biology and Medicine*, 6(1), 163–168.

Harris, W. S., Ginsberg, H. N., Arunakul, N., Shachter, N. S., Windsor, S. L., Adams M, Berglund, L. & Osmundsen, K. (1997). Safety and efficacy of Omacor in severe hypertriglyceridemia. *Journal of Cardiovascular Risk, 4, 385–91.*

Hokanson, J. E. & Austin, M. A. (1996). Plasma triglyceride is a risk factor for cardiovascular disease independent of high density lipoprotein cholesterol: a meta analyses of population based prospective studies. *Journal of Cardiovascular Research*, 3, 213-19.

Hoogeveen R., Gaubatz, J. W., Sun, W., Dodge, R. C., Crosby, J. R., Jiang, J., Couper, D.,…., Ballantyne, C. M. (2014). Small dense low-density lipoprotein-cholesterol concentrations predict risk for coronary heart

disease: the Atherosclerosis Risk in Communities (ARIC) study. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 34(5), 1069–1077.

Ivanova, E., Myasoedova, V., Melnichenko, A., Grechko, A. & Orekhov, A. (2017). Small Dense Low-Density Lipoprotein as Biomarker for Atherosclerotic Diseases. *Oxidative Medicine and Cellular Longevity.* https://doi.org/10.1155/2017/1273042

Jacobson, A., Glickstein, S., Rowe, J., Paresh N. & Soni, P. (2012). Effects of eicosapentaenoic acid and docosahexaenoic acid on low-density lipoprotein cholesterol and other lipids: A review. *Journal of Clinical Lipidology*, 6, 5-18.

Jacobson, T., Ito, M., Maki, K, Orringer, C., Bays, H., Jones, P., McKenney, J., …, Brown, V. (2015). National Lipid Association Recommendations for Patient-Centered Management of Dyslipidemia. *Journal of Clinical Lipidology*, 9, 129–169.

Julius, U. (2017). History of Lipidology and Lipoprotein Apheresis. *Atherosclerosis*. doi: 10.1016/j.atherosclerosissup.2017.05.034

Kastelein, J., Maki, K., Susekov, A., Ezhov, M., Nordestgaard, B., Machielse, B., Kling, D. & Davidson, M. (2014). Omega-3 free fatty acids for the treatment of severe hypertriglyceridemia: The EpanoVa fOr Lowering Very high triglyceridEs (EVOLVE) trial. *Journal of Clinical Lipidology*, 8, 94-106.

Kastelein, J., Maki, K., Susekov, A., Ezhov, M., Nordestgaard, B., Machielse, B., Kling, D. & Davidson, M. (2014). Omega-3 free fatty acids for the treatment of severe hypertriglyceridemia: The EpanoVa fOr Lowering Very high triglyceridEs (EVOLVE) trial. *Journal of Clinical Lipidology*, 8, 94-106.

Krauss, R. & Dreon, D. M. (1995). Low-density-lipoprotein subclasses and response to a low-fat diet in healthy men. *American Journal of Clinical Nutrition*, 62(2), 478Se87S.

Krauss, R. (2010). Lipoprotein subfractions and cardiovascular disease risk. *Current Opinion in Lipidology*, 21(4), 305-411.

Maki, K. C., McKenney, J. M., Reeves, M. S., Lubin, B. C. & Dicklin, M. R. (2008). Effects of adding prescription omega-3 acid ethyl esters to simvastatin (20 mg/day) on lipids and lipoprotein particles in men and women with mixed dyslipidemia. *American Journal of Cardiology*, 102(4), 429-33.

Maki, K. C., Orloff, D. G., Nicholls, S., Dunbar, R., Roth, E., Curcio, D., Johnson, J., Kling, D. & Davidson, M. H. (2013). A highly bioavailable omega-3 free fatty acid formulation improves the cardiovascular risk profile in high-risk, statin-treated patients with residual hypertriglyceridemia (the ESPRIT trial). *Clinical Therapeutics*, 35(9), 1400-11.

Massaro, M., Scoditti, E., Carluccio, M. A. & Caterina, R. (2010). Nutraceuticals and Prevention of Atherosclerosis: Focus on ω-3 Polyunsaturated Fatty Acids and Mediterranean Diet Polyphenols. *Cardiovascular Therapeutics*, 28, e13–e19.

Miller, M., Stone, N. J., Ballantyne, C., Bittner, V., Criqui, M. H., Ginsberg, H. N., Goldberg, A. C.,…, Council on the Kidney in Cardiovascular Disease. (2011). Triglycerides and cardiovascular disease: A scientific statement from the American Heart Association. *Circulation*, 123, 2292–2333.

Mori TA & Woodman RJ. (2006). The independent effects of eicosapentaenoic acid and docosahexaenoic acid on cardiovascular risk factors in humans. *Current Opinion in Clinical Nutrition & Metabolic Care*, 9, 95–104.

National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). (2002). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation*, 106, 3143–3421.

Nordøy, A., Bonaa, K., Nilsen, H., Berge, R., Hansen J. & Ingebretsen, O. (1998). Effects of simvastatin and omega-3 fatty acids on plasma lipoproteins and lipid peroxidation in patients with combined hyperlipidaemia. *Journal of Internal Medicine*, 243(2):163-70.

Nordøy, A., Hansen, J., Brox, J. & Svensson, B. (2001). Effects of atorvastatin and omega-3 fatty acids on LDL subfractions and postprandial hyperlipemia in patients with combined hyperlipemia. *Nutrition, Metabolism and Cardiovascular Diseases*, 11(1), 7-16.

Otvos JD, Collins D, Freedman DS, Shalaurova I, Schaefer EJ, Mc-Namara JR, Bloomfield HE, & Robins SJ. (2006). Low-density lipoprotein and high-density lipoprotein particle subclasses predict coronary events and are favorable changes by gemfibrozil therapy in the Veterans Affairs High-Density Lipoprotein Intervention Trial. *Circulation,* 113, 1556–1563.

Pineo, C.E. & Anderson, J.J.B. (2008). Cardiovascular benefits of the Mediterranean Diet. Nutrition Today. 43:114– 120.

Pirillo, A & Catapano, A. L. (2013). Omega-3 polyunsaturated fatty acids in the treatment of atherogenic dyslipidemia. *Atherosclerosis*, 14, 237-242.

Rizzo, M & Berneis, K. (2006). Low-density lipoprotein size and cardiovascular risk assessment. *Monthly Journal of the Association of Physicians*, 99(1), 1–14.

Schaefer, E. J., Asztalos, I. B., Gleason, J. A., Asztalos, B., Horvath, K., Dansinger, M. & Gillies, P. (2010). Effects of eicosapentaenoic acid, docosahexaenoic acid, and olive oil on cardiovascular disease risk factors. *Circulation,* 122, A20007.

Shen, H., Xu, L., Lu, J., Hao, T., Ma, C., Yang, H., Lu, Z., Gu, Y., Zhu, T. & Shen G. (2015). Correlation between small dense low-density lipoprotein cholesterol and carotid artery intima-media thickness in a healthy Chinese population. *Lipids in Health and Disease*, 29(14):137-142.

Watson, T. D., Caslake, M. J., Freeman, D. J., Griffin, B., Hinnie, J., Packard, C. J. (1994). Determinants of LDL subfraction distribution and concentrations in young normolipidemic subjects. *Arteriosclerosis Thrombosis*, 14(6), 902e10.

Weintraub, H. S. (2014). Overview of prescription omega-3 fatty acid products for hypertriglyceridemia. *Postgraduate Medicine*, 126, 7–18.