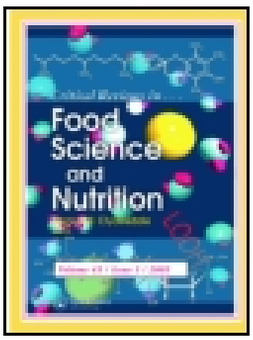

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TITLE

Fish consumption and cardiovascular disease related biomarkers: A review of clinical trials

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ABSTRACT

The purpose of this review is to collect and compare fish intervention studies. Prospective studies have outlined the beneficial effect of frequent fish consumption on cardiovascular incidents that is attributed to n-3 fatty acids incorporated in fish, mainly eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids. This outcome triggered clinical trials to examine the effect of either fish intake or consumption of n-3 fatty acids via capsules on biomarkers related to cardiovascular disease (CVD). The absence of a recent review focusing on clinical trials regarding fish intake and not n-3 fatty acids supplements rendered necessary the composition of this article. In total, 28 studies on healthy volunteers were found to meet the inclusion criteria. With EPA and DHA intake varying between 0.03 to 5g per day, biomarkers, such as triglycerides, high-density lipoprotein and platelet aggregation, tended to ameliorate when daily intake exceeded 1g per day, while the most common inflammatory marker, C-reactive protein, was not affected. In all, fish consumption gives promising results; yet fish micronutrients, total diet fat, as well as other dietary habits may also affect biomarkers. Therefore, all these factors should be considered in future clinical trials in order for one to draw more reliable conclusions.

KEYWORDS

fish consumption, clinical trial, cardiovascular disease, thrombosis, inflammation, n-3 fatty acids

INTRODUCTION

Fish has always been a main ingredient in the diet of many different populations living close to the sea or fresh water (rivers, lakes), representing a great alternative to red meat, especially when the latter is rare or expensive. The immense diversity of the fish family provides people with quality protein, vitamins, essential fatty acids and micronutrients.

Since the Seven Countries Study (Menotti et al. 1999), the scientific community has extensively examined fish consumption in relation to its cardioprotective effect. Although results are still inconclusive, there is an established relation between frequent fish consumption and lower risk of cardiovascular disease (CVD) related incidents (Raatz, Silverstein, et al. 2013). Therefore, either as part of a whole diet, like the Mediterranean Diet, or separately examined, fish intake has been the subject of interest in many cross-sectional and prospective studies.

The beneficial effect of frequent fish consumption is mainly attributed to high content of polyunsaturated fatty acids (PUFA), represented by eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids, without excluding the positive impact of other fish ingredients. American Heart Association (AHA) and European Society of Cardiology (ESC) have both recommended the consumption of two servings of fish per week, with the one meal consisting of fatty fish (Kris-Etherton 2002; Perk et al. 2012), which may provide 1.5-2g of EPA and DHA (Mozaffarian and Rimm 2006) as a preventive step towards CVD.

During the last decades, a considerable number of studies were conducted including cross-sectional, prospective and interventional ones (Panagiotakos et al. 2009; Weichselbaum et al. 2013; Zampelas et al. 2005). Meta analyses on epidemiological studies reveal an association between decreased CVD risk and fish intake (Raatz, Silverstein, et al. 2013), even though many inconsistencies between

the examined studies exist. In regard to clinical trials, multicenter trials focusing on a diet advice scheme, capable of examining a significant part of the population, and smaller ones, focusing on a limited group of volunteers, able to provide fish meals or fish oil supplementation, have been conducted. The significant differences in the studies' design make it almost impossible to export generalized outcomes. Presently, meta-analysis literature is abundant on reviews of observational studies, and also of trials with fish oil supplements, while fish consumption interventions rarely appear among the selected studies for reviewing. Back in 1997, Knapp published a detailed review regarding dietary fats and their effect on haemostasis and thrombosis, including a significant amount of fish intake interventions (Knapp 1997).

The aim of the present review, is to conduct an extensive search in Scopus and PubMed, collect the existing fish intervention trials in healthy volunteers, compare their results and conclude an overall outcome regarding fish consumption effect on markers related to cardiovascular disease such as blood pressure, lipidemic and glycemic profile, inflammatory and thrombotic markers.

METHODS

A thorough search of Scopus and PubMed databases was carried out with the use of the following keywords in multiple combinations: fish consumption/intake, clinical trial, humans, atherosclerosis, inflammation, thrombosis, aggregation, biomarkers. The search was completed in December 2016 and the results were evaluated according to the pre-decided following criteria.

Inclusion/exclusion criteria

Only intervention clinical trials conducted on human subjects with at least one fish-eating group were included in the present review. The review comprises only full-access articles written in English and containing either a group that serves as a control, a run-in period or a follow-up period. Volunteer groups consuming red or white meat, or different types of fish or differently fed fish were also considered as control group. Articles should refer to healthy (non-pregnant) subjects. Overweight and obese volunteers were considered healthy, if the respective authors mentioned a good health status for them. Furthermore, all studies should refer to subjects' adherence to the intervention and also examine biochemical markers' changes apart from EPA and DHA levels.

Regarding the exclusion criteria, studies without control group, as well as abstract-only studies, were the first to be omitted. All studies concerning non-healthy subjects with established diseases were not taken into account and prospective, cohort and case-control studies were also not eligible. Moreover, studies that were only referring to fish-oil intake, whole diet plans, or were advice-only interventions were considered unsuited to be included in the present review. Clinical trials where fish was fortified with n-3 oils or volunteers followed a weight loss diet or exercise routine without the presence of a fish-only intervention group were also excluded. The latter, as well as studies referring only to EPA and DHA levels as an outcome, were not included in data elaboration. However, some of the excluded studies are mentioned separately, as they were of the first fish intervention clinical trials to be conducted, they gave valuable information and they set the base for all the next ones to come.

Comparison criteria

The diversity of research protocols among the studies creates obstacles as far as their comparison is concerned. To facilitate the presentation of the results, the outcomes are classified in these categories: blood pressure, lipidemic profile, glycemic profile, inflammatory markers, bleeding time, aggregation and thrombotic markers. Therefore, most studies are referred multiple times, as they cover more than one of the aforementioned categories. Although most studies state EPA and DHA levels in blood as consumption and absorption indicator, these outcomes are outside the field of interest of this review and only mentioned when a correlation with another biomarker is examined. Table 1 provides essential information of the clinical trials included regarding study design, intervention groups, mean daily intake of EPA and DHA for fish interventions, measured biomarkers, as well as their statistically significant results.

RESULTS

The very first clinical trials on fish consumption did not meet the inclusion criteria, yet triggered a long series of studies. They are characterized by a small number of volunteers (eight in average) and lack of control group. Moreover, they tended to provide excessive amounts of fish with daily intake even reaching 800g. Nevertheless, their promising results raised hopes, especially regarding platelet aggregation. Collagen and adenosine diphosphate (ADP**)** induced platelet aggregation is decreased considerably (Thorngren et al. 1984; Thorngren and Gustafson 1981) followed by reduced thromboxane B_2 production (Bradlow et al. 1983; Siess et al. 1980). Bleeding time is also affected with an increase of around 40% (Thorngren et al. 1984; Thorngren and Gustafson 1981). A significant drop of triacylglycerol (TAG) levels is also observed after 100-200g of fatty fish per day (Pfeuffer et al. 1988; Thorngren

and Gustafson 1981). As expected, the evidence above led scientists to elaborate more detailed trial protocols, results of which are given below.

Included studies

The inclusion criteria were met by 28 clinical trials on healthy volunteers. Less than half of them had a parallel design (13 studies) with the rest being cross-over studies. In addition, fish consumption effect versus the fish-oil intake effect was recorded in 6 out of the 28 studies. The duration of the interventions spanned from 10 days to 24 weeks while the frequency of consumption varied from 2 times per week to daily meals. Regardless the frequency of the meals, the EPA and DHA intake varied from 0.03g/d to 5g/d, depending on the fat content and the fish type. Furthermore, the Atlantic salmon was the most common fatty fish, followed by mackerel and herring, while cod and sole represented the lean fish group. Yet, in many studies, the subjects consumed a variation of fish in the same group, fatty, lean or both. The fish was given fresh, cooked or canned. Last but not least, 2 clinical trials examined the effect of differently fed fish on biomarkers (Hallund et al. 2010; Sofi et al. 2013) and 6 studies concerned overweight, but healthy, subjects (Lindqvist et al. 2007, 2009; Moore et al. 2006; Mori et al. 1994; Neale et al. 2013; Raatz, Rosenberger, et al. 2013).

Blood pressure

A daily consumption of fatty fish seems to have a lowering effect of both systolic (SBP) and diastolic (DBP) blood pressures. The beneficial effect has been clearly shown in a study where 15 healthy volunteers daily consumed canned mackerel (5g/d EPA and DHA) for two weeks, while SBP and DBP remained unchanged for the group consuming canned herring (2.8g/d EPA and DHA)

(Singer et al. 1983). However, in a multi-central trial with mackerel paste (Van Houwelingen et al. 1987) the lowering effect was observed in both fish (4.7g/d EPA and DHA) and control groups. Daily consumption of salmon (Lara et al. 2007) was beneficial for SBP but failed to alter DBP. Healthy men with slightly elevated blood pressure and cholesterol showed an improving trend for both SBP and DBP after receiving 4g/d of EPA and DHA via fish consumption (Beilin et al. 1993). On the other hand, neither a daily intervention with farmed trout (Hallund et al. 2010) nor sparser fish consumption (Grieger et al. 2014) achieved any significant changes regarding blood pressure. In total, an improvement might be observed only when the consumption of EPA and DHA exceeds 2g per day.

Lipidemic profile

As expected, most studies included lipoproteins as a primary outcome regardless the nature of the rest examined markers.

In general, TAG presented either a clean fall (Aadland et al. 2015; Hagen et al. 2016; Lara et al. 2007; Singer et al. 1983; Van Houwelingen et al. 1990) or a trend fall (Ågren et al. 1988, 1990, 1991; Gerhard et al. 1991; Kondo et al. 2010; Marckmann et al. 1991; Rajaram et al. 2009) for all meal frequencies as long as the average daily intake of EPA and DHA was over 0.8g. Regarding clinical trials that included groups also consuming fish oil capsules, a lowering effect of the fish group on TAG, similar to the effect of fish oil capsules, was observed only in one study (Ågren et al. 1996). Nevertheless, the other studies that included fish oil capsules and fish consumption had very low EPA and DHA intake in comparison to the amount taken via fish oil capsules (Brown et al. 1990) and/or only two fish meals per week versus a daily intake of fish oil (Đuričić et al. 2015; Elvevoll et al. 2006). Non-significant effect

on TAG was observed (Din et al. 2008; Grieger et al. 2014) in some cases and especially when overweight subjects were included (Lindqvist et al. 2007, 2009; Moore et al. 2006). In contrary, Mori et al. (1994) noted a beneficial effect of high EPA and DHA (4g/d) on overweight men consuming fish daily, an effect independent of diet's fat percentage. In addition, contradictory results exists regarding fish-oil-fed fish consumption and TAG levels (Hallund et al. 2010; Sofi et al. 2013).

Total cholesterol (TC) levels were not influenced by the increase of fish intake according to most studies examined (Aadland et al. 2015; Ågren et al. 1990, 1991, 1996; Brown et al. 1990; Din et al. 2008; Đuričić et al. 2015; Elvevoll et al. 2006; Grieger et al. 2014; Hagen et al. 2016; Hallund et al. 2010; Kondo et al. 2010; Lara et al. 2007; Lindqvist et al. 2007, 2009; Marckmann et al. 1991; Moore et al. 2006; Rajaram et al. 2009; Van Houwelingen et al. 1990). A lowering effect was observed when EPA and DHA intake reached 5g per day (Singer et al. 1983). One clinical trial regarding differently fed fish also showed a beneficial effect on TC levels but with no significant difference between the interventions, probably due to the fact that portions were calculated to provide 1.6 to 1.8g/d of EPA and DHA in both groups (Sofi et al. 2013). In another study controversial results on TC were reported, depending on the type of fish consumed (Gerhard et al. 1991). Elevated TC have also been reported in a study, a phenomenon that was reversed when the fish intervention was combined with lower total fat content (Ågren et al. 1988). Diet's fat content seems to affect TC, since Mori et al. (1994) observed lower TC levels on volunteers on 30% fat intake, while 40% diet fat provoked higher values compared to baseline.

Concerning lipoprotein levels, the majority of the clinical trials, presented non-significant alternations regardless the daily intake of EPA and DHA(Ågren et al. 1988, 1991; Brown et al. 1990; Din et al. 2008; Đuričić et al. 2015; Elvevoll et al. 2006; Grieger et al. 2014; Hallund et al. 2010; Kondo et al. 2010; Marckmann et al. 1991; Moore et al. 2006; Van Houwelingen et al. 1990). Two recent studies, concerning 0.9g EPA and DHA as maximum daily intake, observed an improvement regarding Very low density lipoprotein (VLDL) particles size (Aadland et al. 2015; Raatz et al. 2016). In one of them, low density lipoprotein (LDL) particle size increased even with an intake of EPA and DHA as low as 0.31g/d (Raatz et al. 2016). A clean drop of LDL levels was recorded in one of the differently-fed fish trials (Sofi et al. 2013). A beneficial trend for both LDL and high density lipoprotein (HDL) levels was detected in one of the first fish clinical trials (Singer et al. 1983) as well. Overweight subjects, consuming 1.2 to 3.4g/d EPA and DHA, were capable of increasing HDL and HDL_2 particles (Lindqvist et al. 2007, 2009). Few other studies also showed an amelioration for HDL levels (Lara et al. 2007; Rajaram et al. 2009) and size (Ågren et al. 1996) as well as a VLDL (Lara et al. 2007) drop regardless EPA and DHA daily intake. The higher diet's fat content (40%) led to LDL increase, while 30% fat intake decreased the LDL levels (Mori et al. 1994) and apolipoprotein B followed the same pattern. The same team also observed a beneficial influence on HDL levels for all subjects in the 40% fat intake intervention groups consuming fish with a simultaneous rise of HDL_2 and fall of HDL_3.

Apolipoproteins A and B (ApoA, ApoB), when measured, had similar behavior with LDL; Apo B increased only in one study where LDL increased as well (Gerhard et al. 1991). In two different studies of the same research team, a decrease of ApoA (Ågren et al. 1988) and ApoB (Ågren et al. 1988, 1990) was observed, while a non-significant effect was presented in studies where LDL did not change (Ågren et al. 1991, 1996; Rajaram et al. 2009).

Glycemic Profile

There was no significant effect, on glycemic markers, namely blood glucose, insulin and HOMA-IR (homeostatic model assessment-insulin resistance) (Hagen et al. 2016; Hallund et al. 2010; Kondo et al. 2010; Lara et al. 2007). The glycemic profile was not affected, even in the occasion of overweight subjects (Mori et al. 1994; Raatz, Rosenberger, et al. 2013).

Inflammatory markers

Although inflammatory markers have been related to cardiovascular disease (He 2009), they are poorly represented in the conducted clinical trials. C-reactive protein (CRP), the interleukins group and tumor necrosis factor-alpha (TNF-α) are most often measured while cell adhesion molecules are also reported in some occasions. Leptin and adiponectin, considered related to inflammatory mechanisms, are examined in few studies too.

CRP was not affected by the fish consumption regardless the amount consumed and the frequency of consumption (Chiang et al. 2012; Hagen et al. 2016; Lara et al. 2007; Lindqvist et al. 2009; Moore et al. 2006; Raatz, Rosenberger, et al. 2013). A very recent clinical trial observed a lowering trend for subjects having a CRP over 3mg/L (Grieger et al. 2014), while similar effect was found in overweight subjects regardless CRP levels (Lindqvist et al. 2007). Another study underlined the negative correlation between EPA and DHA plasma levels and CRP concentration, even if CRP was slightly elevated after salmon consumption (Đuričić et al. 2015). An increasing tendency of CRP was detected after trout consumption (Hallund et al. 2010).

Interleukins were mostly represented by interleukin-6 (IL-6) and interleukin-8 (IL-8), with interleukin-1-beta (IL-1β) and interleukin-10 (IL-10) appearing in few studies. Most fish interventions did not alter IL-6 concentration (Chiang et al. 2012; Grieger et al. 2014; Hallund et al. 2010). Same phenomenon appeared in overweight subjects (Lindqvist et al. 2009; Moore et al. 2006; Raatz, Rosenberger, et al. 2013) and neither IL-1β (Chiang et al. 2012; Grieger et al. 2014) nor IL-10 (Sofi et al. 2013) showed any differences. However, a negative correlation between EPA and DHA change and IL-8 levels was observed (Elvevoll et al. 2006). Moreover, only one clinical trial managed to show a significant drop of both IL-6 and IL-8 after the fish intervention that provided 1.8g/d of EPA and DHA (Sofi et al. 2013).

Regarding TNF-α, only one study reported a slight lowering effect, after a moderate fish consumption (1.2g/d EPA and DHA) as well as a negative correlation between TNF-α levels and EPA and DHA levels (Elvevoll et al. 2006). The rest of the trials did not show any significant change of TNF-α levels (Chiang et al. 2012; Moore et al. 2006; Sofi et al. 2013), while in one of them, TNF-α levels were below the detection threshold of the measuring method (Grieger et al. 2014).

Cell adhesion molecules have an essential role in connecting blood cells, mostly leukocytes and platelets, with the activated endothelium. Intercellular Cell Adhesion Molecule 1 (ICAM-1) in its soluble form (sICAM) (Đuričić et al. 2015; Lara et al. 2007; Lindqvist et al. 2009) remained generally the same after fish intervention, with only one study giving a lowering effect after moderate fish consumption (0.8g/d EPA and DHA) (Chiang et al. 2012). A no-significant effect was observed for soluble Vascular Cell

Adhesion Molecule 1 (sVCAM-1) as well (Đuričić et al. 2015; Hallund et al. 2010). In healthy subjects' studies, E-selectin was taken into account in only one occasion and the fish intervention did not modify its levels (Chiang et al. 2012).

Leptin and adiponectin were measured only in one and four studies respectively, therefore the results cannot be easily compared. The one study including leptin had healthy, non-obese subjects and leptin levels did not alter significantly after the fish intervention (Kondo et al. 2010). As for adiponectin, one study failed to show any change (Hagen et al. 2016), while an increasing trend after fish consumption was observed both in normal-weight volunteers (Lara et al. 2007), especially female (Kondo et al. 2010), and obese volunteers (Neale et al. 2013).

Bleeding time, aggregation and thrombotic markers

The studies examining coagulation parameters are few, most of them being carried out more than ten years ago. In addition, the newer ones usually focus on specific factors while neglecting others.

Bleeding time measurements were quite popular in the first fish intervention trials, and an important increase of bleeding time was only observed when the subjects consumed about 200g of fatty fish, giving more than 2.2g of EPA and DHA daily (Van Houwelingen et al. 1987; Wander and Patton 1991). Yet, this was not always the case since, 5 fish meals per week (Lindqvist et al. 2009; Nelson et al. 1991), did not significantly alter bleeding time even with an average daily intake of 3.4g EPA and DHA.

Two interventions, with salmon consumption giving 1.2 to 3.4g/d EPA and DHA, resulted in increased platelet size (Elvevoll et al. 2006; Nelson et al. 1991) while their count either decreased (Nelson et al. 1991) or remained stable (Brown et al. 1990; Elvevoll et al. 2006).

On the other hand, platelet aggregation is quite more explored with a variety of aggregating agents. In the case of collagen, being the most popular agent, volunteers eating 4 or more fish meals per week (more than 0.8g/d EPA and DHA) revealed lower ability of platelets to aggregate (Ågren et al. 1990, 1997; Wander and Patton 1991). Yet, this was not always the case, as other studies of similar EPA and DHA intake showed either a falling trend (Van Houwelingen et al. 1989) or no effect at all (Mann et al. 1997; Nelson et al. 1991). A resembling effect appears when ADP is used as aggregation agent, since 0.4-3.4g/d EPA and DHA tend to suppress platelet aggregation (Ågren et al. 1990; Nelson et al. 1991; Wander and Patton 1991), though there are also cases where no change is observed (Ågren et al. 1991, 1997). Mori et al. (1997) included platelet aggregation induced by collagen and platelet activating factor (PAF). Both fish and fish oil interventions achieved a significant comparable reduction of collagen-induced aggregation in both 40% and 30% fat diets. Concerning PAF-induced aggregation, all interventions resulted in lower aggregability but fish oil intake seemed more potent. In a more recent study, where flow cytometry was used to measure platelet-monocyte aggregation, a weekly consumption of 500g of mackerel, providing 1g/d EPA and DHA reduced the aggregation by 35% after 4 weeks (Din et al. 2008). Aggregation induced by arachidonic acid (AA) was ameliorated in one study (Nelson et al. 1991) while thrombin-induced aggregation was not influenced by frequent salmon consumption (Mann et al. 1997; Nelson et al. 1991). In addition, levels of thromboxane B_2 (TxB_2) significantly fell when daily intake of EPA and DHA exceeded 0.8g, provided by more than 3.7 fish meals per week (Ågren et al.

1988, 1990; Honstra et al. 1990; Mann et al. 1997; Mori et al. 1997; Wander and Patton 1991), while a lowering trend of TxB_2 was observed even with 0.7-1.2g/d EPA and DHA (Ågren et al. 1991; Elvevoll et al. 2006) and the dehydrogenated analog 11-D-TxB_2 was also less produced in one occasion (Chiang et al. 2012).

Fibrinogen, being the essential component of blood clots and consequently of atherosclerotic plaque and thrombosis, has been a subject of interest among studies. However, none of the interventions managed a significant effect on fibrinogen level either of normal weight (Ågren et al. 1997; Brown and Roberts 1991; Elvevoll et al. 2006; Lara et al. 2007; Marckmann et al. 1991) or overweight volunteers (Lindqvist et al. 2007; Moore et al. 2006), with only one study observing a significant increase of fibrinolytic activity (Brown and Roberts 1991). Fewer studies have also examined whether fish consumption could lower the plasminogen activator inhibitor-1 (PAI-1) activity and levels and, therefore, enhance fibrinolysis via tissue plasminogen activator (tPA) activity. Relatively low to moderate EPA and DHA intake, 0.6-1.2g/d, did not have any significant effect on PAI-1 levels (Elvevoll et al. 2006) or activity (Moore et al. 2006), while a daily fish consumption providing 4.7g/d EPA and DHA increased PAI-1 levels by 45% without altering plasminogen and tPA activity (Emeis et al. 1989). In another intervention with EPA and DHA intake reaching 3.4g/d (Marckmann et al. 1991), the intervention did not alter tPA or PAI-1 activity.

Regarding other coagulating factors, Factor VII_C was not affected by fish consumption in any of the studies (Ågren et al. 1997; Brown et al. 1990; Marckmann et al. 1991; Moore et al. 2006). Factor X was measured only in one case, demonstrating a significant decrease of its activity when EPA and DHA intake reached 1g/d (Ågren et al. 1997).

Last but not least, a daily consumption of mackerel, with EPA and DHA intake as high as 4.7g/d, significantly increased the urinary excretion of prostacyclin (PGI_2) metabolite, 6-keto-$PGF_{1\alpha}$, implying an increase of its circulation in blood (Honstra et al. 1990). However, when daily fish consumption provided 2.9g/d EPA and DHA, the levels of PGI_2 remained the same (Mann et al. 1997). Ågren et al. (1990) also measured 6-keto-$PGF_{1\alpha}$ in two different studies, and a decreasing trend of the metabolite secretion was detected when EPA and DHA were more than 0.8g/d. The decrease was amplified by a fat reduction in the diet but the change remained insignificant (Ågren et al. 1988).

DISCUSSION

At first glance, the results derived from this review seem to follow the uncertainty of the existing literature, providing a promising yet weak beneficial influence of frequent fish consumption. For the current analysis, an important number of eligible clinical trials was collected and studied, reaching 28 interventions in total. The great diversity among the experiment protocols rendered a quantitative comparison almost impossible. Differences in type of fish, frequency of meals and duration of the trial were considered the most important. EPA and DHA daily intake appears as an important factor but does not always justify the results. It should also be taken into account that all subjects were healthy and therefore all measured biomarkers appeared to be within normal range before the clinical trials took place with the exception of one clinical trial with slightly elevated blood pressure and cholesterol (Mori et al. 1994).

Daily fish consumption providing more than 2.4 g/d EPA and DHA resulted to a falling trend of both systolic and diastolic blood pressure (Beilin et al. 1993; Lara et al. 2007; Singer et al. 1983). It has been suggested that n-3 fatty acids have an antihypertensive

action in reference to supplements' trials (He 2009), a comment that agrees with the present outcomes, where significant amounts of EPA and DHA are consumed almost daily. These fatty acids tend to inhibit the production of vasoconstrictors such as thromboxane A_2 while favoring the production of potent vasodilators such as prostacyclin PGI_3, derived from EPA (Maehre et al. 2015; Poudyal et al. 2011). Moreover, ion channel function may also be affected when n-3 fatty acids are incorporated in lipid membranes (Mozaffarian and Wu 2011). Taking in to account that the aforementioned clinical trials usually do not reflect an average fish intake, a research more focused on a realistic dietary plan could shed light to whether a moderate consumption may lead to similar results as well.

An overall subtle beneficial effect was observed on lipidemic profile measurements. TAG generally followed at least a lowering trend even with only two fish meals per week and moderate EPA and DHA average daily intake (0.8g/d, though in a few cases TAG were unaffected (Din et al. 2008; Grieger et al. 2014; Lindqvist et al. 2007, 2009; Moore et al. 2006). EPA and DHA are associated with both TAG synthesis and clearance in in vitro and in vivo experiments (Mozaffarian and Wu 2011; Poudyal et al. 2011). Even though a clear explanation has not been given yet, EPA and DHA may influence transcription factors that influence the expression of enzymes connected to TAG metabolism leading to the underexpression of proteins that favor synthesis (Harris et al. 2008) and may also regulate apolipoprotein E action, a molecule essential for TAG clearance (Poudyal et al. 2011).

Total cholesterol was almost unaffected; diet fat content seems to be the major contributor concerning TC levels in comparison to fish consumption (Ågren et al. 1988; Gerhard et al. 1991; Mori et al. 1994). At the same time, the variety of lipoproteins does not follow the same pattern. VLDLs have not been sufficiently studied to draw safe conclusions. LDL is more unaffected by the beneficial

impact, while HDL increases not only in concentration but also in size. Enlargement of HDL molecules was observed in the studies where they were measured, followed by a drop of HDL_3 levels (Ågren et al. 1996; Mori et al. 1994). The last evidence may introduce another point of view regarding the examination of the lipidemic profile. Hence, one should carefully consider including lipoproteins size in the outcomes of a new study, and also appropriate measurements of their functionality. From a mechanistic perspective, there is evidence that n-3 fatty acids affect lipoprotein lipase activity and interact with peroxisome proliferator-activated receptors (PPARs) that are multifunctional transcription factors (Poudyal et al. 2011). Furthermore, n-3 fatty acids seem to influence key stages of HDL synthesis and maturation, such as promoting apolipoprotein A-I, though the exact mechanisms still remain unclear (Pizzini et al. 2017).

Recent reviews have linked n-3 fatty acids intake to their action against inflammation, tightly connected to initiation and progression of atherosclerosis (Calder 2012; He 2009). Nonetheless, these studies are mainly aiming on supplementation clinical trials. In the present attempt, the few clinical trials that included inflammatory markers did not present significant alternation. A decrease of IL-6 was only observed in one case (Sofi et al. 2013), providing 1.6-1.8g/d EPA and DHA. Probably, biomarkers regarding inflammation may need excessive amounts of n-3 fatty acids in order to be altered significantly, a quantity that could not be achieved only via fish consumption.

As a matter of fact, there is strong evidence, mostly from in vitro experiments, that EPA and DHA may exert anti-inflammatory action since they compete with AA as substrates for COX and LOX (Maehre et al. 2015), inhibit cytokines formation and down-regulate

inflammatory transcription factors (Calder 2014). Furthermore, n-3 fatty acids suppress in vitro PAF, a potent inflammatory lipid mediator (Calder 2014).

Platelet aggregation, endothelial function and thrombosis markers seem to be the converging point between existing literature and the present study. Back in 1997, Howard Knapp published a review regarding the effect of dietary fatty acids in human thrombosis and haemostasis, including the already important, and representing n-3 fatty acids, EPA and DHA (Knapp 1997). Although it's almost twenty years ago, the main outcomes are similar to the ones of this review. Platelet aggregation and function appear to be significantly and positively influenced by fish consumption. Collagen induced aggregation is the first to be affected, with other agents following, such as ADP, PAF and arachidonic acid. Yet, only 8 studies in total actually provided this information, leaving a promising but not certain beneficial effect of regular fish intake. TxB_2 follows aggregation pattern by undergoing a significant lowering trend after the intervention. Ka He tried to define the possible mechanism assuming that polyunsaturated fatty acids, especially n-3 chains, may induce the production of vasodilators and weak aggregating/inflammatory agents while suppressing the potent molecules of inflammation and thrombosis (He 2009). Not many studies carried out measurements of inflammatory and thrombotic markers and statistically significant results are too sporadic to be considered eligible and important. Overall, platelets are the most affected, also regarding a lower platelet count and a bigger platelet volume; yet, in recent trials, researchers do not focus on platelet aggregation.

Apart from n-3 fatty acids, other fish lipid micronutrients have been reported to inhibit PAF-induced aggregation in vitro (Nasopoulou et al. 2007, 2008; Nomikos et al. 2006). These results suggest that not only EPA and DHA but also other lipids as well are responsible

for this antithrombotic effect either by affecting platelets through agonist receptors or modulating platelet signal transduction or membrane density.

After cumulative assessment of the results, one may consider that frequent fish consumption can actually have a positive influence in several biomarkers, despite the differences between the studies. The beneficial effect fades out when the fish consumption is less than two portions per week, related to a low EPA and DHA daily intake of less than 0.8g/d and a return to baseline levels is observed when there is a follow up period (Din et al. 2008; Lara et al. 2007). Even though most clinical trials contribute scattered significant results, data from prospective studies favor the cardioprotective effect of habitual fish consumption (Burr et al. 1989; Panagiotakos et al. 2009; Zampelas et al. 2005), while major meta-analyses have difficulty finding a strong protective effect of n-3 fatty acids, due to the differences among the relevant studies (Weichselbaum et al. 2013). It is important to underline that volunteers already consuming fish frequently may not show any beneficial effects in case they participate in a clinical trial either with fish consumption or n-3 fatty acids oral supplements (Watanabe et al. 2009).

From a different point of view, fish influence should be included in a complete dietary plan. Not only Mediterranean Diet, but also nutritional schemes coming from Scandinavia are being increasingly studied. NORDIET, for instance, is inspired by various other diets such as the Mediterranean Diet and Dietary Approaches to Stop Hypertension (DASH) and includes a variety of fruits and vegetables and low fat dairy along with fatty fish consumption. A clear amelioration of the lipidemic profile can be attributed to a synergistic effect of many beneficial ingredients of the diet (Adamsson et al. 2011). This indication is supported by the five year

follow up of ATTICA study, where the most favored group was the one closer to the Mediterranean Diet pattern (Panagiotakos et al. 2009). Similarly, in the Sysdimet study which combined fatty fish, bilberries and whole grain products, the glucose impaired volunteers improved various markers of inflammation and endothelial function (de Mello et al. 2011).

The present review was limited to fish only interventions, ignoring the effect of volunteers' overall diet. A comparison of fish intervention trials with those regarding fish oil supplements was outside the limits of interest during the composition of this review. Yet, clinical trials including both intervention observed that fish consumption provided better incorporation of EPA and DHA than intake of fish oil supplements (Elvevoll et al. 2006; Visioli et al. 2004). In addition, other ingredients of fish flesh may also contribute to the beneficial effect of its consumption, such as amino acids, trace elements and other micronutrients (He 2009; Maehre et al. 2015). Still, fish interventions remain disadvantaged due to the simplicity of fish oil capsules, allowing n-3 fatty acids intake even by people averted by fish. In this case, one could develop new ways so as to extract both n-3 fatty acids and other bioactive micronutrients.

Undoubtedly, fish consumption should be more studied in regards to its effect on CVD related biomarkers. Upcoming clinical trials are advised to take more details into consideration, such as fat intake, medication and overall eating habits of the volunteers. One should also be more careful regarding the results by including statistical correlation in their extraction, so as to seek for important connections among biomarkers and consumed active ingredients like n-3 fatty acids. Last but not least, including fish in a healthy diet plan could lead to more promising results as volunteers would be benefited both by fish intake and other nutritional ingredients.

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Table 1: Clinical Trials on healthy subjects with or without fish-oil intervention

Study	Design	Intervention Groups	Average grams of EPA+DHA/day	Measured Markers	Significant Results
(Singer et al. 1983)	Crossover 2weeks 15 normalweight 3months washout	G1. 2 cans mackerel/day G2. 2 cans herring/day	G1: 5g G2: 2.80g	SBP, DBP, TAG, TC, LDL, HDL, ApoA, ApoB	G1: ↓ SBP, DBP, TAG, TC
(Emeis et al. 1989; Honstra et al. 1990; Van Houwelingen et al. 1987,1989, 1990)	Parallel 6 weeks 84 normalweight 2 weeks run-in	G1. 135g mackerel paste/day G2. 135g meat paste/day	G1: 4.70g	SBP, DBP, TAG, TC, LDL, HDL, ApoA, ApoB, tPA activity, tPA levels, PAI-1 activity, PAI-1 levels, pl.ag.(col), TxB_2	G1: : ↑ HDL, bl.T, PAI-1, ↓TAG, no of platelets, TxB_2
(Ågren et al. 1988; Hanninen et al. 1989)	Parallel 15 weeks 62 normalweight	G1. 3.7 MF meals/w G2. 3.7 MF meals/w (↓%fat) G3. control	G1 & G2: 0.80g	TAG, TC, ApoA, ApoB, 6-keto-PGF, TxB_2	G1: ↑ TC ↓ TAG, ApoA, ApoB, TxB_2, 6-keto-PGF G2: ↓ TAG, TC, ApoA, ApoB, TxB_2, 6-keto-PGF
(Ågren et al. 1990; Hanninen et al. 1989)	Parallel 12 weeks 100 normalweight	G1. control G2. 0.9 meals MF/w G3. 1.5 meals MF/w G4. 2.3 meals MF/w G5. 3.8 meals MF/w	G2: 0.20g G3: 0.40g G4: 0.48g G5: 0.88g	TAG, TC, ApoA, ApoB, 6-keto-PGF, pl.ag.(col, ADP), TxB_2	G3: ↓ TAG, ApoB, pl.ag.(ADP) G4: ↓ TAG, ApoB G5: ↓ TAG, ApoB, TxB_2, 6-keto-PGF, pl.ag.(col, ADP)
(Brown et al. 1990; Brown and Roberts 1991)	Crossover 6weeks 12 normalweight 6 weeks washout	G1. control G2. 200g LF, 5meals/w G3. 200g LF, 5meals/w and 5g f-o/d	G2: 0.40g G3: 0.40g +5g f-o	TAG, TC, VLDL, LDL, HDL, HDL_2, HDL_3, FacVIIc, fibrinogen, fibrinolytic act, no of platelets	G2: ↑ fibrinolytic act. G3: ↓ TAG, VLDL, no of platelets, ↑ fibrinolytic act.
(Ågren et al. 1991)	Parallel 14 weeks 99 normalweight	G1. control G2. 3.5 MF meals/w G3. exercise G4. 3.5 MF meals/w and exercise	G2 & G4: 0.75g	TAG, TC, LDL, HDL, ApoA, ApoB, 6-keto-PGF, pl.ag.(ADP), TxB_2	NS results for fish intervention (without exercise)
(Gerhard et al. 1991; Wander and Patton 1991)	Crossover 18 days 21 normalweight 3 weeks washout	G1. 200g sole/day G2. 200g salmon/day G3. 200g sablefish/day	G1: 0.55g G2: 2.22g G3: 1.90g	TAG, TC, LDL, HDL, ApoB, bl.T, pl.ag.(col, ADP), TxB_2	G1: ↓ HDL, TXB_2 G2: ↑ ApoB, LDL, ↓ TC, TXB_2, pl.ag (ADP),↑ bl.T G3: ↑ ApoB, LDL, TC, ↓ HDL, TXB_2, pl.ag.(col, ADP)

Table 1: Clinical Trials on healthy subjects with or without fish-oil intervention

Study	Design	Intervention Groups	Average grams of EPA+DHA/day	Measured Markers	Significant Results
(Marckmann et al. 1991)	Crossover 10 days 12 normalweight 18 days washout	G1. 210g FF daily G2. lean meat	G1: 3.40g	TAG, TC, HDL, tPA activity, tPA levels, PAI-1 activity, PAI-1 levels, FacVIIc, fibrinogen	NS results for fish intervention
(Nelson et al. 1991)	Crossover 40 days 9 normalweight 20 days run-in no washout	G1. control G2. salmon 5 meals/w	G2: 3.4g	bl. T, pl. size, no of platelets, pl.ag.(col, ADP, AA, thrombin), TxB_2, prt.T	G2: ↓ no of platelets, pl.ag. (ADP, AA), ↑ pl. size, prt.T
(Beilin et al. 1993; Mori et al. 1994, 1997)	Parallel 12 weeks 120 overweight	40% fat G1. control G2. MF daily G3. 6 f.o.c./d G4. MF and 6 f.o.c/d G5. 12 f.o.c/d 30% fat G6. control G7. MF daily	G2 & G7: 4g	SBP, DBP, TAG, TC, LDL, HDL, HDL_2, HDL_3, ApoB, glucose, insulin, pl.ag.(col, PAF), TxB_2	G2 (MF, 40% fat): ↓TAG, pl.ag. (PAF, col), TxB_2, ↑TC, LDL, HDL, HDL_2 G7 (MF, 30% fat): ↓TAG, TC, LDL, HDL_3, pl.ag. (PAF, col), TxB_2, ↑HDL_2 Overall: different % of fat influences TC, LDL, HDL, HDL_3 but not HDL_2 when there is fish consumption
(Ågren et al. 1996, 1997)	Parallel 15 weeks 55 normalweight	G1. control G2. 4.3 MF meals/w G3. f-o daily G4. DHA daily	G2:1.05g	TAG, TC, VLDL, LDL, HDL, HDL_2, HDL_3, ApoA, ApoB, FacVIIc, FacX, fibrinogen, pl.ag.(col, ADP)	G2: ↓ TAG, VLDL, HDL_3, pl.ag (col), FacX, ↑ HDL, HDL_2
(Mann et al. 1997)	Crossover 2 weeks 29 normalweight 1 week run-in 3 weeks washout	G1. white meat G2. red meat G3. salmon daily	G3: 2.90g	PGI_2, pl. size, no of platelets, pl.ag.(col, thrombin), TxB_2	G3: ↓ PGI_2, TxB_2
(Elvevoll et al. 2006)	Parallel 8 weeks 71 normalweight	G1. control G2. 400g smoked salmon/w G3. 400g cooked salmon/w G4. 400g cod/w G5. clo/d	G2: 1.20g G3: 1.20g G4: 0.03g	TAG, TC, HDL, TNF-α, IL-8, tPA levels,, PAI-1 levels, fibrinogen, pl. size, no of platelets, TxB_2	G3: ↑ pl. size

Table 1: Clinical Trials on healthy subjects with or without fish-oil intervention

Study	Design	Intervention Groups	Average grams of EPA+DHA/day	Measured Markers	Significant Results
(Moore et al. 2006)	Parallel 24 weeks 134 overweight	G1. control G2. 50g LF with RS G3. 50g LF with SF G4. 50g FF with RS G5. 50g FF with SF 2-5: 2 meals/w	G2 & G3: 0.07g G4 & G5: 0.64g	SBP, TAG, TC, LDL, HDL, glucose, insulin, CRP, TNF-α, IL-6, PAI-1 activity, FacVIIc, FacX, fibrinogen, leptin	NS results, only ↓ trend for TAG in FF groups
(Lara et al. 2007)	Crossover 4 weeks 48 normalweight 4 weeks follow-up (41stayed)	G1. 125g salmon/day	G1: 2.4g	SBP, DBP, TAG, TC, VLDL, LDL, HDL, CRP, fibrinogen, sICAM, adiponectin	G1: ↓ SBP, TAG, VLDL, ↑ HDL, ↑ adiponectin, ↓25% CHD risk
(Lindqvist et al. 2007)	Crossover 4 weeks 13 overweight 2 weeks washout	G1. lean meat G2. 150g herring 5meals/week	G2: 3.4g	TAG, TC, VLDL, LDL, HDL, HDL_2, HDL_3, ApoA, ApoB, CRP, fibrinogen	G2: ↑ HDL
(Din et al. 2008)	Parallel 4 weeks 28 normalweight 4 weeks follow-up	G1. control G2. 500g mackerel/w	G2: 1g	TAG, TC, LDL, HDL, pl.ag. (flow cytometry)	G2: ↓ pl.ag. (flow cytometry)
(Lindqvist et al. 2009)	Crossover 6 weeks 35 overweight 12 weeks washout	G1. lean meat G2. 150g herring 5meals/week	G2: 1.2g	SBP, DBP, TAG, TC, LDL, ox-LDL, HDL, HDL_2, HDL_3, CRP, IL-6, IL-18, sICAM, bl. T	G2: ↑ HDL, HDL_2
(Chiang et al. 2012; Rajaram et al. 2009)	Crossover 4 weeks 25 normalweight 2 days washout	G1. control G2. walnut 6 meals/w G3. 113g salmon 2 meals/w	G3: 0.78g	TAG, TC, LDL, HDL, ApoA, ApoB, CRP, TNF-α, IL-1β, IL-6, PGEM, sICAM, sE-selectin, 11-D-TxB_2	G3: ↓ TAG, 11-D-TxB_2, PGEM, sICAM, ↑ HDL
(Hallund et al. 2010)	Parallel 8 weeks 65 normalweight	G1. control G2. 150g trout(fed with f-o) daily G3. 150g trout(fed with v-o) daily	G2 & G3: 2.9g	SBP, DBP, TAG, TC, LDL, HDL, glucose, insulin, CRP, IL-6, sVCAM-1	NS results for both fish interventions

Table 1: Clinical Trials on healthy subjects with or without fish-oil intervention

Study	Design	Intervention Groups	Average grams of EPA+DHA/day	Measured Markers	Significant Results
(Kondo et al. 2010)	Crossover 8 weeks 17 normalweight 4 weeks follow-up	G1. 230 or 170g MF 5 meals/w (male or female)	G1: 2g	TAG, TC, LDL, HDL, glucose, insulin, HOMA-IR, adiponectin	G1: ↑ adiponectin for female subjects
(Neale et al. 2013)	Parallel 4 weeks 28 overweight 2 weeks run-in	G1. MF 6 meals/w G2. f-o daily	G1: 1.86g	adiponectin	NS results for fish intervention
(Raatz, Rosenberger, et al. 2013; Raatz et al. 2016)	Crossover 4 weeks 19 overweight 4-8 weeks washout	G1. 90g salmon, 2 meals/w G2. 180g salmon, 2 meals/w G3. 270g salmon, 2 meals/w	G1: 0.31g G2: 0.62g G3: 0.93g	TAG, TC, VLDL, VLDL particles size, LDL, LDL particles size, HDL, HDL particles size, glucose, insulin, HOMA-IR, hsCRP, IL-6	↓ TAG, ↑ HDL ↓ VLDL particles' size in all groups ↑ LDL particles' size, dose dependent
(Sofi et al. 2013)	Crossover 10 weeks 20 normalweight 15 days run-in no washout	G1. Sea bream (f-o) 4 meals/w (630g/w) (Group A first) G2. Sea bream (f-o & pp) 4 meals/w (630g/w) (Group B first)	G1: 1.8g G2: 1.64g	TAG, TC, LDL, HDL, TNF-α, IL-6, IL-8, IL-10	Group A (f-o): ↓ TC, LDL, TAG, IL-6, IL-8 Group B (f-o): ↓ TC, IL-6
(Grieger et al. 2014)	Parallel 8 weeks 80 normalweight (>64y)	G1. control G2. MF 4 meals/w	G2: 0.8g	SBP, DBP, TAG, TC, LDL, HDL, CRP, TNF-α, IL-1β, IL-6	NS results for fish intervention
(Aadland et al. 2015)	Crossover 4 weeks 20 normalweight 3 weeks run-in 5 weeks washout	G1. control G2. LF daily	G2: 0.82g	TAG, TC, VLDL, VLDL particles size, LDL, HDL, ApoB	G2: ↓ TAG, VLDL vol.
(Đuričić et al. 2015)	Crossover 8 weeks 33 normalweight 6 months washout	G1. Smoked salmon 2 meals/w G2. f-o daily	G1: 0.95g	TAG, TC, LDL, HDL, CRP, sICAM, sVCAM-1	NS results for fish intervention

Table 1: Clinical Trials on healthy subjects with or without fish-oil intervention

Study	Design	Intervention Groups	Average grams of EPA+DHA/day	Measured Markers	Significant Results
(Hagen et al. 2016)	Parallel 4 weeks 45 normalweight	G1. 750g/w salmon 5 meals/w G2. 750g/w cod 5 meals/w G3. 750g/w chicken 5 meals/w	G1: 1.3g G2: 0.31g	TAG, TC, LDL, HDL, glucose, insulin, CRP, adiponectin	G1: ↓ TAG, ↑ HDL

"Design" column gives information in the following order: study design, intervention period, number of participants and condition, run-in period (if applicable) and washout period (if applicable). "Intervention groups" column gives information regarding the number and intervention plan of all groups. Abbreviations: 1. "Design" column: y (year). 2. "Intervention Groups" and "Average grams of EPA+DHA/day" columns: clo (cod liver oil), d (day), DHA (docosahexaenoic acid), f-o (fish oil either as fish feed or dietary supplement), f.o.c. (fish oil capsules), FF (fatty fish), g (gram), LF (lean fish), MF (mixed fish), pp (plant proteins as fish feed), RS (rapeseed oil consumed by subjects), SF (sunflower oil consumed by subjects), v-o (vegetable oil as fish feed), w (week),. 3. "Measured Markers" and "Significant Results" columns: 6-keto-PGF (6-keto prostaglandin F), AA (arachidonic acid), ADP (adenosine di-phosphate), ApoA and ApoB (apolipoproteins A and B), bl.T (bleeding time), CHD (coronary heart disease), col (collagen), CRP (C-reactive protein, also as hsCRP: high sensitivity), DBP (diastolic blood pressure), f-o (fish oil either as fish feed or dietary supplement), FacVIIc (factor VIIc), FacX (factor X), FF (fatty fish), HDL (high density lipoprotein and also particles HDL_2, HDL_3), HOMA-IR (homeostatic model assessment-insulin resistance), IL-# (interleukin and #:corresponding number 1β, 6, 8, 10, 18), sICAM (intracellular adhesion molecule, soluble form), LDL (low density lipoprotein also in oxidized form as ox-LDL), no (number), LF (lean fish), MF (mixed fish), NS (non-significant results), PAI-1 (plasminogen activator inhibitor 1), PAF (platelet activating factor), PGEM (prostaglandin E_2 metabolite), PGI_2 (prostaglandin I_2), pl.ag. (platelet aggregation- aggregating method/agent in the parenthesis), pl. size (platelet size), prt.T (prothrombin time), SBP (systolic blood pressure), TAG (triacylglycerols), TC (total cholesterol), TNF-α (tumor necrosis factor-alpha), tPA (tissue plasminogen activator), TxB_2 (thromboxane B_2 and its dehydrogenated analog 11-D), sVCAM-1 (vascular cell adhesion molecule 1, soluble form) VLDL (very low density lipoprotein), vol (volume).