Chapter 1: Beneficial effects of omega-3-fatty acids in cardiovascular disease

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Abstract

This chapter discusses the chemistry, metabolism, and biological properties of omega-3-fatty acids (ω-3 PUFAs), mainly eicosopentaenoic acid (EPA) and docosahexaenoic acid (DHA). Specifically, the current state of knowledge on effects of ω-3 PUFAs on plasma lipids, atherosclerosis, metabolic syndrome and diabetes, and obesity, plateled function, blood pressure and cardiac arrhythmia at molecular level is provided. This review also discusses the evidence from basic and clinical studies that propose the use of ω-3 PUFAs as an adjunct therapy in the treatment of cardiovascular disease (CVD). The use of ω-3 PUFAs in CVD may be recommended, but caution should be kept in mind when used with other drugs.

I. Introduction

Cardiovascular diseases (CVD) are the principal cause of mortality in many economically developed countries, and its incidence is increasing rapidly in emerging economies. Since an appropriate diet assures the supply of essential nutrients for the regulation of diverse physiological functions, a nutritional imbalance favors the risk of cardiovascular and metabolic diseases. Despite some countries establish healthy lifestyle policies, implementation is not always easy as persons are not sufficiently motivated to make recommended modifications. Parallel strategies are directed to explore the use of nutraceuticals to promote cardiovascular health, as some specific components of diet have been described to possess therapeutic roles in human health. Specifically, a higher consumption of fish as source of essential fatty acids (EFAs) is promoted, they could prevent the development of CVD such as dyslipidemias, arrhythmias, arterial hypertension and atherosclerosis, among others.

A. Chemistry and metabolism of essential fatty acids

EFAs are a group of organic compounds that have a carboxylic group (-COOH) at one end, and methyl (H_3C-) at another end. The nature of the rest of the molecule, a chain of hydrocarbons, determines the chemical and biological characteristics of the different fatty acids. Structural differences in the hydrocarbon chain are fundamentally based on the number of carbon atoms, absence or presence of double bonds in saturated and unsaturated fatty acids and location of these bonds *cis* or *trans* in their configuration. Fatty acids are classified by the length of the carbon chain (long chain, n-20 to 22; intermediate chain, n-18) and the number of double bonds (saturated, monounsaturated, polyunsaturated) (DeFilippis and Sperling 2006).

Long chain polyunsaturated fatty acids, known as PUFAs, are present in all mammalian tissues. However, mammals cannot directly synthesize these fatty acids because they lack the enzymes to make double bonds at some position in the fatty acid chain. Therefore, long-chain PUFAs should be consumed with diet and are, therefore, "essential" fatty acids (EFAs) (Dimitrow and Jawien 2009).

PUFAs comprise two main classes: ω-6 PUFA and ω-3 PUFA, also known as ω-6 and ω-3, respectively. These two families of PUFAs distinguish themselves in the position of the first double bond, counted from the end methyl group of the molecule. Major sources of ω-6 PUFAs are vegetable oils such as corn, safflower and soybean oil, and they are derived from linoleic acid (LA; C18:2 ω-6), while ω-3 PUFAs are derived from α-linolenic acid (ALA; C18:3 ω-3) and their main sources are fish, such as salmon, trout and tuna (De Caterina et al. 2003). Unsaturated fatty acids also include the ω-9 series, derived from oleic acid (OA, C18:1 ω-9) and the ω-7 series, derived from palmitoleic acid (C16:1 ω-7), are not essential (Das 2006, Wallis, Watts, and Browse 2002).

Once consumed, these EFAs are further metabolized within mammalian cells by the same set of enzymes to their respective long-chain metabolites (Figure 1.1). The main limiting step in the biosynthesis of ω-6 and ω-3 PUFAs is the microsomal Δ^6 desaturation of LA and ALA (Bernert and Sprecher 1975, Sprecher, Chen, and Yin 1999). As a result of this metabolic reaction, the λ-linolenic (GLA; 18:3n-6) and stearidonic (SDA; 18:4n-3) acids are obtained, which are elongated respectively to dihomo-λ-linolenic (DGLA; 20:3n-6) and eicosatetranoic (ETA; 20:4n-3) acids. Both PUFAs, Δ^5-desaturase substrates generate arachidonic (AA; 20:4n-6) and eicosapentaenoic acids (EPA; 20:5n-3), which undergo two consecutive elongation processes to their respective products 24:4n-6 and 24:5n-3. A second microsomic Δ^6 desaturation takes place again in the biosynthesis of PUFAs (Baker et al. 2016). The products of this desaturation, 24:5n-6 and 24:6n-3, are then converted by enzymatic β-oxidation to 22:5n-6 and docosahexanoic acid (DHA; 22:6n-3), respectively.

Insert Figure 1.1

As can be seen, both EPA and DHA, the two ω-3 PUFAs with greater biological activity are obtained from the same precursor, ALA (Robinson and Stone 2006). However, the effectiveness of conversion of ALA into EPA and DHA is limited in humans. Thus, in humans ALA can be converted in EPA up to 8%, while ALA to DHA conversion rate is estimated to be less than 4% (Burdge, Jones, and Wootton 2002, DeFilippis and Sperling 2006). Although the regulation of this process is poorly understood, EPA and DHA have been reported to play a vital role in brain development and cardiovascular health, and have anti-inflammatory and other effects (Das 2006, Wang et al. 2006).

The biochemical processes described above regulate a delicate balance between ω-6 and ω-3 fatty acids. These PUFAs are powerful regulators of cellular functions and constitute the main lipid mediators with biological activity. It should be noted that these two types of PUFAs are not inter-convertible, metabolic and functionally distinct, and often have opposite physiological functions (Simopoulos 1991). Thus, the pathways described for ω-3 PUFAs and the biosynthesis of LA-derived metabolites are obtained through the concerted action of elongases and desaturases. Evidence from several *in vivo* and *in vitro* studies indicates that these two PUFAs families not only share these enzymes, but also establish a system of competition. It is known that the affinity of LA and ALA for Δ^6-desaturase is different; in fact, in order to inhibit up to 50% of GLA formation, a concentration of ALA approximately 10 times greater than LA is required (Mohrhauer et al. 1967). These findings suggest that in the presence of higher LA concentration, as occurs in a living system, the pathway leading to the synthesis of AA is preferred. For this reason, the LA/ALA ratio of dietary components is important, because mammalian cells lack the ω-3 enzyme desaturase, they are unable to convert ω-6 into ω-3 fatty acids.

Unhealthy eating is one of the factors that can be attributed to the pandemic of CVD affecting most countries in the world. Compared to our Paleolithic ancestors, for whom consumption of products rich in ω-3 was higher; the modern western diet is considered to be up to 20 times richer in ω-6. In relation to the consumption rate, excessive amounts of ω-6 PUFAs and a very low proportion of ω-3/ ω-6 has been described to promote the pathogenesis of many diseases, including CVD, cancer and inflammatory and autoimmune diseases.

B. Beneficial effects of ω–3 PUFAs

The beneficial effects that the ω–3 PUFAs, in particular EPA and DHA, provide protection against CVD, have been supported by an important number of clinical, experimental and epidemiological studies (Mozaffarian and Wu 2011). A universally known fact is that, in the primary prevention of CVD, a ratio of 4/1 (ω–3/ ω–6) is associated with a 70% reduction in total mortality (de Lorgeril et al. 1994, Simopoulos 2006). It is important to consider that this relationship may vary when the objective is to prevent other diseases. In general, ω–6 fatty acids are described as pro-inflammatory and capable of triggering thrombosis and contributing to the formation of atherosclerotic plaque. Whereas, ω–3 fatty acids are described to have anti-inflammatory and cardioprotective effects. Although this is generally accepted, the scientific community continues to generate data to establish the variables associated with the biological potential of PUFAs (Szostak-Wegierek et al. 2013). In 2002, the American Heart Association (AHA) published a scientific statement "Fish Consumption, Fish Oil, Omega-3 Fatty Acids, and Cardiovascular Disease" (Kris-Etherton et al. 2003). The main observation is summarized as the capacity of EPA+ DHA to reduce fatal cardiac events. Further studies are suggested to confirm these findings and to define the health benefits of ω–3 PUFAs supplementation in primary and secondary prevention of CVD. Since then, there have been numerous studies in humans and experimental models to define the impact of ω–3 PUFAs on reducing cardiovascular risk. Some studies have focused on understanding their effects and possible mechanisms of action. The aim of this chapter is to review the evidence for the beneficial effects of fatty acids, particularly ω–3 PUFAs on cardiovascular risk factors.

II. Effects on plasma lipids and lipoproteins

Even though historically, risk management of coronary heart disease (CHD) has focused primarily on low-density lipoproteins (LDL) targets; recently, greater emphasis has been

placed on non- high-density lipoproteins (HDL) and apolipoprotein B (*apo* B) as more robust predictors of CHD death. Also, high plasma triglyceride (TG) levels represent a risk factor for atherosclerosis and related cardiovascular disease (Labreuche, Touboul, and Amarenco 2009), and treatment with ω–3 PUFAs is an established intervention to reduce TGs (Harris 2013, Mori 2014). The role of TGs in the development of CHD is a function of several pathophysiological factors such as: **(1)** In normal conditions, TGs are transported mainly by TG-rich lipoproteins, such as very low-density lipoproteins (VLDL) derived from the liver and chylomicrons (CMs) derived from the intestine. After lipoprotein lipase (LPL) mediated triglyceride hydrolysis to free fatty acids (FFAs), the remaining lipoproteins including LDL are formed. Thus, it is considered that hypertriglyceridemia can generate an excessive amount of highly atherogenic LDL (Goldberg, Eckel, and McPherson 2011, Schwartz and Reaven 2012). **(2)** In hypertriglyceridemic conditions, the metabolism of VLDL shifts from a system dominated by apolipoprotein E (*apo* E), and characterized by its rapid elimination from vascular circulation, to a system dominated by apolipoprotein C III (*apo* C III), which is characterized by a preferential conversion of TG-rich lipoproteins into small amounts of highly dense atherogenic LDL.

ω-3 PUFAs from both marine and plant sources have been shown to reduce the risk of death from CHD. It is believed that this beneficial cardiovascular effect is due in part to its anti-atherosclerotic properties, that is associated with a reduction in TG levels. It has been proposed that ω-3 PUFAs exert these TG-lowering effects via a number of mechanisms (Backes et al. 2016): (1) by suppression of the expression of protein-1c, and thus a reduction of liver lipolysis. (2) by inhibition of phosphatidic acid phosphatase and diacylglycerol acyltransferase (DGAT), key enzymes in the hepatic synthesis of TGs. (3) by an increase in the oxidation of fatty acids, resulting in a reduction of the available

substrate required for TG and VLDL synthesis, (4) by increments in LPL expression, a key component of the TG-rich lipoproteins biosynthetic pathways, leading to greater removal of TG from circulating VLDL and CM particles.

Many of the mechanisms involved in PUFAs-mediated reduction of TGs consist in regulating the gene expression of key proteins in lipid metabolism (Figure 1.2). PUFAs suppress the nuclear abundance of several transcription factors involved in lipid and carbohydrate metabolism, including sterol regulatory element binding proteins (SREBPs) (Horton, Goldstein, and Brown 2002) and carbohydrate responsive element binding protein (ChREBP) (Postic et al. 2007).

Insert Figure 1.2

There are 3 isoforms of SREBP: SREBP-1a and -1c, which originate from the same gene, and SREBP-2. All of them are activated in response to a decrease in free cholesterol content in the endoplasmic reticulum (ER). SREBP-2 stimulates primarily transcription of genes related to cholesterol biosynthesis and LDL receptor (rLDL). SREBP-1c is the predominant form of SREBP-1 in most tissues, and together with SREBP-1a, regulate the synthesis of FAs, TGs and phospholipids. Most of the lipogenic effects of insulin depend upon the induced expression of SREBP-1c and the subsequent stimulation of the FA synthesis pathway. The expression of SREBP-1c is also stimulated by the liver X receptor (LXR), through two LXR binding sites (LXRE) present in the SREBP-1c promoter (Desvergne, Michalik, and Wahli 2006) (Figure 1.2). In contrast to these signaling events, high levels of PUFAs suppress the expression of SREBP-1c and ChREBP, the latter being a sensitive factor for glucose concentration and promotes lipogenesis from carbohydrates. However, these transcription factors are not regulated by the direct binding of fatty acids

but rather respond to changes in gene transcription. For example, PUFAs decrease the transcription of SREBP-1c and ChREBP secondary to competition with oxysterols, a positive regulator of the SREBP-1 and ChREBP genes, that bind to the LXR (Ou et al. 2001).

In addition to the LXR, PUFAs bind to the ligand binding domain of several nuclear receptors expressed in the liver, including peroxisome proliferator-activated receptors (PPAR α, β, γ1 y γ2) (Xu et al. 1999), farnesoid X receptor (FXR) (de Urquiza et al. 2000), and hepatic nuclear factor-4α (HNF4α) (Wisely et al. 2002, Yuan et al. 2009). All receptors, except HNF4α, form heterodimers with the nuclear receptor retinoid X receptor (RXR) to regulate gene expression. Fatty acids are hydrophobic which function as hydrophobic hormones (steroids and thyroid) to control nuclear receptor function (Davidson 2006).

As mentioned above, the expression of the SREBP-1c gene is partly regulated by the LXR/RXR receptor complex. Data suggest that PUFAs inhibit binding of the LXR/RXR heterodimer to the LXR response elements (LXREs) in the SREBP-1c promoter, a crucial process for the expression of SREBP-1c. The inhibitory magnitude order of each long chain fatty acid over the expression of SREBP-1c is as follows: AA> EPA> DHA> LA> SA = 0 (Yoshikawa et al. 2002). Other mechanisms have been proposed for explaining the SREBP-1c downregulation induced by PUFAs. For example, in rat hepatocytes decay of transcription SREBP-1c can accelerate, and PUFAs can also interfere with the maturation process of the SREBP-1c protein, decreasing its activity (Yoshikawa et al. 2002).

The lipogenic activity of LXR/RXR is due to the positive regulation it exerts on the above-mentioned SREBP-1c, as well as to the direct regulation of several genes in the SREBP-1c downstream, including those that encode the synthesis of DGAT, fatty acid synthase (FAS)

and acetyl CoA carboxylase (Mozaffarian and Wu 2011). Through this pathway, synthesized FAs can be used in the synthesis of TGs, which get integrated with cholesterol particles in the VLDLs. This mechanism could explain how LXR agonists produce increased plasma concentration of TGs. Thus, PUFAs cause their hypotriglyceridemic effects by the coordinated suppression of liver lipogenesis through the blockage of LXR activation and suppression in the processing of SREBP-1c and other lipogenesis-related proteins.

PUFAs and products derived from these so called natural ligands for peroxisome proliferator-activated receptors (PPARs), another transcription factor that plays a crucial role in fatty acid oxidation. Although a possible influence on the concentration of circulating TGs has been described for all PPARs, it is only with PPARα that a hypotriglyceridematic effect is regularly observed. PPARα receptors reduce the availability of FA for TGs synthesis by regulating specific genes including LPL (Michaud and Renier 2001), and apolipoprotein A I (*apo* A I) (Vu-Dac et al. 1994), a key structural element in HDL. In addition, PPARα has been shown to increase the liver expression of apolipoprotein A V (*apo* A V), which stimulates LPL-mediated lipolysis (Prieur, Coste, and Rodriguez 2003). As a result, ω–3 PUFAs stimulate PPARα pathways and increase signaling that lower TG levels and increase HDL-C levels.

The binding of fatty acid to HNF-4 (α and γ) was documented by X-ray crystallographic analysis of HNF4 binding domains expressed in bacteria (Dhe-Paganon et al. 2002). *In vitro* studies report that fatty acids are irreversibly bound to HNF-4 (Wisely et al. 2002), while others report that binding of LA to HNF-4α is reversible (Yuan et al. 2009). It has also been observed that, while saturated fatty acids stimulate the transcription of HNF-4α, PUFAs inhibit the effects of HNF-4α on gene transcription (Clarke 2001, Pegorier, Le

May, and Girard 2004). Proteins involved in lipoprotein metabolism, including apo C III, A I, A IV, are encoded by this receptor (Jump and Clarke 1999). However, in contrast to the direct regulation of FAs over other nuclear receptors, the occupation of HNF-4α does not appear to significantly affect their transcriptional activity, and are considered indirect mechanisms. For example, PUFA-activated PPARs compete with HNF-4α for binding to the *apo* C III promoter.

According to these mechanisms, in samples of fasting subjects showing moderate to severe hypertriglyceridemia (TGs of 500 to 2000 mg/dL), a 12-week treatment with ω–3 PUFAs revealed a significant reduction in plasma *apo* C III levels (Morton et al. 2016). Another randomized, double-blind, crossover study with 8-week treatment periods using high-dose EPA + DHA, demonstrated potential mechanisms by which ω–3 PUFAs may decrease the risk of coronary heart disease by reducing atherogenic apolipoproteins in individuals with elevated TGs. Treatment with 3.4 g/d of EPA + DHA significantly reduced the concentrations of both *apo* C III and *apo* B, and tended to a modest reduction of lipoprotein associated phospholipase A2 (Lp-PLA2) (Skulas-Ray et al. 2015). The effect of ω–3 PUFAs on *apo* B 48 has also been evaluated in animal and *in vitro* models where it has been shown to suppress its synthesis, with the consequent reduction of CM rich in TGs. It has been considered that this inhibition may be due to a decrease in the expression of *apo* B 48 and/or increased post-translational degradation (Levy et al. 2006).

Apo C III is believed to contribute to the progression of atherosclerosis and CVD through a number of mechanisms, including the activation of pro-inflammatory pathways (Sacks, Zheng, and Cohn 2011). This apolipoprotein is a key factor in hypertriglyceridemia, mainly due to its inhibitory actions on LPL. It also inhibits receptor-mediated uptake of VLDL,

CM and their remnants, slows down their purification rate, and promotes the formation of small, dense, highly atherogenic LDL particles.

Even though, ω–3 PUFA therapy reduces cholesterol and TGs carried by VLDL particles, a parameter which is rarely measured in clinical practice. According to experimental findings, ω–3 PUFAs are associated with reductions in plasma TGs levels of approximately 25% to 34% (Harris 1997), but these findings vary at baseline. Studies in individuals with severe hypertriglyceridemia (TGs 5.65 mmol /L [500 mg / dL]) reported reductions in TGs from 40% to 79% with EPA+DHA intake of 3 g/day (Harris 1997, Phillipson et al. 1985). When baseline TGs levels are ≥2.0 mmol/L (≥177 mg / dL), the reduction rate is approximately 34%, while for subjects where baseline TGs levels are below 2.0 mmol/L, the reduction is up to 25%. With administration of ω–3 PUFAs in patients with CVD and TGs ≥1.70 mmol/L (150 mg/dL), reduction of up to 30% of TGs has been observed. Two of the three studies in patients with dyslipidemia reported a 20% to 33% reduction in TGs. With the administration of EPA and DHA in patients with diabetes, reduction of TGs between 25 and 45% has been observed, resulting in a dose-dependent effect (Nettleton and Katz 2005). In middle-aged and elderly subjects, a diet rich in marine ω–3 PUFAs has been observed to decrease concentrations of lipoproteins rich in TGs (VLDL) by direct catabolism of the *apo* B 100, and it is reasonable to expect an increase in LDL levels (Ooi et al. 2012). In an unfavorable, but to some extent expected degree, ω–3 PUFAs produce elevations in cLDL levels between 5% and 11% (Harris 1997). However, this increase in cLDL levels from ω–3 PUFAs supplementation appears to be due to an increase in LDL particle size rather than to the number of LDL molecules. These nutritional supplements modify the composition of LDL cholesterol by increasing apolipoprotein B and decreasing lipoprotein levels, resulting in a less atherogenic molecule (von Schacky 2000). Small LDL

particles are considered as an important cardiovascular risk factor (Hulthe et al. 2000), and correlated with subclinical atherosclerosis as measured by the thickening of the middle intima (Lahdenpera et al. 1996).

Other findings in the field of dyslipidemias have shown that ω–3 PUFAs significantly reduce liver fat by 18% in women with hepatic steatosis (Cussons et al. 2009) and in obese dyslipidemic men with insulin resistance, combined ω–3 PUFAs and statins therapy provided the optimal change in lipid profile and HDL cholesterol (Chan et al. 2002). However, the impact of ω–3 PUFAs on HDL is variable. Some studies report discrete elevations in HDL cholesterol of approximately 1% to 3% (Harris WS. 1997), while other authors report a decrease in HDL of approximately 8%-14% (Weintraub 2014).

Considerations for the use of PUFAs in the treatment of dyslipidemias include:

(1) Long-chain omega-3 fatty acids may be a well-tolerated and effective alternative to fibrates and niacin, yet further large-scale clinical studies are required to evaluate their effects on cardiovascular outcomes and CVD risk reduction in patients with hypertriglyceridemia (Davidson et al. 2014). (2) Since they have different mechanisms of action and the efficacy profile against the different lipid components varies between ω–3 PUFAs and statins, combined therapy is expected to provide complementary benefits over lipid profile when given together. However, further long-term studies with clinical end-points are needed to confirm the synergistic benefits of statins and omega-3 supplements on cardiovascular incidence and mortality (Minihane 2013). (3) As for accepted therapeutic uses, the 2016 ESC/EAS Guidelines for the Management of Dyslipidemias, suggest that if TGs are not controlled by statins or fibrates, addition of ω–3 PUFAs may be considered, and these combinations are safe and well tolerated (Authors/Task Force et al. 2016). (4) Despite findings that ω–3 PUFAs supplements (fish oil) is highly effective in the treatment

of hypertriglyceridemia, omega-3 dietary supplements are not approved by the Food and Drugs Administration (FDA) for this purpose (Jellinger et al. 2017).

III. Effects on Atherosclerosis

Atherosclerosis is an inflammatory disease of the vascular system and is a major cause of cardiovascular and cerebrovascular diseases. When considering the pathophysiological process of atheroma plaque development, it can be inferred that ω-3 PUFAs have beneficial effects, given their ability to modify both lipid profile and inflammatory process. However, variations in methodological designs of both experimental and clinical studies show controversial results in relation to ability of ω-3 PUFAs in reversing or preventing atherosclerosis.

It is well known that the principal constituents of an atheroma plaque are LDLc, and some studies have demonstrated that, in addition to reducing TGs and VLDL, ω-3 PUFAs increase the concentration of floating and large LDL particles, while reducing the concentration of dense atherogenic particles of LDL. In relation to this property, in a study conducted in low-density lipoprotein receptors knockout mice (LDLR-/-) subjected to a diet rich in cholesterol, fish oils supplements inhibited the development of atherosclerosis after 20 weeks of treatment (Zampolli et al. 2006). Using the model of apolipoprotein E knockout mice (*apo* E -/-), administration of 1% fish oil for 14 weeks did not modify atherogenesis (Xu et al. 2007). In contrast, another study using both models, where LDLR -/- and *apo* E -/- mice were administered a cholesterol-rich diet, treatment for 12 weeks with high concentrations of EPA decreased the plaque size (Matsumoto et al. 2008).

Although LDLs play a key role in the development of atheroma plaque, studies carried out in other animal models have shown that the development of atherosclerosis may be significantly inhibited by diet rich in ω-3 PUFAs, without this effect being associated with

a reduction in lipids (Davis et al. 1987, Weiner et al. 1986). These findings lead us to consider other aspects related to the pathogenesis of plaque formation, which may be involved in the cardioprotective effects described for EPA and DHA. This forces us to reconsider the inflammatory nature of this vascular disease, in which the excess of LDL deposited in the vascular intima layer induces excessive recruitment of monocytes, which are different from macrophages and allows elimination of the excess of sub-endothelial cholesterol. This process gives rise to the generation of foam cells capable of secreting pro-inflammatory mediators that characterize the pathogenesis of atherosclerosis and, precisely, one of the biological effects of ω-3 PUFAs is to alter the course of the inflammatory process.

The anti-inflammatory effects of ω-3 PUFAs are partly related to their attenuating effects on inflammatory prostanoids and leukotrienes. For example, in intestinal microvascular endothelial cells, DHA has been shown to reduce cyclooxygenase-2 (COX-2) expression and limits the production of PGE_2 and leukotriene B_4 (LTB_4) (Ibrahim et al. 2011). Similar effects have been observed in rats with colitis, where administration of fish oil rich in EPA and DHA inhibited the production of PGE_2 and LTB_4.

Monocyte adhesion to endothelial cells is mediated by adhesion molecules, such as intracellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1). In the models described above by Ibrahim et. al. (2011), inhibition of eicosanoids was accompanied by reduction in VCAM-1 expression. In humans, fish oil (EPA + DHA) supplements have been found to lower circulating VCAM-1 levels in elderly subjects (Miles et al. 2001). In patients with metabolic syndrome (Met-S), EPA administration decreased plasma concentrations of soluble ICAM-1 and VCAM-1 (Yamada et al. 2008).

However, more recently in order to assess the effects of ω-3 PUFAs supplements on plasma concentrations of soluble adhesion molecules, a meta-analysis of randomized controlled trials was carried out. This study revealed that ω-3 PUFAs supplements did not affect plasma concentrations of VCAM-1, but instead, in both healthy and dyslipidemic patients, they did significantly reduce ICAM-1 (Yang et al. 2012).

In relation to other components of the atherosclerotic process, high concentrations of EPA and DHA are known to modulate the expression of critical genes associated with inflammation, including interleukin-1-alpha (IL-1α), interleukin-1-beta (IL-1β), and tumor necrosis factor alpha (TNF-α) (Calder 2001, Honda et al. 2015, Zainal et al. 2009). TNF-α is an inflammatory cytokine that plays an important role in the development of atherosclerotic lesions. TNF-α is associated with up-regulation of monocyte chemotactic protein-1 (MCP-1) and VCAM-1 expression, in addition to inducing expression of early growth response protein 1 (Egr-1), capable of inducing ICAM-1 production (Maltzman, Carmen, and Monroe 1996). Additionally, the IKK / NF-κB pathway (IkB kinases/nuclear factor κB) regulates the genes involved in inflammation, oxidative stress and endothelial dysfunction through TNF-α activation (Kumar et al. 2004, Rimbach et al. 2000).

ω-3 PUFAs regulate TNF-α activity by activating the G-coupled protein receptor 120 (GPR120). This receptor intervenes in the activation cascade of NF-κB mediated by the TNF-α receptor (TNFR). Post-activation signaling pathways of the GPR120 receptor in adipocytes inhibit the pro-inflammatory activity of TNF-α (Gregor and Hotamisligil 2011). Additionally, in macrophages inflammasome activation is inhibited by DHA via GPR120 (Free Fatty Acid 4; FFA4), an effect that also appears to be mediated by inhibition of NF-

κB. Thus, blocking the TNF-α pathway mediated by ω-3 PUFAs-related receptors is crucial in explaining the regulation of the formation and stability of atheroma plaque.

Emerging evidence shows that uncontrolled inflammation is a prominent characteristic of many cardiovascular diseases, and that atherosclerosis may be seen as a state of failed resolution of inflammation (Serhan et al. 2007). Thus, it is known that from essential fatty acids endogenous chemical mediators are biosynthesized in different phases of inflammatory response, where resolution is one of them. In this sense, the production of a new class of lipid mediators, called resolvins (products of interaction in the resolution phase), have also been associated with the anti-inflammatory effects of ω-3 PUFAs (Figure 1.3).

Insert Figure 1.3

Resolvins of series E (RvE) and series D (RvD), are obtained from EPA and DHA, respectively (Serhan et al. 2002). RvE and RvD-resolvins are believed to have a similar function, which is primarily to block the migration and infiltration of neutrophils and monocytes, protecting tissues from damage by immune system cells (Serhan et al. 2002). Both types of resolvins, like their precursors, can inhibit the NF-κB by a receptor-dependent mechanism activated by PPARγ in addition to the involvement of membrane receptors (Liao et al. 2012).

It has been shown that RvE1 can act as an agonist of the ChemR23 receptor, also known as Chemokine-like receptor 1 (Kaur et al. 2010) (Figure 1.4). Rv-E1-mediated signaling on ChemR23 plays a role in mononuclear cellular migration to inflammed tissue, as well as in resolving inflammation (Serhan, Yacoubian, and Yang 2008). Actions mediated by Rv-E1 through ChemR23, block signaling of NF-κB induced by TNF-α (Arita et al. 2005) and

increase phagocytosis of apoptotic neutrophils (Ohira et al. 2010). In addition to ChemR23, it has been described that RvE1 interacts with leukotriene B4 receptor type 1 (BLT_1), a receptor of LTB_4, and serves as a local buffer of BLT_1 signals in leukocytes (Arita et al. 2007).

Insert Figure 1.4

Meanwhile, the actions of RvD1 are mediated by two G-protein coupled receptors (GPCR), ALX / FPR2 and GPR32, which also regulate specific microRNAs (miRNAs) and their target genes in new resolution circuits. Interaction with ALX / FPR2 signals could control infiltration of polymorphonuclear neutrophils (PMN) and stimulate macrophagocytosis of apoptotic PMNs (Chiang et al. 2006), while it is suggested that the GPR32 also plays a key role in mediating the effects of RvD1 on human macrophages (Schmid et al. 2016). Despite numerous findings that document the molecular actions associated with the anti-inflammatory effects of ω-3 PUFAs, crucial aspects of signaling mechanisms and downstream interactions remain to be elucidated.

The anti-inflammatory effects of ω-3 PUFAs seem to play an important role in both the prevention and stabilization of the plaque. This has been demonstrated in patients with symptomatic carotid atherosclerotic disease, where ω-3 PUFAs supplements are associated with less macrophage infiltration into the plaque and a firmer fibrous lamina, suggesting a greater plaque stability (Thies et al. 2003). Similarly, it has been found that patients with atherosclerotic disease, waiting for a carotid endarterectomy, receiving ω-3 PUFAs, specifically EPA which is incorporated into atherosclerotic plaques and is associated with a reduction of foam cells, T cells, decreased inflammation and increased plaque stability.

It is known that plaque stability is determined by the production of matrix metalloproteinases (MMPs), proteases which are capable of degrading extracellular matrix

proteins. The presence of MMPs generated from foam cells plays a definite role in the stability of atheroma plaque, as they are associated with degradation of the fibrous layer and rupture of the plaque. ω-3 PUFAs, evaluated in different cellular models, have shown that they are capable of decreasing expression of these pro-inflammatory markers (MMP-7, MMP-9, MMP-12) (Cawood et al. 2010), and these findings may represent an important mechanism by which ω-3 PUFAs reduce ischemic cardiovascular events by inducing plaque stabilization. However, marine ω-3 PUFAs in human studies show no effects on plasma MMP-9 levels, determined in patients with myocardial infarction (Aarsetoy et al. 2006) or in subjects at risk of coronary heart disease (Furenes et al. 2008), which again highlights inconsistencies between experimental observations and clinical findings.

Endothelial dysfunction, through increased inflammatory and thrombogenic molecular changes, has also been associated with atherosclerosis. Endothelial dysfunction is characterized by the alteration of the bioavailability of main endothelial derived relaxation factor, which affects endothelial dependent vasodilation. Decreased bioavailability of nitric oxide (NO) has been associated with decreased expression of endothelial NO synthase (eNOS), reductions in eNOS activity and further degradation of NO by reactive oxygen species (Rossier, Bochud, and Devuyst) (Harrison 1997, Wilcox et al. 1997).

On the other hand, in certain disease states, endothelial dysfunction can also produce higher levels of eicosanoids and free radicals, which on the one hand affects the bioavailability of NO and on the other promotes abnormal contraction of blood vessels (Abeywardena 2003, Higashi et al. 2009).

ω-3 PUFAs when incorporated into phospholipids, modulate both the composition and fluidity of the cell membrane. The endothelial cell membrane harbors lipid caveolaes and lipid rafts where various receptors that regulate cell function are concentrated. ω-3 PUFAs

modulate the composition of the caveolae, causing an increase in NO production and a reduction in pro-inflammatory mediators. In particular, DHA has been shown to improve endothelial dependent vasorelaxation of aortic rings by increasing NO liberation (Lawson et al. 1991), and to increase the production of IL-1β -induced NO in vascular smooth muscle cells (Hirafuji et al. 2002). In addition to increasing NO production, ω-3 PUFAs reduce oxidative stress, as a result of a direct modulatory effect on the sources of ROS. In hypercholesterolemic conditions and diabetes, omega-3 fatty acids improve endothelial function and reduce vasoconstrictor response of vascular smooth muscle.

Although experimental findings appear to be consistent with the cardioprotective properties of ω-3 PUFAs, clinical data are not always convincing. In some studies, consumption of ω-3 PUFAs-enriched diet improved various functional biomarkers of endothelial activity, for example, increased expression of the eNOS gene expression. Similarly, in studies carried out in the elderly, the circulatory biomarkers of endothelial dysfunction were also reduced. However, the general evidence of ω-3 PUFAs' effects on endothelial function is inconsistent, showing wide variations in the doses administered, characteristics of the mixtures (DHA + EPA) used as supplements, treated populations and evaluated parameters. Thus, adhesion molecules were affected in a variable manner, as discussed above. In healthy young adults, neither EPA nor DHA administered for a short period of time altered endothelin-1 (ET-1) or soluble E-selectin, VCAM-1, or ICAM-1 concentrations. Interestingly, a combination of EPA + DHA reduced both soluble E-selectin and VCAM-1.

Although there are contradictions between the studies published to date, most authors conclude that ω-3 PUFAs are a complement in preventing both the development as well as progression of atherosclerosis.

IV. Effects on metabolic syndrome, diabetes, and obesity

Metabolic syndrome is a group of coexisting and interrelated risk factors that include abdominal obesity, impaired glucose tolerance, hypertriglyceridemia, decreased serum HDL cholesterol and/or hypertension. The first observation related to this syndrome was insulin resistance, however, given the complexity of the syndrome, the focus has been on its use as an epidemiological tool related to cardiovascular risk (Tune et al. 2017). Although its definition as a disease generates debate, it is considered to be a determining pathological stage in the development of diseases such as type 2 DM and atherosclerotic disease (Tune et al. 2017).

On the other hand, DM is a group of diseases characterized by elevated blood glucose levels due to insufficient production or action of insulin. This elevation of plasma glycemic level generates characteristic micro- and macro-vascular complications that include retinopathy, neuropathy, nephropathy, AMI and stroke (Asmat, Abad, and Ismail 2016). Both syndromes are associated with obesity, which besides being a triggering factor, is characterized by excessive accumulation of adipose tissue particularly in the abdomen (Gonzalez-Muniesa et al. 2017). The relationship between these diseases is based on epidemiological studies, which have established a parallelism in terms of prevalence and increase in last decades (Grundy 2008).

It is recognized that in all these pathologies, there are genetic factors involved that determine their predisposition, however the environmental factors have great influence on their development, especially sedentary life style and excess calories intake, where a positive energy balance leads to an increase in fat deposits in the adipose tissue with the consequent development of obesity and alterations in adipocytes functions (Rask-Madsen and Kahn 2012).

Obese individuals present a chronic low-level inflammation, which has been associated with a chronic stage of oxidative stress, resulting from an imbalance between mechanisms of production and elimination of ROS, which are byproducts of the respiratory chain within mitochondria, organelle responsible for energy production, carbohydrates, amino acids and lipids metabolism, and apoptosis. Other enzyme systems such as NADPH oxidase and peroxisomes can also generate ROS (Carrier 2017).

In addition to this, the increase in the bioavailability of FFAs increases their cellular uptake inducing mitochondrial β-oxidation, which in turn generates a blockage at the substrate binding site, accumulation of toxic lipid intermediates and cellular dysfunction. This predominance of lipid metabolism at the expense of glucose utilization, reduces the uptake of the last substrate and the synthesis of glycogen in the skeletal muscle, resulting in a chronic state of hyperglycemia thus decreasing the sensitivity to insulin (Verma and Hussain 2017).

This rise in glycaemia, together with compensatory hyperinsulinemia, has been associated with insulin resistance; while the intolerance to glucose conduces to the glycation of circulating proteins and the formation of so-called advanced glycation end products (AGEs). The progression of these events causes a detriment in the secretory function of beta pancreatic cells, and ultimately to apoptosis (Verma and Hussain 2017, Carrier 2017).

Ravussin et al. (2002), showed that an increase in dietary fats favors the lipid storage within the liver, skeletal muscle and pancreas, unobserved under normal conditions (Ravussin and Smith 2002).

All of the above suggests the importance of the inflammatory process as a basis for Met-S, diabetes and obesity in which the production of different pro-inflammatory cells from immune system such as monocytes, macrophages, natural killer (NK) cells and

lymphocytes induce cytokines secretion that perpetuate systemic inflammation. A large number of cytokines are related to the inflammatory process, however IL-1β, TNF-α, IL-17 and IL-6 are more important in this process (Pirola and Ferraz 2017). Given the relevant role of the accumulation of adipose tissue in these conditions and their function as a regulating organ on energetic homeostasis, it is described that adipokines are released from the adipose tissue, some of which have pro-inflammatory activity (TNF-α y IL-6), while others generate anti-inflammatory actions (adiponectin).

Given the close pathological relationship between the described diseases and the elevated cardiovascular risk to patients, it is established that some lifestyle modifications such as a healthy diet, weight reduction and increased physical activity significantly reduce their development and progression (Rochlani et al. 2017).

In relation to diets, ω-3 PUFAs have demonstrated their beneficial effects in metabolic diseases, therefore the utilization of ω-3 PUFAs as a supplement constitutes a strategy for treating these pathologies (Carpentier, Portois, and Malaisse 2006, Tortosa-Caparrós et al. 2017).

As diabetes, Met-S and obesity have common etiological factors (increased of FFA, hyperglycemia, increased adipose tissue, increased free radicals and inflammation), the mechanisms by which ω-3 PUFAs exert their positive effects can be explained by three main points: a) lipid metabolism, b) inflammation, and c) energetic metabolism.

I. *lipid metabolism*: these effects have been described above in section of plasma lipid effect, where emphasis was placed on molecular actions leading to the reduction of TGs, a widely recognized effect of ω-3 PUFAs. These actions are summarized in Figure 1.2.

II. *Inflammation*: as previously stated, another relevant aspect in metabolic diseases is their interrelation with the inflammatory process, latter being an essential component in organism's response to infections or aggressions (Henson 2005). The beneficial effects of ω-3 PUFAs on obesity and metabolic syndrome are due to their direct anti-inflammatory actions confirmed by experimental and clinical studies.

It is recognized that saturated fatty acids (SFAs) stimulate directly the expression of inflammatory mediators such as TNF-α and IL-6, through binding to Toll-like receptor 4 (TLR-4) (Lee et al. 2001) and Toll-like receptor 2 (TLR-2) (Lee et al. 2004), mainly in adipocytes. The stimulation of these receptors has been associated with the activation of the NF-kB pathway, which is involved in diverse inflammatory pathologies (Lira et al. 2010). In this sense, the administration of ω-3 PUFAs has been shown to exert potent anti-inflammatory actions through the following mechanisms (Box 1.1).

Box 1.1. Mechanism of anti-inflammatory actions of ω-3 PUFAs
Facilitating the production of mediators known as resolvins ◖ Resolvins, metabolic products of PUFAs, generate potent anti-inflammatory actions at the sites of inflammation (Serhan et al. 2002). ◖ Anti-inflammatory effects include inhibition of NF-κB, blocking neutrophil migration, downregulating of adhesion molecules, reduction of respiratory burst, and promoting neutrophil apoptosis and clearance of apoptotic bodies by macrophages (Fritsche 2015, Liao et al. 2012, Capo et al. 2017).
Affecting lipid rafts of the plasma membrane of immune system cells. ◖ ω-3 PUFAs induce changes in the size as well as the composition of the cell membrane (cholesterol and sphingomyelin) and, therefore, modify its biological properties, with intracellular signaling being significantly altered. ◖ ω-3 PUFAs suppress T-cell activation in murine models, and have an anti-inflammatory effect (Stillwell et al. 2005, Turk and Chapkin 2013). Transduction signals by T cells mediated pro-inflammatory mechanism, such as epidermal growth factor (EGF), Ras activation (Isoform H) and PLCγ.
Stimulation of Free Fatty acid receptor 120 (FFA4 or GPR120) ◖ GPR120 belongs to the family of GPCR, which is expressed in various mammalian tissues, and under physiological conditions it is predominantly activated by intermediate and long chain fatty acids; however, it has greater affinity for DHA and EPA (Miyamoto et al. 2016).

- GPR120 probably couples mainly with Gq/11 proteins, whose signaling mechanism is associated with an increase in intracellular Ca^{2+} in both human and mice cells (Moniri 2016, Katsuma et al. 2005).
- Activation of GPR120 receptor is associated with the reduction of COX-2 gene expression and production of PGE_2, a mechanism involved in inflammatory response (Li, Yu, and Funk 2013).
- Studies with murine macrophage cell cultures showed that DHA generates a significant reduction in inflammatory response. In these cells, receptor activation leads to β-arrestine-2 recruitment forming a complex (DHA- GPR120- β-arrestine-2) that binds to the TAK-1 binding protein (TAB-1), which interferes with the phosphorylation and activation of TAK-1 (kinase 1 activated by TGF-β) (Oh et al. 2010, Im 2016, Song et al. 2017).

Affecting hormones and mediators in adipose tissue

- In relation to the effect on leptin, a study in insulin-resistant animal model, found that the ω-3 PUFA-treated group showed an increase of 75% in plasma leptin levels compared to the control group. ω-3 PUFA treatment increases expression of GLUT4 transporters in adipose tissue (Peyron-Caso et al. 2002).
- Findings related to animal studies were described by Kondo et. al. (2010), who developed a study in a small group of non-obese patients, determining that administration of PUFAs increases adiponectin levels; this effect being more pronounced in women than in men (Kondo et al. 2010).
- Incubation of human adipocytes with EPA and DHA increased adiponectin levels, an anti-inflammatory cytokine, without modifying leptin secretion. Only DHA showed a decrease in TNF-α levels (Romacho et al. 2015).

III. *Energy metabolism*: as metabolic diseases such as diabetes and obesity have an imbalance between food intake, energy expenditure and fatty tissue accumulation, it is understandable that this factor should be improved. In this sense, the interest has been focused on adipose tissue, and the GRP120 receptor is postulated as a fundamental target in the regulation of metabolism in this tissue, due to its wide expression. In a diet-induced obesity study (high-fat diet; HFD) in mice, activation of the GRP120 receptor by ω-3 PUFAs or the GW9508 agonist was shown to be associated with insulin sensitization and improvement in glucose intolerance. The same authors using cell cultures of 3T3-L1 adipocytes and primary adipose tissue, reported a significant increase in activation of PI3K/Akt receptor pathway, which triggered the translocation of GLUT4 and increasing the cellular uptake of glucose

(Oh et al. 2010). These findings are reinforced by the publication of Liu et al. (2012), who report a reduction in the expression of GLUT4 and insulin receptor substrate (IRS) in 3T3-L1 cells with low expression of GRP120 (Liu et al. 2012). On the other hand, Ichimura et. al. (2012), in GPR120 double knockout mice and fed with HFD, reported the development of hyperglycemia, glucose intolerance and insulin resistance as compared with the control group (Ichimura et al. 2012).

An important observation in relation to the supply of HFD is the phenomenon of upregulation in the expression of the GPR120 and PPARγ receptors, which is suggested to provide a regulatory pathway for energy metabolism (Song et al. 2017). This pathway of GPR120-PPARγ also appears to intervene in the upregulation of vascular endothelial grow factor-A (VEGF-A) in adipocytes 3T3-L1, effect that is related to the decrease in glucose intolerance and insulin sensitization in mice fed with HFD (Sun et al. 2012).

The demonstrated increase in GPR120 expression in several studies is crucial because of its significant effects on adipogenesis, however, it has been reported that obese patients have a reduction of both receptor and its mRNA in visceral fatty tissue as compared with lean subjects, these results being contradictory with the rest of the studies (Rodriguez-Pacheco et al. 2014). In addition, animal models demonstrated that receptor functionality was essential for proper adipogenesis, therefore, obese people may be considered to lack functional receptors, and HFD consumption worsens this dysfunction and insulin resistance (Rodriguez-Pacheco et al. 2014, Ichimura, Hara, and Hirasawa 2014). Unfortunately, at present, there are no tools available to measure the functionality of the receptor in patients and therefore this assumption should be corroborated.

As mentioned above, the activation of the GRP120 receptor facilitates adipogenesis and lipogenesis within the adipose tissue, guaranteeing metabolic homeostasis. In relation to the expression of this receptor in other tissues of metabolic relevance, there are studies that indicate its existence in murine and human pancreatic islets, in addition to β-cell lines (Taneera et al. 2012, Fritsche 2015). The activation of this receptor by DHA in β-cells increases insulin secretion, although the subtype of GPR40 receptor is considered to be mainly responsible for this action. Taking into account its signaling mechanism, the GPR120 receptor implies an increase of intracellular calcium ([Ca^{2+}i]) levels, an insulinotropic effect would be plausible (Ozdener et al. 2014).

This aspect was considered by Zhang et al. (2017), who in both animal models and cell cultures, unequivocally demonstrated for the first time the expression of these receptors in β-pancreatic cells where their function is to modulate the release of insulin through PLC/Ca^{2+} dependent signaling pathway. In this study, the authors demonstrated that oral administration of GPR120 receptor agonists in mice with DM-2 improves postprandial hyperglycemia and increases insulin secretion in the oral glucose tolerance test (OGT) (Zhang et al. 2017).

These findings can be extrapolated to other GPR120 agonist such as ω-3 PUFAs. In fact, the administration of DHA while increased the insulinotropic effect in obese non-diabetic mice (Raimondi et al.), it reduced in those with DM-2. In view of these findings, the authors propose that hyperlipidemia in OND and hyperglycemia in DM-2 could affect PPARγ expression in β-cells through different pathways, these changes being responsible for modifications in the expression of GPR120 (Liu et al. 2012).

The regulatory role in β-cell survival is another effect described for this receptor. Activation of the receptor in different cells such as enteroendocrines, adipocytes, macrophages and hypothalamic neurons induce Akt/ERK phosphorylation (Katsuma et al. 2005, Fritsche 2015); this signaling in β-cells is closely related to their functionality and survival (Wijesekara et al. 2010). In this sense, Zhang et. al. (2017), observed in rodent islets, that stimulation of GPR120 protects the cells against glucotoxicity and was dependent on the Akt/ERK pathway, which was increased in OND islets but reduced in diabetic islets. These results suggest the importance of cell survival in pathological conditions and the role that ω-3 PUFAs can play in their utilization (Zhang et al. 2017).

Despite the functions already described for this receptor, it is expected that new roles will be elucidated which may be associated with the expression of the receptor in particular tissues as well as the signaling mechanisms underlying its activation.

The evidence for the beneficial effects of ω-3 PUFAs in improving obesity, Met-S and diabetes has been provided from studies in experimental animals. This is a limiting factor because there are differences among species in accumulating these fatty acids in the cell membranes, which depends directly on the amount ingested. Because their positive actions in these pathologies are related to the agonist effect on GRP120, very high doses will be required which is impractical, for this reason a long term daily consumption of ω-3 PUFAs is the highly recommended (Huang et al. 2016).

V. Effects on platelet function and thrombosis

Both endothelial function and platelet activation determine acute thrombotic events. Decades of inflammatory vascular damage increases progressively the likelihood of

triggering events, platelet adhesion and aggregation, and thrombus formation (Strong et al. 1999).

Agonists such as adenosine diphosphate (ADP), serotonin, thrombin, epinephrine and AA participate in the platelet activation process (Li et al. 2010). Arachidonic acid, the most important ω–6 PUFAs physiologically, is required as a constituent of membrane phospholipids and is the precursor of "Series 2" eicosanoids (PGD_2, PGE_2, PGF_2, PGI_2, or A_2 thromboxane (TXA_2)). Prostanoids production pathway begins with the mobilization of AA from membrane phospholipids by cytosolic A_2 phospholipase ($cPLA_2$). The next step is the production of PGH_2 prostaglandin endoperoxide from AA, conversion mediated by endoperoxide prostaglandin H synthase-1 or -2 (PGHS-1 or -2), also known as cyclooxygenase-1 or -2 (COX-1 or -2) (Simmons, Botting, and Hla 2004, Smith 2005). The process of synthesis of eicosanoids culminates in the isomerization of PGH_2 into AA-derived "Series 2" products. The "3-series" prostanoids, including PGD_3, PGE_3, PGF_3, PGI_3 and TxA_3, are derived from EPA and DHA. Both ω–6 PUFAs and ω–3 PUFAs use the same metabolic pathways, which include specific enzymes such as lipoxygenase (LOX) (Brash 1999, Kuhn et al. 2005) and cytochrome P450 (CYP450) (Capdevila and Falck 2002), in addition to the COX pathway described above (Figure 1.5). Eicosanoids obtained by the metabolic pathway of PUFAs participate in different ways in this platelet aggregation process. Thus, while TXA induces amplification of the platelet response, prostaglandin I (PGI) acts by inhibiting platelet activation (FitzGerald 1991).

Insert Figure 1.5

The influence of the ratio ω–6/ω–3 on arterial thrombosis risk and the progression of atherosclerosis has been investigated using animal models (Yamashita et al. 2005). These studies demonstrated that, in addition to reducing TGs and LDL levels, the lower

proportion of ω–6/ω–3 tested was more effective in suppressing thrombotic and atherosclerotic parameters.

The antithrombotic properties observed with ω–3 PUFAs have been attributed to the incorporation of EPA and DHA in platelet phospholipids, displacing ω-6 PUFAs such as AA (Smith 2005). EPA has been shown to induce profound changes in the biosynthesis of prostanoids and leukotrienes by competing with AA for COX and LOX binding sites. The efficiencies of prostanoids production can be significantly lower with EPA compared to AA, considering that COX-1 and COX-2 produce EPA oxygenation at only 10 and 30% of the rates obtained with AA (Wada et al. 2007).

In relation to the biological properties of prostanoids, it was initially proposed that TXA_2, originating from the AA pathway, was a potent pro-aggregator; whereas TXA_3, originating from the EPA-derived series 3, was inactive. However, more recent studies have shown that both TX series have similar binding and stimulation capabilities of the thromboxane receptor (TP) (Wada et al. 2007). This does not allow us to sustain the theory of competition between EPA and AA for the production of pro-aggregant TXA. Nor does prostaglandins appear to tip the scales in favor of one or the other route, since both AA-derived (PGI_2) and Series 3 (PGI_3) prostacyclins generated from EPA have similar ability to inhibit platelet activation. The preferable route in favor of EPA's protective effects is the production of leukotrienes. While AA is metabolized by 5-LOX to the "4-series" of leukotriene (LTB_4), which induces inflammation and is a powerful chemoattractant agent of neutrophils. The same pathway produces LTB_5 from EPA, a metabolite of the "5-series", which is at least 30 times less potent than LTB_4 (Terano, Salmon, and Moncada 1984). This competition between AA and EPA for the production of leukotrienes provides an explanation for the anti-inflammatory effect of dietary ω-3 PUFAs, which, in turn, could

also be considered in pro-thrombotic protection. In addition to competing with COX and LOX enzymes, ω-3 PUFA derivatives also compete with AA for other enzymatic system involved in the synthesis of lipid derivatives with pro-inflammatory activity. EPA and DHA are also the parental fatty acids of new classes of lipid mediators, called resolvins, which have very potent anti-inflammatory properties and can play an essential role in protecting against various inflammatory diseases (Serhan, Chiang, and Van Dyke 2008). Atherosclerosis, which in turn is associated with pro-thrombotic and atherogenic processes, would also be included in this group of inflammatory diseases.

On the other hand, CYP450 enzymes catalyze hydroxylation and epoxidation of AA in what was recently established as the "third branch" of the eicosanoid cascade (Capdevila and Falck 2002). These enzymes catalyze the conversion of AA into epoxyeicosatrienoic acids (EETs). The CYP450 isoforms that metabolize AA also bind efficiently to EPA and DHA to produce epoxyeicosatetraenoic acids (EEQs) and epoxydocosapentaenoic acids (EDPs), respectively. These interactions can have important physiological implications and provide a novel insight into the mechanisms of vascular and cardiovascular protective effects of ω-3 PUFAs. Thus, it has been observed that the EET produced by AA epoxidation are inhibitors of thrombocyte adhesion to the vascular wall. However, in a ristocetin induced thrombocyte aggregation model, inhibitory potency was found to be 5 times higher for EPA and DHA derivatives than for AA-derived EETs. These results provide new data on the very specific role of CYP450-dependent eicosanoids in preventing thromboembolic events. In addition, they suggest that the formation of EEQs and EDPs may contribute to the antithrombotic effects of ω-3 PUFAs (Jung et al. 2012).

Over the past two decades, several studies have reported the effects of ω-3 PUFAs on composition of platelet lipid and platelet function (Kristensen, Schmidt, and Dyerberg

1989). Dietary ω-3 fatty acids have been shown to inhibit both platelet aggregation and release of platelet TXA in response to collagen and ADP (Brox et al. 1983, Hirai et al. 1980). Other studies have shown an inhibition of thrombin-induced platelet aggregation (Ahmed and Holub 1984) and adrenaline-induced platelet aggregation (Kristensen et al. 1987). In the presence of ω-3 PUFAs, the platelet response has also been inhibited by the addition of platelet-activating factor (PAF) (Codde et al. 1987). However, ω-3 PUFAs do not appear to affect the platelet aggregation induced by AA (Knapp et al. 1986).

These findings are complemented by other observations, aimed at evaluating the activity of ω-3 PUFAs on endothelial activity, as the role of these cells in the production of vasoactive substances is recognized. Prostacyclins and NO, mediators produced by vascular endothelium, are factors that promote the relaxation of vascular smooth cells and inhibition of the platelet activation pathways involved in the maintenance of homeostasis. In this way, endothelial function plays a protective or limiting role in the development of thrombosis, particularly in arterial thrombus formation.

In this sense, it is known that in human coronary endothelial cells, DHA regulates the function of eNOS and promotes NO synthesis by activating Akt (Stebbins et al. 2008). Therefore, it could be hypothesized that DHA-induced increases in bioavailability of NO, improving endothelial function and blood flow, in addition to counteracting the pro-thrombotic effects of eicosanoids derived from active platelets.

These findings suggest that a reduction in platelet-vascular wall interaction may result from a diet rich in ω-3 PUFAs. However, it is clear that the mechanisms are complex and encompass more than a simple imbalance between eicosanoid derivatives with pro-aggregant or anti-aggregant properties. In any case, experimental data correlate with the

effect of a diet rich in EPA and DHA on platelet phospholipid fatty acid composition, platelet aggregation and bleeding time.

In accordance with experimental data, one study conducted in healthy subjects, the EPA diet prolonged bleeding time (42%) and decreased platelet aggregation (Thorngren and Gustafson 1981). This study also showed a decrease in sensitivity to ADP that persisted for several weeks after stopping the ω-3 PUFAs-rich diet. Similarly, in humans with high cardiovascular risk, it was observed that consumption of ω-3 PUFAs at an average dose of 3.65 g/d, is capable of reducing platelet aggregation induced by different agonists, such as collagen, PAF and TXB_2 (Mori et al. 1997). There are other studies that support the antiplatelet activity of ω-3 PUFAs. For example, a reduction in platelet reactivity after DHA supplements for two weeks was reported in healthy male volunteers (Guillot et al. 2009). In addition, other experimental studies conclude that low ω-3 PUFA supplements lead to a state of platelet overactivity, increased blood viscosity and a tendency to thrombus development (Wan et al. 2010). Similarly, another study was conducted on healthy volunteers who received 3.4 g/d of EPA+DHA where clinical hematological parameters were measured along with collagen-stimulated platelet activation and protein phosphorylation. Treatment with ω-3 PUFAs produced a significant attenuation of collagen-mediated platelet signaling events and α-granule secretion (Larson et al. 2011). These results based on platelet function, suggests that ω-3 PUFAs incorporated into the diet generate a broad spectrum of favorable effects on factors associated with thrombosis risk. However, other data reported are not consistent with the antithrombotic properties described previously.

In human trials, it has been reported that ω-3 PUFAs consumption has no effect on platelet aggregation and clotting factors (Balk et al. 2004, Wang et al. 2004). Recent study,

demonstrated that even though ω-3 PUFAs increase of von Wilbrand factor (vWF) release in an inflammation model using human endothelial cells (Burgin-Maunder, Brooks, and Russell 2013). Same authors reported that administration of ω-3 PUFAs in hypertensive patients does not reduce plasma concentration or binding activity of vWF to collagen (Burgin-Maunder et al. 2015).

Based on the biological properties of ω-3 PUFAs described above, the debate on their safety in patients susceptible to bleeding is still open. This may be particularly relevant in view of the specific recommendations for people with a medical history of cardiovascular events or patients in preparation for surgery, who frequently use antithrombotic drugs. In this sense, there has been no excess risk of clinical bleeding with fish or fish oil consumption, including among patients undergoing surgery or percutaneous intervention (Eritsland et al. 1995). On the other hand, in a study using 4 g/day doses of fish oil, change in bleeding time or number of bleeding episodes was not observed in patients on aspirin or warfarin therapy after coronary artery bypass (Eritsland et al. 1995). In a more recent analysis that included 8 clinical studies conducted with enteral nutritional products containing fish oil as a source of ω-3 PUFAs, adverse events related to bleeding and effects on key clotting parameters were assessed. The authors found that there was no evidence of increased risk of bleeding with the use of fish oil-enriched medical nutrition in patients with moderate to severe disease (Jeansen et al. 2017). Despite these findings, it is suggested that due to the possibility of prolonged bleeding time, patients receiving warfarin therapy should be monitored and the dosage of anticoagulant adjusted if necessary.

VI. Effects on blood pressure and endothelial function

High blood pressure (HBP), defined as the sustained rise in blood pressure higher than 140/90 mmHg, is the leading modifiable cause of death associated with cerebrovascular

disease and, is responsible for severe disability worldwide (WHO 2013). Based on its etiology, HBP is usually classified as primary or secondary, the latter being of a specific origin such as primary hyperaldosteronism, pheochromocytoma, use of drugs, etc.; while the primary or essential hypertension has multifactorial causes that include genetic and environmental factors and alterations in the blood pressure regulating systems (Rossier, Bochud, and Devuyst 2017, Shrout, Rudy, and Piascik 2017).

Blood pressure (BP) determined by cardiac output (CO) and peripheral vascular resistance (PVR), is a physiological variable that depends on the heart, blood vessels and extracellular fluid volume, whose functions are regulated by the activity of the central nervous system (CNS), autonomic nervous system, kidneys, and circulating hormonal factors such as the renin-angiotensin-aldosterone system (RAAS) (Lawton and Chatterjee 2013).

Due to the many factors responsible for the regulation of BP and considering that the alteration of these factors is involved in the development of HBP, the therapeutic approach includes a large number of drugs with different mechanisms of action, which are aimed at modifying the underlying alterations and control the increase in BP, while at the same time reducing the harmful effects of the pathology on target organs such as heart, blood vessels, kidney and brain.

This pharmacotherapy has shown, overtime, a significant reduction in cardiovascular morbidity and mortality (Chobanian 2009, Ettehad et al. 2016), therefore it is imperative that the hypertensive patients receive medication, in addition to lifestyle changes, which have also been shown to decrease BP (James et al. 2014).

Despite the existence of drugs with clinical efficacy and demonstrated safety, control of HBP is inadequate, as several authors point out that a large percentage of patients do not

reach the established target (<140/90 mm Hg) and thus maintain an elevated cardiovascular risk (Laurent 2017, Ferdinand and Nasser 2017).

As indicated above, there is a renovated interest in the search for adjunct therapies that guarantee a better antihypertensive response in patients. One of these possibilities is the use of nutritional supplements, which have been shown to demonstrate positive effects of diet on health. Among this group of agents are ω-3 PUFAs which have pleiotropic properties in CVD (Turner and Spatz 2016).

In this regard, animal, randomized and, observational studies indicate that intake of ω-3 PUFAs modifies BP. However, the reduction of BP observed with these fatty acids is mild, but in other cases the results are inconsistent (Cabo, Alonso, and Mata 2012).

Since BP is the result of CO and PVR, these fatty acids would be expected to exert their antihypertensive effect by modifying both parameters and the regulating variables. For this reason, studies have been conducted to evaluate the effects of ω-3 PUFAs on the following parameters that determine the BP.

A. Effect of ω-3 PUFAs on cardiac output

In relation to this parameter, a meta-analysis found a significant reduction in heart rate by 1.6 beats per minute (bpm) associated with the consumption of ω-3 PUFAs. The effect on CO appears to be independent of amount ingested; however, the treatment period is determinant, and the most pronounced effect has been observed in studies with periods over 12 weeks of treatment (Mozaffarian et al. 2005). Despite this observed effect, its mechanism is not yet defined and may, therefore, be related to the ability of these compounds to modulate cardiomyocyte activity, associated with an increase in parasympathetic activity in the myocardium (Mozaffarian and Wu 2011). This reduction in HR could favor the diastolic filling function, causing an increase in systolic volume, effect

that are evident from the beginning of supplementation (Cabo, Alonso, and Mata 2012). Considering that the effect on CO is minimal, it can be stated that the antihypertensive effect associated with ω-3 PUFAs is more related to the modification of PVR rather than with its actions on CO.

B. Effect of ω-3 PUFAs on peripheral vascular resistance

This parameter is determined by the caliber of the resistance blood vessels, where the smooth muscular layer and vascular endothelium are most important

1. *Endothelial function:* ω-3 PUFAs increase the production and disposition of NO from endothelial cells and the effect on this vasodilatory mediator is due to following actions:

a) Alterations in the composition of phospholipids in the endothelial cell membrane, as a result of their incorporation. Within these cells there are the caveolae that contain eNOS bound to caveolin-1, a protein complex capable of inhibiting enzymatic function.

   In cell cultures incubation with EPA, eNOS activity is increased due to the dissociation of the enzyme from caveolin-1 (Zanetti et al. 2015). Another complementary study shows that EPA stimulates the phosphorylation of eNOS dependent pathway of adenosine monophosphate-activated protein kinase (AMPK), which increases NO. This surge in AMPK activity was related to the higher expression of the mitochondrial proton transporter (UCP-2), which has a regulatory impact in the production of NO (Enre Y 2010, Wu et al. 2012).

   In the case of DHA, some authors indicate similar effect on NO production, however, its mechanism is by promoting the interaction between eNOS and

Heat Shock Protein-90 (HSP-90) which activates the PKB/AKt pathway generating consecutive phosphorylation of eNOS (Stebbins et al. 2008).

b) Reduction of asymmetric dimethylarginine (ADMA). A study carried out by Raimondi et. al. (2005), in spontaneously hypertensive older rats reported that EPA-DHA supplementation reduced the concentration of ADMA, an endogenous inhibitor of eNOS, intimately associated with HBP and other pathologies. This effect on ADMA is related to an increase in NO availability (Raimondi et al. 2005) .

c) eNOS gene expression. Various studies in laboratory animals have shown that administration of ω-3 PUFAs induces an increase in the eNOS gene expression which increases NO availability.

2. *Antioxidant effect:* This effect has been demonstrated by the ability of these fatty acids in decreasing the formation of reactive oxygen species such as peroxynitrite by modulating the activity of NADPH oxidase and inducible nitric oxide synthase (iNOS).

3. *Anti-inflammatory effect:* ω-3 PUFAs reduce the activity of various inflammatory mediators, such as NFκB, IL-6, IL-1 and C-reactive protein (CRP), associated with endothelial dysfunction, proliferation of vascular smooth muscle cells, and production of free radicals (Wang, Chen, et al. 2011). The neutralization of these mediators would increase NO levels, to confer the ω-3 PUFAs with a vasodilatory and antihypertensive effects.

4. *Vascular smooth muscle cells:* the capability of DHA to produce hypotensive effects in mice after intravenous administration has been described, which is

associated with its ability to directly activate calcium-dependent and voltage-activated K+ (BK) channels (Hoshi, Wissuwa, et al. 2013). These play an important role in vascular tone since their activation induces hyperpolarization thus closing calcium-dependent-voltage channels, resulting in vasodilation. This action on BK was confirmed by *in vitro* studies in coronary smooth muscle cells, arteries and cell culture, where the application of DHA increases this current, inducing a vasodilatory effect (Lai et al. 2009, Hoshi, Tian, et al. 2013). In the case of DHA and its antihypertensive effect, it has been proposed that this effect may depend upon its conversion to active metabolites, EDPs, which have a greater hypotensive effect and better stability than eicosatrienoic acids (ETrAs) which are derivatives of AA (Wang, Chai, et al. 2011, Fer et al. 2008). Based on the above, Ulu et al., (2014) investigated the effect of an epoxy DHA, 19,20 epoxy-docosapentaenoic acid (19,20-EDP), on an angiotensin II-induced hypertension in mice, and demonstrated that administration of 19,20-EDP was involved in the antihypertensive effect of DHA (Ulu et al. 2014).

Despite the considerable evidence from experimental results, the effect of ω-3 PUFAs on BP in humans is not conclusive since the interpretation of epidemiological studies is difficult, especially because of the accurate estimation of fish intake in the population (Mori 2006). In this respect, the INTERMAP study that evaluated 4680 individuals from Asia and Western countries, demonstrated an important direct association between the consumption of these fatty acids and the reduction of BP (Ueshima et al. 2007).

From these observations, several studies have evaluated the effect of ω-3 PUFAs *vs* placebo in order to establish their antihypertensive effect. Thus, in early studies, intake of 3 g of ω-3 PUFAs in untreated hypertensive patients, report reductions of –5.5 mmHg in systolic

blood pressure (SBP) and –3.5 mmHg in diastolic blood pressure (DBP) (Turner and Spatz 2016).

A recent meta-analysis of 70 randomized controlled trials (RCTs) published by Miller et al. (2014), considering all possible sources of EPA and DHA administered in an average dose of 3.8 g/d, reported a significant reduction of BP in both hypertensive and normotensive subjects. In this meta-analysis, the effect was more pronounced in hypertensive patients, as shown in earlier studies, but no dose-dependent relationship (Miller, Van Elswyk, and Alexander 2014).

Given the effect shown in clinical studies, other prospective studies have evaluated the possible impact of diet supplemented with these fatty acids on the development of HBP in normal subjects, indicating a significant reduction in the risk of developing HBP compared with subjects with low ω-3 PUFAs intake (Colussi et al. 2017).

With these results, it has been demonstrated that the effect of ω-3PUFAs on BP is minimal and is manifested mainly in hypertensive patients. However, its ability to decrease the progression of this disease could be relevant for the population. In addition, there is a need for further studies to evaluate more broadly the antihypertensive efficacy of these agents using more strict criteria related to populations, sources and quantity of ω-3 PUFAs, duration and treatment modalities.

VII. Effects on cardiac arrhythmias

Cardiac arrhythmias are defined as an alteration in the onset or form of electrical impulses transmitted in the myocardium. They constitute a relevant health problem since, apart from being related to other cardiovascular pathologies such as heart failure (HF), myocardial

ischemia and acute myocardial infarction, they can be responsible for the sudden cardiac death (SCD) (Gaztanaga, Marchlinski, and Betensky 2012).

There are different types and ways of classification of cardiac arrhythmias. However, the most commonly used form is the conventional, which is based on heart rate or pacemaker disturbance. In relation to the latter criteria, arrhythmias are divided into: a) supraventricular arrhythmias, those originating in the atrium or above the atrioventricular node (AVN); and b) ventricular arrhythmias that occur in the ventricular tissue. Within this division, there are sub classifications, some of which do not require treatment, however, others due to implicit risk of fatality, require a strict pharmacological treatment that guarantees the suppression of the altered rhythm (Li 2015).

Two mechanisms involved in the genesis of arrhythmias are described: (1) increased normal and abnormal automaticity, (2) decreased driving performance.

Both mechanisms, in turn, are determined by cardiac electrophysiology that depends on the different ionic currents, a determining factor of the cardiac action potential. Therefore, the most antiarrhythmic agents act by modifying these ionic currents or, in some particular cases, membrane receptors.

Within the conventional classification, three types are of particular clinical interest: atrial fibrillation (AF), ventricular tachycardia (VT) and ventricular fibrillation (VF), in which cases combined therapy is required, ranging from anticoagulant drugs to utilization of electrical cardioversion.

It is recognized that AF is the most common arrhythmia in the world population with an estimated prevalence of 3% in adults, and is associated with factors such as: age, valvular heart disease, coronary artery disease, diabetes mellitus (DM) and chronic kidney disease,

among others (Gomez-Outes, Suarez-Gea, and Garcia-Pinilla 2017, Kanaporis and Blatter 2017).

Meanwhile, TV and VF are potentially lethal arrhythmias that can develop during AMI or as clinical sequelae of advanced cardiomyopathy, and are often associated with SCD (Jong-Ming Pang and Green 2017). Because these types of arrhythmias generate important morbidity and mortality and despite the existence of available treatments, in recent decades, there is a need for additional more effective strategies that provide better results for the patients. In this sense, epidemiological data from studies of more than 40 years have demonstrated an inverse association between diets with a high content of fish fat and cardiac mortality (Daviglus et al. 1997).

Additionally, the main components of this diet, ω-3 PUFAs, in clinical trials had shown a significant reduction in mortality in post-infarction patients (Marchioli et al. 2002) or patients with high cholesterol levels receiving conventional treatment, indicating its safety in combination therapy and their antiarrhythmic potential (Yokoyama et al. 2007).

Based on the above, a large number of studies have been conducted in animals including rats, pigs, monkeys, and cell cultures, to determine the precise mechanism of action by which ω-3 PUFAs produce their antiarrhythmic effect (McLennan 2014). These studies indicate that ω-3 PUFAs possess modulating properties of ionic currents in the cardiomyocyte, which are responsible for maintaining electrophysiology of the heart and are involved in the origin of arrhythmias (Roy and Le Guennec 2017, Endo and Arita 2016). It has been proposed that the ability to modify the sodium, potassium and calcium channels can be a direct or indirect effect, the latter being due to the ability of ω-3 PUFAs to alter the constitution and function of phospholipids in cellular membrane (Reiffel and McDonald 2006). Other studies have reported that the administration of these agents does

not cause changes in electrocardiographic parameters, making it difficult to explain, in a congruent manner, the observed molecular effects on ion channels and their relationship with changes in electrocardiogram (Roy and Le Guennec 2017).

Despite these discrepancies, research has continued, giving more emphasis on modulation of sodium, potassium and calcium channels.

A. Effects on sodium currents

Cellular electrophysiological studies in animals, on the effects of acute administration of ω-3 PUFAs, indicate an inhibitory effect of the ventricular sodium current, denoting a reduction in potential amplitude, reduction of the channel inactivation voltage and rapid increase of its inactivation and an increase in the activation threshold (Reiffel and McDonald 2006, Roy and Le Guennec 2017). These effects are evident in adult and neonatal cardiomyocytes.

In non-heart cells transfected with mutant sodium channels, Xiao et al. (2006) reported that ω-3 PUFAs induced their potent blockage. The effects of ω-3 PUFAs on $I_{Na\ late}$ was greater than that on $I_{Na\ peak}$. It had been demonstrated that an increase in persistent I_{Na} with a consequent increase on $[Ca^{2+}]i$ level, can cause arrhythmias and irreversible cell damage. Thus, the inhibition of $I_{Na\ late}$ by ω-3 PUFAs might have a potential therapeutic value in certain patients with ischemia-induced arrhythmias. This action was limited to ω-3 PUFAs and was not produced by monounsaturated or saturated fatty acids (Xiao et al. 2006).

On the other hand, the longer exposition to these compounds in ventricular myocytes isolated from pigs and rats feeding with a diet rich in ω-3 PUFAs, has not shown significant effects on sodium current. However, the antiarrhythmic effect is maintained, which may suppose that the effect on sodium channels is not the main mechanism for this therapeutic

effect (Moreno et al. 2012). Additionally, Li et al. (2009) using human atrial myocytes, demonstrated similar inhibitory effects on this current, this effect being more pronounced with EPA than with DHA (Li et al. 2009).

B. Effects on potassium currents

In cardiomyocytes, there are different types of potassium currents which are responsible for the repolarization process during the action potential, therefore, the modulation of these currents generates significant effects on the electrical activity of the heart. The principal potassium currents in the cardiac action potential are: transient outward current, ultra-fast, fast and slow repolarizing (I_{Kur}, I_{Kr} and I_{Ks}) and rectifying inward (I_{Kir}) current. These currents have a varied distribution according to the type of cardiac tissue, for example I_{to} and I_{Kur} are located mainly in atria (Li et al. 2009); while I_{Ks}, I_{Kr} and I_{Kir} predominate in the ventricle (Song et al. 2013).

Despite the large number of potassium currents, few studies have been conducted to determine the effect of ω-3 PUFAs on these currents. The most information has been obtained from cellular studies with acute administration of these compounds, without evaluating the effect of dietary supplements and their incorporation into the membranes(McLennan 2014). Thus, DHA demonstrated an inhibitory effect on I_{to} current in transfected Chinese hamster ovary cells(Singleton et al. 1999). This effect was also observed in isolated human atrial cardiomyocytes, where treatment with DHA and EPA significantly inhibited this current(Li et al. 2009).

Another study in rat ventricular myocytes reported that DHA significantly inhibited repolarizing currents with the consequent prolongation of the duration of action potential (APD) and effective refractory period (ERP), effects that determine their ability of eliminating reentry inputs and cardiac arrhythmias(Song et al. 2013). The same study

showed that DHA had no effect on I_{Kir}, indicating that DHA does not modify the resting potential in these cells.

Taking into consideration the inhibitory effect on different potassium currents, it can be inferred that this effect may explain, in part, the low incidence of supraventricular and ventricular arrhythmias associated with the use of ω-3 PUFAs (Mozaffarian et al. 2004, Leaf et al. 2005).

C. Effects on calcium channels

Two calcium currents are present in the heart: the transitory calcium current (I_{CaT}) present exclusively in the nodes, and the slow calcium current (I_{CaL}) expressed in all cells. The latter has the characteristics of generating depolarization phase in nodal cells and sustaining the plateau during cardiac action potential in atrial and ventricular cells, allowing these cells to raise the $[Ca^{2+}]i$ levels necessary for mechanical contraction function. Moreover, the elevation in the level of this ion has been related to different arrhythmias such as those induced by digitalis, adrenergic stress and anoxia, among others (Landstrom, Dobrev, and Wehrens 2017). In this sense, acute and long-term treatment with ω-3 PUFAs in rat prevented transient elevation and calcium overload in cardiomyocytes, in an associated effect with blockage of these ionic currents (Rinaldi et al. 2002, Jahangiri et al. 2006). Several authors using mammalian cardiomyocytes have shown that the effect on calcium levels is due to the blocking of I_{CaT} and I_{CaL} currents, which reduces transitory elevations of this ion, shortens the plateau of action potential and decreases the occurrence of arrhythmias (McLennan 2014).

Another important aspect of $[Ca^{2+}]i$ is the function of the sodium-calcium exchanger (NCX) responsible for a current (I_{NCX}) that can lead to the accumulation of calcium inside

the cell, which can generate arrhythmias. Xiao et. al., in HEK293t cell lines, demonstrated that ω-3 PUFAs suppress this activity. This finding is relevant because during ischemia, the accumulation of hydrogen ions stimulates the activity of the sodium/hydrogen exchanger, which leads to the accumulation of intracellular sodium and consequently increases the activity of NCX, causing the increase of calcium (Xiao et al. 2004).

Given the intimate relationship between calcium overload and arrhythmias, it is clear that blocking calcium currents is an important antiarrhythmic mechanism of ω-3 PUFAs.

Other mechanisms: it is recognized that these fatty acids accumulate in the cell membrane and modify its constitutive and functional properties. From this point of view, the functionality of membrane-attached proteins (ionic channels) can be altered without having a direct interaction between ω-3 PUFAs and these channels, thus affecting the ionic currents involved in cardiac action potentials (Mozaffarian and Wu 2011, 2012).

As regards to the alteration of membrane composition, some authors suggest that the incorporation of ω-3 PUFAs in the cardiomyocyte membrane reduces the release of inositol triphosphate (1,4,5-triphosphate, IP3) in response to stimulation of alpha-adrenergic receptors in porcine cardiomyocytes (Nair, Leitch, and Garg 2000) and rat hearts in response to ischemia (Anderson et al. 1996). This mediator, IP3, is a second messenger in signals resulting from the activation of PLC and whose function is to increase the release of intracellular calcium, consequently its reduction could have an implication in the antiarrhythmic effect.

All the mechanisms described above, have been established mainly using isolated cardiomyocytes and cell cultures, however in some similar *in vitro* and *in vivo* models, these results are inconsistent, therefore, a definite mechanism has not been established and cannot be extrapolated to humans. These differences may be due to different cell types and

the composition of the membrane, which could alter the effect of ω-3 PUFAs. Another factor to consider is the administration of these compounds, which may be acute (*in situ*) intravenously or on a long-term basis (supplied in the diet), where direct and indirect action (incorporation into cell membranes) may play a relevant role within their actions (McLennan 2014, Roy and Le Guennec 2017, Trimarco 2012).

In relation to the possible antiarrhythmic effect of ω-3 PUFAs in humans, several clinical studies show evidence of their capacity to reduce the development of supraventricular, ventricular arrhythmias and SCD in different populations (patients with ischemia, with implanted defibrillators or AMI convalescent). However, there are also contradictory studies in this respect, for this reason the antiarrhythmic potential should be carefully considered (Borghi and Pareo 2012).

These conflicting results can be explained in part by the findings of Hu et al. (2016), who showed that there was no difference in the prevalence of AMI compared the Inuid population in Canada *vs* United States and Canadian general populations. Authors associate this observation with levels of methylmercury (MeHg) present in the fish diet of the Inuid Canadian population. Thus, the intake and accumulation of mercury can favor lipid peroxidation counteracting the beneficial effect of ω-3 PUFAs (Hu, Laird, and Chan 2017).

A recent publication by Roy et al. (2015), indicates that the antiarrhythmic effect of DHA is related to the non-enzymatic oxidation carried out by ROS, where neuroprostanes are generated. These mediators are recognized biomarkers of oxidative stress, therefore, would be expected to have a harmful effect. However, these researchers demonstrated experimentally that neuroprostanes, primarily 4RS-4F4t-neuroprostane and 10(S)-10-F4t-neuroprostane, can regulate the function of the ryanodine receptor (RyR2), decreasing calcium efflux from the sarcoplasmic reticulum leading to a reduction in calcium sparks

and the risk of arrhythmias (Roy et al. 2015). Under this premise, it would be expected that in chronic conditions of oxidative stress, which are common in cardiovascular diseases (ischemia, atherosclerosis, AMI, cardiac post-surgery, etc.) (Luscher 2015, Islam et al. 2016, Yalta and Yalta 2017), ω-3 PUFAs generate the production of neuroprostanes which participate in the antiarrhythmic effects induced by these fatty acids.

Because of many factors and variables involved that can modify the results of preclinical and clinical studies with the use of these compounds, it is necessary and imperative that the new studies consider all these factors, so that their mechanism of action and their application as antiarrhythmic agents can be more precisely underpinned.

VIII. Conclusions

Recent developments confirm and extend the concept that ω-3 PUFAs are beneficial in the prevention of cardiovascular disease and sudden cardiac death. In experimental studies and animal models, ω-3 PUFAs modulate a variety of relevant biologic pathways, with several lines of evidence suggesting at least some differential benefits.

During regular consumption of ω-3 PUFAs, a potential decrease on endothelial dysfunction and prevent CVD has been described through effects on endothelial metabolism, inflammation, thrombosis, and arrhythmia. On the other hand, the effects shown on vascular function and inflammation, could explain the antiatherogenic properties of these fatty acids. Such is the consideration of this effect that if in the treatment of dyslipidemia, the LDL-c target cannot be achieved by lifestyle changes or treatment with statins, it may be considered to add supplementation with ω-3 PUFAs, in order to help reduce the cardiovascular risk. Associated with effects on smooth muscle and endothelial cells, the administration of ω-3 PUFAs has been shown to lower blood pressure in both animal models and hypertensive patients.

There is no question that research on ω-3 PUFAs has made significant progress in a number of areas. However, there is a lack of conclusive data from clinical and mechanistic studies on the potential benefits of ω-3 fatty acids for primary and secondary prevention in CVD. On the basis of the available literature, ω-3 PUFAs should be considered as important components of a healthy diet and as a potential therapeutic modality in patients with coronary artery disease, particularly in populations at heightened risk of cardiovascular disease. These patients should be advised to eat a healthy dietary pattern that includes fatty fish. For individuals with low consumption of fish rich in ω-3 PUFAs, fish oil capsules as a source of EPA and DHA can be considered, given their long history of safety and the relationship between benefit and risk.

In conclusion, research on the biology of ω-3 PUFAs and its role in cardiovascular health is developing rapidly. However, harmonization of clinical criteria and parameters will be necessary in order to achieve consensus on beneficial cardiovascular effects of ω-3 PUFAs.

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