UNIVERSITY OF SÃO PAULO

Faculty of Pharmaceutical Sciences

Graduate Program in Food Science

Area of Experimental Nutrition

Effect of long-term consumption of oxidized polyunsaturated fatty acids on atherosclerotic lesion in LDL receptor knockout mice.

Marina Sayuri Nogueira

São Paulo

2020

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Marina Sayuri Nogueira

Effect of long-term consumption of oxidized polyunsaturated fatty acids on atherosclerotic lesion in LDL receptor knockout mice.

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“Science is nothing but perception.”

Plato.

“we don’t look backwards for very long. We keep moving forward, opening up new doors and doing new things, because we’re curious… and curiosity keeps leading us down new paths.”

Meet the Robinsons.

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Resumo

Nogueira, M.S. **Efeito do consumo crônico de ácidos graxos poliinsaturados oxidados na aterosclerose em camundongos knockout para receptor de LDL.** 2020. 128p. Tese (Doutorado) -Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2020.

Os ácidos graxos poliinsaturados (PUFA) são suscetíveis à oxidação enzimática e não enzimática, levando à produção de compostos secundários que apresentam diferentes efeitos fisiológicos. Entre os PUFA, os produtos formados a partir da oxidação dos ácidos graxos ômega 6 (n-6 FA) e ômega 3 (n-3 FA) podem modular a inflamação, dislipidemia e estresse oxidativo, impedindo ou reduzindo a progressão da aterosclerose. De fato, o efeito da ingestão crônica de óleos contendo produtos da oxidação de ácidos graxos poliinsaturados (POPs) na aterosclerose ainda é controverso. Em geral, os POPs dos n-6 FA têm um perfil mais pró-inflamatório do que os POPs dos n-3 FA. Assim, o objetivo deste estudo foi comparar a ingestão crônica de POPs provenientes de óleos ricos em n-6 FA e n-3 FA em biomarcadores de aterosclerose. Inicialmente, seis óleos ricos em n-6 FA e n-3 FA foram submetidos a condições oxidativas, simulando as etapas de transporte, armazenamento e consumo. Observou-se que a reação oxidativa iniciou-se em todos os óleos desde a primeira etapa e, no momento do consumo, alguns marcadores oxidativos estavam fora da faixa legal sugerida pelas agências reguladoras. Além disso, foi possível identificar o tipo de produto secundário formado a partir de cada óleo precursor, fornecendo melhores informações para o controle de qualidade dos óleos. Após esta etapa, os óleos de peixe e soja foram escolhidos como óleos ricos em n-3 FA e n-6 FA, respectivamente. Utilizando camundongos $LDLr^{(-/-)}$, o efeito de três níveis oxidativos de óleo de soja foi avaliado após 24 semanas de suplementação. Os animais alimentados com o óleo com maior nível de oxidação (óleo frito e de reuso) não apresentaram ganho de peso corporal, sugerindo que os POPs do óleo de soja nesse nível de oxidação pudessem promover um efeito de "Browning" no tecido adiposo branco, aumentando a expressão de UCP-1. Este grupo também mostrou a maior concentração de lipoproteínas no plasma. No entanto, essas diferenças metabólicas não aceleraram a aterosclerose nos animais. Finalmente, o efeito de POPs da oxidação de óleos ricos em n-3 FA e n-6 FA foi comparado também usando camundongos $LDLr^{(-/-)}$, como modelo para aterosclerose experimental. Algumas alterações observadas após a suplementação com óleo de peixe fresco, como aumento do peso hepático, IL-6, SONPC, 8-HETE e 15-F_2-IsoP e diminuição da BAT e glicose, foram revertidas por seus POPs. Além disso, os POPs do óleo de soja causaram aumento de LDL e 5-HETE. Como observado no estudo anterior, essas alterações metabólicas não foram suficientes para prevenir ou acelerar a aterosclerose, medida pela análise histológica do tamanho da lesão na aorta. Esses resultados sugerem que, embora uma quantidade significativa de POPs esteja sendo consumida pela dieta, seus efeitos metabólicos não influenciaram as placas ateroscleróticas no modelo animal. Porém, além da área de lesão nas aortas, novos estudos também devem avaliar a estabilidade das placas.

Palavra chave: omega 3, omega 6, aterosclerose, oxidação, camundongo.

Abstract

Nogueira, M.S. **Effect of long-term consumption of oxidized polyunsaturated fatty acids on atherosclerotic lesion in LDL receptor knockout mice.** 2020. 128p. Thesis (PhD) – Faculty of pharmaceutical Sciences, University of São Paulo, São Paulo, 2020.

Polyunsaturated fatty acids (PUFA) are susceptible to enzymatic and non-enzymatic oxidation, leading to the production of secondary compounds that present different physiological effects. Among the PUFA, the products formed from Omega 6 (n-6 FA) and Omega 3 (n-3 FA) fatty acids oxidation can modulate inflammation, dyslipidemia and oxidative stress preventing or reducing the atherosclerosis progression. In fact, the effect of chronic intake of edible oils containing products of polyunsaturated fatty acids oxidation (POPs) on atherosclerosis is still controversial. In general POPs from n-6 FA have a more pro-inflammatory profile than POPs from n-3 FA. Thus, the objective of this study was to compare the chronic intake of partially oxidized n-6 FA and n-3 FA rich oils on atherosclerosis biomarkers. Initially, six edible oils containing a higher amount of n-6 and n-3 FA were submitted to oxidative conditions, simulating the steps of transport, storage and consume. It was observed that oxidative reaction started in all oils since the first step and at the moment of consumption, some oxidative chemical markers were out the legal range suggested by the Official Agencies. In addition, it was possible to identify the type of secondary product formed from each precursor oil, providing a better information for oils quality control. After this step, fish and soybean oils were chosen as n-3 FA and n-6 FA rich oils, respectively. Using $LDLr^{(-/-)}$ mice, the effect of three oxidative levels of soybean oil was evaluated after 24 weeks of supplementation. Animals fed with the oil with the highest level of oxidation (fried and reused oil) showed no body weight gain, suggesting that POPs from soybean oil at this level could promote a browning effect on white adipose tissue by increasing UCP-1 expression. This group also showed the highest concentration of lipoproteins in plasma. However, these metabolic differences did not accelerate atherosclerosis in the animals. Finally, the effect of POPs from n-3 FA and n-6 FA oxidation were compared also using $LDLr^{(-/-)}$ mice as model for experimental atherosclerosis. Some alterations observed after n-3 FA supplementation, such as the increase of liver weight, IL-6, SONPC, 8-HETE and 15-F_2-Isop and the decrease of BAT and glucose, were reversed by their POPs. In addition, POPs from n-6 FA caused increased of LDL and 5-HETE. As observed in the previous study, these metabolic alterations were not enough to prevent or accelerate atherosclerosis, as measured by histological analysis of the lesion size in the aorta. These results suggest that although a significant amount of POPs are being consumed by diet, their metabolic effects did not influence atherosclerotic plaques in the animal model. However, besides lesion area in the aortas, new studies should also evaluate the plaques stability.

Key-words: omega 3, omega 6, atherosclerosis, oxidation, mice.

Abbreviations

4-HNE (4-Hydroxynonenal)
ADH (Alcohol dehydrogenase)
AKR (Aldoketo reductase)
ALA (alpha-Linolenic acid)
ALDH (Aldehyde dehydrogenase)
ApoB (Apolipoprotein B)
ARA (Arachdonic acid)
BAT (Brown adipose tissue)
COX (Cyclooxygenase)
CVD (Cardiovascular disease)
DHA (Docosahexaenoic acid)
EPA (Eicosapentaenoic acid)
GLA (gamma-Linolenic acid)
GSH (Reduced glutathione)
GST (Glutathione-S- transferase)
HDL (High-density lipoprotein)
HODA-PC (9-hydroxy-12-oxododec-10-enoyl-PC)
HOHA-PC (4-hydroxy-7-oxohept-5-enoyl-PC)
HOOA-PC (5-hydroxy-8-oxooct-6-enoyl-PC)
IsoF (Isofuran)
IsoLG (Isolevuglandin)
IsoP (Isoprostane)
KOdiA-PC (7-carboxy-5-oxo-hept-6E-enoyl-PC)
LDL (Low-density lipoprotein)
LNA (Linoleic acid)
LOX (Lipoxigenase)
LTB_4 (Leukotriene B4)
LTB_5 (Leukotriene B5)
MCP-1 (Monocyte chemoattractant protein-1)
MDA (Malondialdehyde)
mLDL (Modified low-density lipoprotein)
n-3 FA (Omega-3 fatty acid)
n-6 FA (Omega-6 fatty acid)
NADPH (Nicotinamide adenine dinucleotide phosphate)
NOX (NADPH oxidase)
Nrf2 (Nuclear factor erythroid 2-related factor 2)
oxLDL (Oxidized low-density lipoprotein)
OxPL (Oxidized phospholipids)
PAPC (1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidyllcholine)
PAzPC (1-palmitoyl-2-azelaoyl- sn-glycero-3-phosphocholine)
PC (Phosphatidylcholine)
PG (Prostaglandin)
PGE_2 (Prostaglandin E_2)
PGE_3 (Prostaglandin E_3)
$PGF_{2\alpha}$ (Prostaglandin $F_{2\alpha}$)
PGPC (1-palmitoyl-2-glutaroyl- sn-glycero-3-phosphocholine)
PL (Phospholipids)
PLPC (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine)
PONPC (1-palmitoyl-2-(9-oxo)nonanoyl- sn-glycero-3-phosphocholine)
POP (PUFA oxidation product)

POVPC (1-palmitoyl-2-(5-oxovaleroyl)- sn-glycero-3-phosphocholine)

PPAR-α (Peroxisome proliferator-activated receptor alpha)

PUFA (Polyunsaturated fatty acid)

ROS (Reactive oxygen species)

SAPC (1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine)

SAzPC (1-stearoyl-2-azelaoyl- sn-glycero-3-phosphocholine)

SDA (Stearidonic acid)

SGPC (1-stearoyl-2-glutaroyl- sn-glycero-3-phosphocholine)

SLPC (1-stearoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine)

SMC (smooth muscle cells)

SONPC (1-stearoyl-2-(9-oxo)nonanoyl- sn-glycero-3-phosphocholine)

SOVPC (1-stearoyl-2-(5-oxovaleroyl)- sn-glycero-3-phosphocholine)

TXA_2 (Tromboxane A2)

UCP1 (Uncoupling protein 1)

VLDL (Very low-density lipoprotein)

WAT (White adipose tissue)

Introduction.

Cardiovascular disease (CVD) is the leading cause of mortality in many countries, accounting for 15.6 million deaths annually (Lozano *et al.*, 2012). Although several advances in the pharmacological and surgery proceedings alternatives have been achieved in the last years, the projected annual number of worldwide deaths from CVD in 2030 will be 23.6 million (Laslett *et al.*, 2012). Among many types, ischemic heart disease and stroke are the two more prevalent fatal events, being both associated to the atherosclerotic process (Lozano *et al.*, 2012).

Atherosclerosis, a chronic non-resolving inflammatory disorder, is the pathological process that underlies the CVD (Libby *et al.*, 2011; Kasikara *et al.*, 2018). The process starts early in life and usually manifests in the adulthood, as myocardium infarction, angina, stroke and other forms (Berenson and Srinivasan, 2003). In summary, the atherosclerosis genesis is associated to the infiltration of excess of low density lipoprotein (LDL) particles in the first layer of inflamed endothelium, where they will be oxidized by reactive species of oxygen and phagocyted by macrophages forming foam cells. This process stimulates the migration of smooth muscular cells from media to intima layer, leading to an increased production of extracellular matrix and, consequently, the formation of a fibrous cap that will alter the artery morphology (Hansson and Libby, 2006). The formed atherosclerotic plaque can suffer rupture, forming a thrombus that block the blood stream, causing an infarction or stroke (Libby, 2002). While the relation between oxidative stress and atherosclerosis is still under investigation, the implication of inflammation and cholesterol excess have been well stablished by clinical studies such as CANTOS and the 4S study respectively (the Scandinavian Simvastatin Survival Study (4S), 1994; Ridker, 2013).

Among the several risk factors involved in the atherosclerosis, the consumption of saturated and trans-fatty acids is one of the most important. Saturated and trans-fatty acids, present in dietetic fats, are able to increase the LDL concentration in the blood circulation by the reduction of LDL receptors synthesis in the liver and other tissues, and also to promote inflammation via Toll like receptor-4 pathway (TLR-4) (Mattson and Grundy, 1985; Mensink and Katan, 1992; Simopoulos, 1999; Lee *et al.*, 2001; Suganami *et al.*, 2007). Epidemiological studies have showed the high correlation

between the saturated and trans-fatty acids consume and CVD risk (Willett *et al.*, 1993; Oomen *et al.*, 2001; Hunter *et al.*, 2010). For this reason, international food agencies and health guidelines have strongly recommended the replacement of saturated and trans-fatty acids by mono and polyunsaturated fatty acids (Amine *et al.*, 2002; Mendis *et al.*, 2005; Alwan, 2011). The problem is that monosaturated fatty acids are present just in the very expensive oils, such as olive oil and high oleic vegetal oils, while polyunsaturated fatty acids (PUFA) are extremely susceptible to oxidative damage (Frankel, 2005; Spiteller, 2005; Kanner, 2007).

Oxidation is a normal reaction that occurs when PUFA present in the oils are exposed to oxygen, light, heat and transition metals. As consequence, the PUFA form some secondary compounds that present an unpleasant odor and potential toxicity (Esterbauer, 1993; Frankel, 2005; Awada *et al.*, 2012; Böttcher *et al.*, 2015). When highly oxidized, the aggressive odor often associated to “solvents” and “fish” inhibits the oils consume. For this reason, there is just one reported case of human intoxication caused by the intake of oxidized fried noodles in Japan (Inagaki, 1966; Gotoh *et al.*, 2006). On the other side, secondary compounds are being formed since the beginning of oxidative reaction and there is no information if the chronic consume of this low amount of potentially toxic molecules could contribute to increase the oxidative stress and atherosclerosis progression. In fact, there is very scarce data about the oxidative condition of the oils and foods consumed by humans. Depending of the oil industry practices and also the way by which consumers prepare fried foods and stock the oils used in the culinary proceedings, the level of oxidation of the oils can be higher than the expected values. This fact, associated to the increase of PUFA consume stimulated by international agencies and health guidelines recommendation, has risen new scientific concerns about the quality of the oils, supplements and foods in terms of their oxidative stability, as well as the mechanism involved in the absorption and excretion of these potentially toxic secondary compounds (Esterbauer, 1993; Kubow, 1993; Staprans *et al.*, 1996; Spiteller, 2005). Some of these secondary products, such as 4-Hydroxy-nonenal (4-HNE) and malonaldehyde (MDA), form adducts with lysine present in the apolipoprotein B (ApoB) protein in the LDL particle, contributing to the recognition of these modified LDL by specific receptors in the macrophages

membrane, promoting atherosclerosis (Esterbauer *et al.*, 1992; Staprans *et al.*, 1993; Staprans *et al.*, 1994; Staprans *et al.*, 1996).

Omega 6 (n-6 FA) and omega 3 (n-3 FA) fatty acids represent two classes of PUFA with different effects in terms of *in vivo* inflammation and oxidative stress. While n-6 FA have been associated to a more inflammatory profile, several studies have reported the anti-inflammatory effect of n-3 FA. Actually, the beneficial effect attributed to n-3 FA would be a consequence of the enzymatic (COX, LOX) and non-enzymatic (ROS) oxidation of these molecules, usually represented by EPA and DHA present in marine oils (Calder, 2012b; Machado *et al.*, 2012; Calder, 2015).

Although the higher consume of n-3 and n-6 FA is already occurring in most of countries, there is scarce information about: (1) which are the specific secondary products of oxidation of the oils containing high amount of these two classes of fatty acids; and (2) the effect of chronic intake of products from n-6 and n-3 FA oxidation, at a level that does not inhibit their consumption, on atherosclerotic process. These information are extremely important, since it is necessary to identify specific chemical markers to follow the oxidative reaction according to the fatty acids precursors and it is also necessary to better know about the long time benefit involved in the replacement of saturated and trans-fatty acids by n-3 and n-6 FA, at the real conditions by which the oils are processed, commercialized and consumed.

Review.

Atherosclerosis

Atherosclerosis is a non-resolving chronic inflammatory disease of the arterial wall and the leading cause of death and loss of productive life years worldwide. It is characterized by the accumulation of lipids and fibrous elements in large arteries (Libby *et al.*, 2011; Kasikara *et al.*, 2018). In general, the inflammation is prevalent in regions where there is turbulence in the blood flow, such as the root of the aorta, aortic arch curvature, carotid bifurcation and others (Rezvan *et al.*, 2011). The high concentration of total cholesterol, triglycerides and LDL or now specifically denominated ApoB lipoproteins as well as the low concentration of high density lipoprotein (HDL) are considered important risk factors for coronary heart disease,

since the deposition of lipoproteins in the sub endothelial layer consists in a key step for onset and progression of atherogenesis (Stocker and Keaney, 2004; Houston *et al.*, 2009; Tabas *et al.*, 2015). The deposited ApoB particles in the sub endothelial react with the reactive oxygen species (ROS) produced by the vessel, forming oxidized particle of LDL (oxLDL). In the atherosclerotic process, oxLDL particles increase the expression of cell adhesion molecules (like vascular cell adhesion molecules-1 [VCAM-1], P- and E- selectins) on the endothelial cells, leading to leukocyte recruitment (mainly monocytes and T-lymphocytes) into the sub-endothelial space. The monocytes penetrate the intima endothelial monolayer (**Figure 1**), where they differentiate into macrophages that express scavenger receptors, such as CD36+, capable of recognizing and phagocyte oxLDL, forming foam cells (Rocha and Libby, 2009; Kattoor *et al.*, 2017).

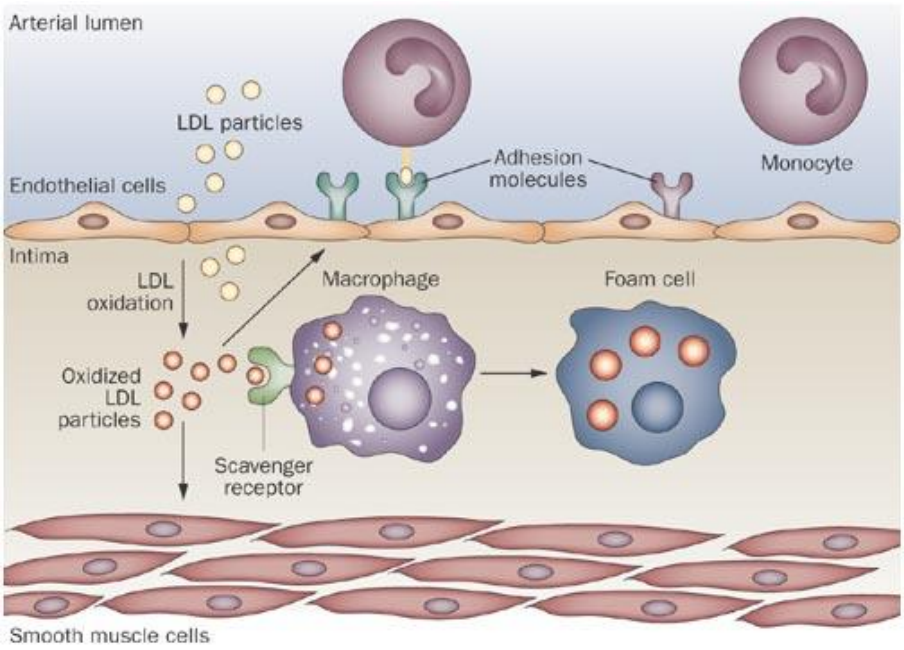

Figure 1: The excess of LDL particles circulating in the arterial lumen infiltrates the intima monolayer, where they undergo oxidation mediated by ROS. Once oxLDL, there is activation of adhesion molecules, internalization of monocytes that differentiate into macrophages and phagocyte the oxLDL particles forming foam cells, thereby initiating the formation of atherosclerotic plaque. Adapted from Rocha and Libby (2009).

The lipid accumulation and the increased release of inflammatory cytokines lead to histological changes, with considerable individual variability. The presence of foam cells in the intima contributes to increase the synthesis of inflammatory cytokines, causing an increase in the chemotaxis of leukocytes in the site, contributing to exacerbate the inflammatory response. In addition, smooth muscle cells migrate

from the media monolayer to the intima, leading to an increased production of extracellular matrix and, consequently, the formation of a fibrous cap (Libby *et al.*, 2011). Vascular smooth muscle cell can differentiate into macrophage-like cells in an environment full of cholesterol by the regulation of the transcipition factor Kruppel-like factor 4. These differentiated vascular smooth muscle cells also form foam cells when phagocyte oxidized lipids present in the atherosclerotic site (Chistiakov *et al.*, 2017). Thus, excess of LDL, inflammation and oxidative stress are conditions associated with the genesis and progression of the atherosclerotic process.

Polyunsaturated fatty acids and Atherosclerosis

PUFA are important constituents of phospholipids of cell membranes, where they play a role in regulating cell signaling, cellular function, gene expression and serve as substrate for the synthesis of lipid mediators (Calder, 2012b). The n-6 fatty acids (n-6 FA) are characterized by the presence of at least 2 carbon-carbon double bonds, with the first bond at the sixth carbon counted from the terminal methyl group. Linoleic acid (C18:2 n-6; LNA), the primary dietary n-6 FA, is found in higher concentration in vegetable oils such as corn, sunflower, safflower and soybean. LNA is converted to arachidonic acid (C20:4 n-6; ARA), which is the substrate for production of a wide variety of eicosanoids. Some are proinflammatory, vasoconstrictive, and/or proaggregatory, such as prostaglandin E_2 (PGE_2), thromboxane A_2 (TXA_2), and leukotriene B_4 (LTB_4). However, others are anti-inflammatory and/or antiaggregatory, such as prostacyclin (PGI_2), lipoxin A_4 (LXA_4) and epoxyeicosatrienoic acids, suggesting that n-6 FA are associated with inflammatory modulation (Harris *et al.*, 2009). The higher presence of n-6 FA in the diet leads a large amount of eicosanoids derived from ARA, specifically prostaglandins, thromboxanes, leukotrienes, which contribute to the development of inflammatory disorders, formation of thrombi and atheroma. Thus, a diet rich in n-6 FA can contribute to a prothrombotic and proaggregatory physiological condition, increasing blood viscosity and vasoconstriction (Simopoulos, 1999). Despite n-6 FA have characteristic of a proinflammatory fatty acids, clinical trials reviewed by (Al-Khudairy *et al.*, 2015) showed that the consumption of n-6 did not elevated the

blood pressure or increased the cholesterol levels in the studies, which make of n-6 FA a healthier source of FA than saturated FA.

The n-3 fatty acids (n-3 FA) are characterized by the presence of at least 3 carbon-carbon double bonds, being the first bond at the third carbon counted from the terminal methyl group. Major n-3 FA are alpha-linolenic acid (C18:3 n-3; ALA), stearidonic acid (C18:4 n-3; SDA), eicosapentaenoic acid (C20:5 n-3; EPA) and docosahexaenoic acid (C22:6 n-3; DHA). ALA can be found in plants such as flaxseed, chia, camelina; while SDA is naturally found in plants seeds such as *Echium*, hemp, blackcurrant and corn gromwell (Mozaffarian, 2005; Guil-Guerrero, 2007). The main sources of EPA and DHA are fish and algae oils, respectively (Geleijnse *et al.*, 2010). ALA can be converted to SDA, EPA and DHA (**Figure 2**), but this conversion is limited by desaturases and enlongases viability (Barceló-Coblijn and Murphy, 2009). Only 5% of ALA is converted to EPA (De Caterina, 2011). The initial conversion of ALA to SDA by the action of Δ6-desaturase is the rate limiting reaction of the pathway. The affinity of Δ6- desaturase for ALA is greater than for LNA, but the higher concentration of LNA in western diets results in a greater conversion to γ- linolenic acid (C18:3; GLA) and later to ARA (Burdge and Calder, 2005). SDA use to be considered a pro-EPA, because of the advantaged position of SDA with respect to ALA in the metabolic pathway that leads to EPA and DHA (Calder, 2012a) (**Figure 2**).

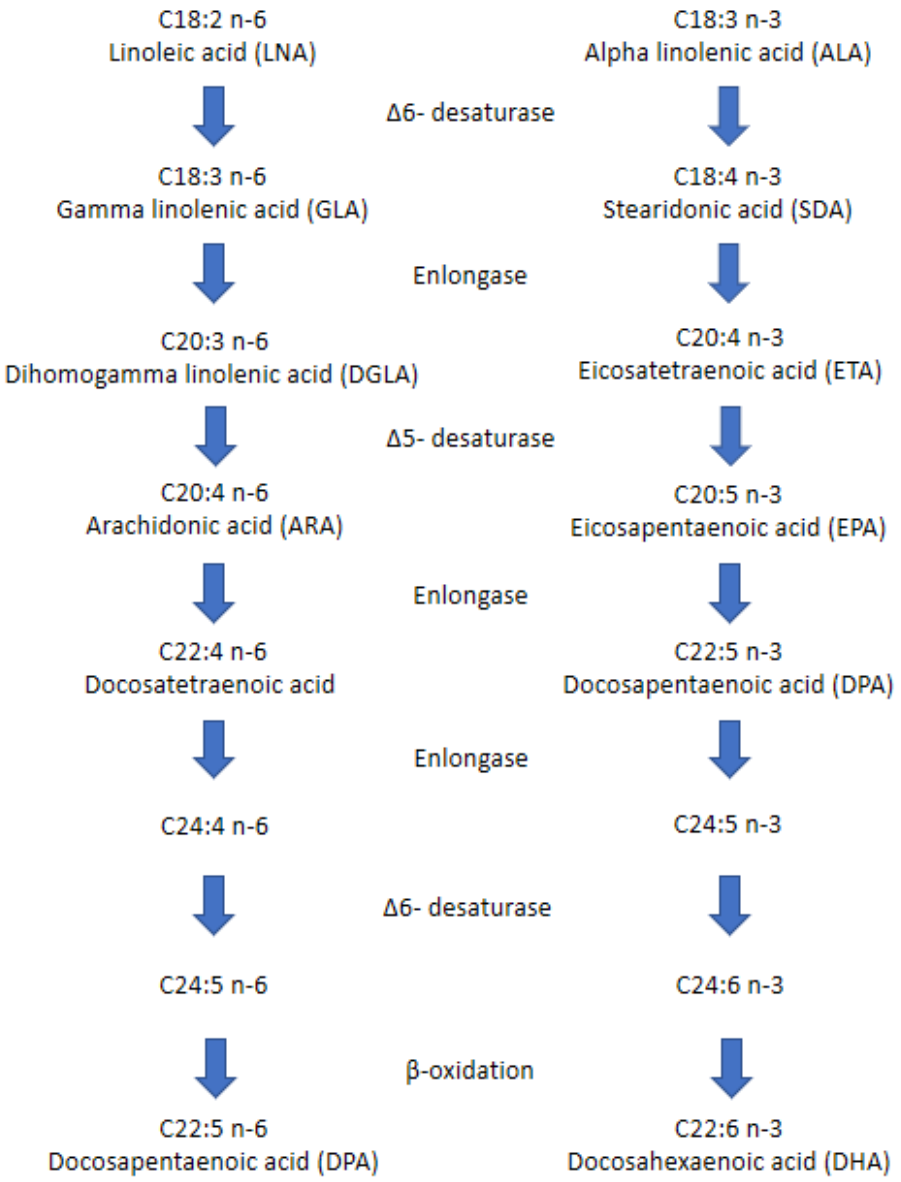

Figure 2: Elongation and desaturation of n-6 and n-3 FA. Adapted from Simopoulos (2008).

EPA and DHA are substrates for the production of a wide variety of lipid mediators (Calder, 2012a). EPA acts as an inhibitor of arachidonic acid metabolism via cyclooxygenase (COX) and lipoxygenase (LOX) enzymes and produces PGE_3 and LTB_5, that have a different structure from the arachidonic acid-derived mediators. Thus, eicosanoids derived from EPA are biologically less active than those produced from arachidonic acid (Calder, 2009). Another group of lipid mediator is the "resolvins and protectins". EPA and DHA give rise to resolvins (resolvin E_1 and resolvin D_1) and DHA to protectins (protectin D_1) through complex pathways involving COX and LOX enzymes

(Calder, 2009; 2012b). These mediators have been demonstrated to be anti-inflammatory and inflammation resolving, vital in limiting tissue damage (Calder, 2009; 2012b). N-3 FA not only act as an anti-inflammatory but also have a hypotriglyceridemic effect, promoting the increase of lipolysis and decreasing of lipogenesis (Mozaffarian,2011). The synthesis of eicosanoids is summarized in **Figure 3**.

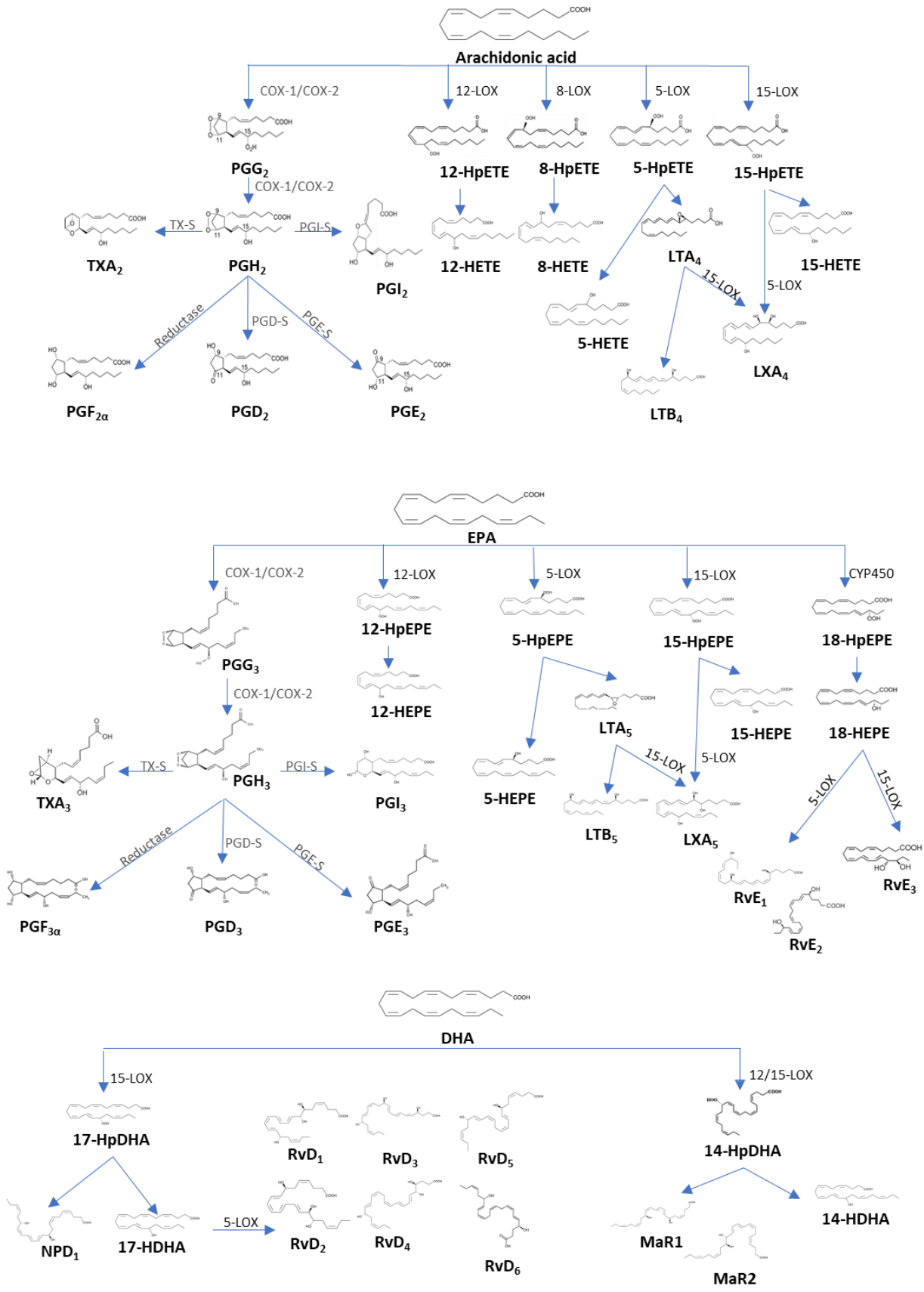

Figure 3: Formation of eicosanoids from ARA, EPA and DHA via COX and LOX. Abbreviations: PG. prostaglandins; TX, thromboxane; LT, leukotriene; LX, lipoxin; Rv, resolvins; MaR, maresin; NPD, neuroprotectin D. HETE, hydroxyeicosatetraenoic acid; HEPE, hydroxyicosapentaenoic acid ; Adapted from Layé *et al.* (2018)

EPA and DHA can activate the Nuclear factor erythroid 2-related factor 2 (Nrf2) that plays a major role in cellular response to oxidative stress. Nrf2 is present in aortic endothelial cells, where its activation inhibits inflammatory signaling (Majkova *et al.*, 2011). EPA and DHA may also reduce the expression and activity of NADPH oxidase (NOX) in the endothelium, resulting in a lower production of ROS (Balakumar and Taneja, 2012). Besides enzymatic PUFA oxidation, n-3 FA and n-6 FA are also substrate to non-enzymatic *in vivo* oxidation mediated by ROS. The products resulting from this reaction, such as Isoprostanes (IsoP), are associated with many diseases, including atherosclerosis (Morrow and Roberts, 1997; Galano *et al.*, 2013). Different isomers can be formed according to the fatty acid precursor. For example, LNA forms F_{15}-isoprostanes, ALA can form F_1-phytoprostanes, while EPA is able to form F_3-isoprostanes and DHA F_4-isoprostanes, also known as neuroprostanes (Galano *et al.*, 2013). Although they are considered the "gold biomarker" for *in vivo* oxidative stress, their physiological effects are still being investigated.

Oxidative susceptibility of PUFA

The susceptibility of PUFA to oxidation increases according to the number of double bounds present in the fatty acid chain (Gray, 1978). Therefore, the presence of a double bond between two carbon atoms in the fatty acid chain favors the abstraction of the methylenic hydrogen by the reactive species, due to the lower energy required to take the hydrogen from this position (Frankel, 2005). In summary, lipid oxidation can be described in three steps (**Figure 4**), known as initiation, propagation and termination.

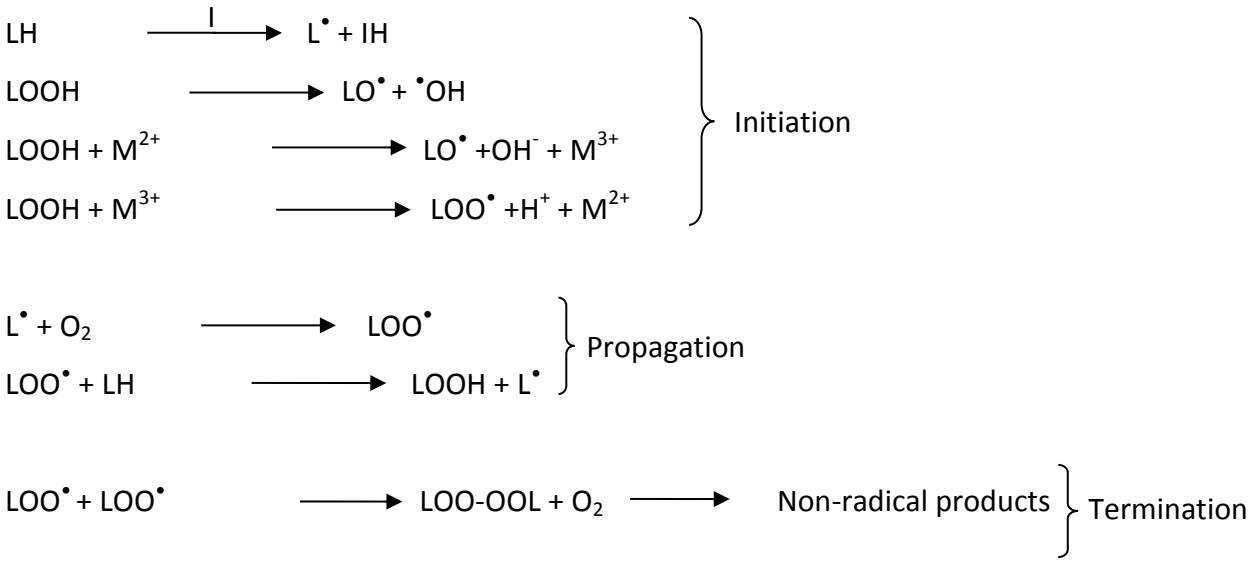

Figure 4: Steps of lipid oxidation. I: initiator (reactive species), LH: fatty acid, L •: fatty acid having the carbon centered radical; $LOO^{\bullet}$ /$LO^{\bullet}$: peroxyl radical and alkoxyl radical, LOOH: hydroperoxide, M: metals. Adapted from Frankel (2005).

The oxidation of PUFA can occur by different mechanisms being autoxidation, photooxidation and thermoxidation the most common. The oxidation of lipids does not only produce undesirable flavors in foods, but it can also decrease their nutritional quality and safety by the formation of secondary products. The PUFA oxidation products (POPs) can be formed during processing, transportation, storage and after the use of these oils to cook or to fry foods (Frankel, 2014).

POPs differ according to the fatty acid precursor and the type of inductor (Frankel, 2005). For example, linoleic acid (LNA) oxidation produces a mixture of four hydroperoxides during autoxidation (*cis, trans*-9-OOH; *trans,trans*-9-OOH; *cis,trans*-13-OOH and *trans,trans*-13-OOH) and other four hydroperoxides from photooxidation (*trans,cis*-9-OOH; *cis,trans*-13-OOH; *trans,cis*-10-OOH and *cis,trans*-12-OOH). At the same way, linolenic acid (ALA) oxidation produces a mixture of four hydroperoxides during autoxidation (trans,*cis,cis*-9-OOH; *cis,trans,cis*-13-OOH; *cis,trans,cis*-12-OOH and *cis,cis,trans*-16-OOH) and other six hydroperoxides from photooxidation (*trans,cis,cis*-9OOH; trans,*cis,cis*-10-OOH; *cis,trans,cis*-12-OOH; *cis,trans,cis*-13-OOH; *cis,cis,trans*-15-OOH and *cis,cis,trans*-16-OOH) (Esterbauer, 1993; Frankel, 2005). The decomposition of these hydroperoxides can form different secondary products such as

epoxyhydroxy, epoxyhydroperoxy, ketones and aldehydes, being most of them volatile (Frankel, 2014). Thus, the profile of the products found after the PUFA oxidation strongly depends on the fatty acids composition and the type of induction.

Some of those volatile compounds are associated not only with unpleasant odor in foods but also can cause potential adverse health effects (Esterbauer, 1993; Frankel, 2005). For example, an important product of LNA oxidation is the 4-HNE derived from the decomposition of 9-OOH and 13-OOH (**Figure 5**). 4-HNE has been identified in vegetable oils heated at frying temperatures and reported as cytotoxic. 4-HNE has also been found in oxLDL, the lipoprotein associated to atherosclerosis genesis and progression (Esterbauer, 1993; Frankel, 2005; 2014).

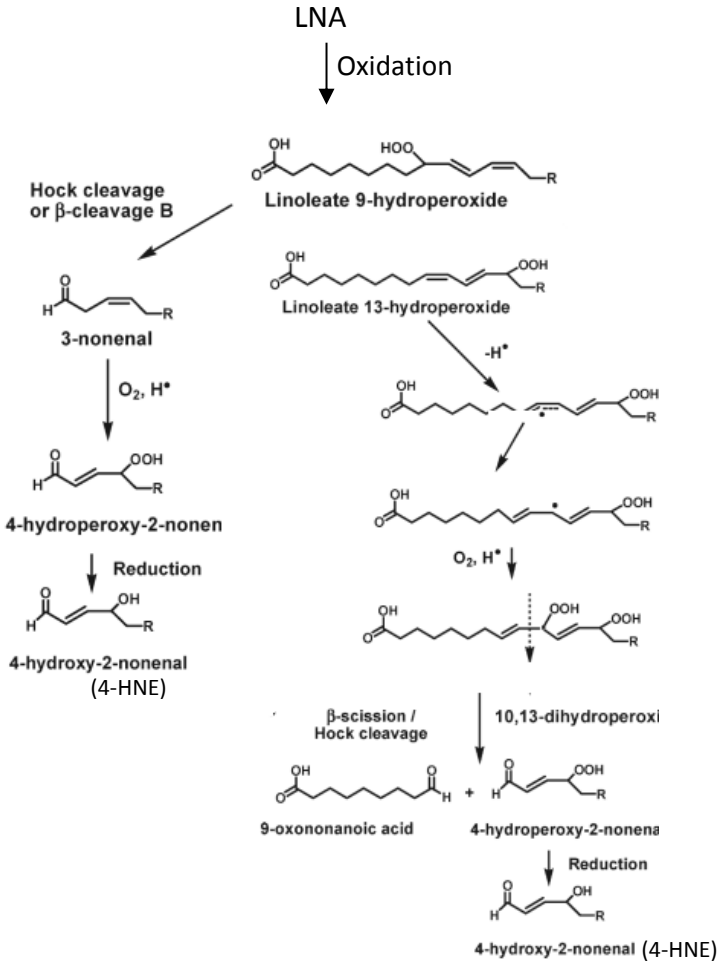

Figure 5: 4-HNE formation from hydroperoxide of linoleic acid. Adapted from Frankel (2005).

Another example of an important product of fatty acid oxidation is the MDA, that is formed by the oxidation of any PUFA containing more than two double bounds

(**Figure 6**) (Frankel, 2014). MDA can be formed by the physiological metabolism of the PUFA as a side product by enzymatic processes during the biosynthesis of thromboxane A2 (Ayala *et al.*, 2014) and has been associated with several diseases such as cancer, preeclampsia, diabetes and cardiovascular disease. Furthermore, MDA is highly mutagenic and also has been considered a biomarker of oxidative stress (Del Rio *et al.*, 2005).

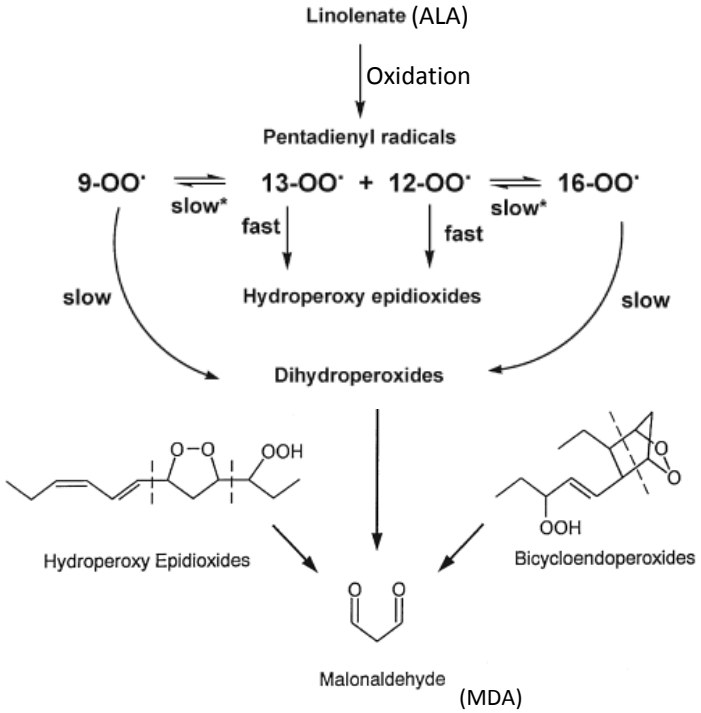

Figure 6: Malondialdehyde (MDA) formation from hydroperoxide of linolenic acid. Adapted from Frankel (2005).

The cleavage of hydroperoxides from EPA and DHA oxidation produces complex mixtures of volatile compounds, responsible by unpleasant off-flavors, that includes heptenal, 2-hexenal, 2,4-decadienal, decatrienal and 3-hexen-1-ol (Frankel, 2005; 2014). The *in vivo* effect of those volatiles compounds is still unknown.

After intake, the metabolism of POPs follows the same pathway of PUFA metabolism (Staprans *et al.*, 1996). Both are absorbed by the enterocytes and are carried by the chylomicrons via thoracic duct to the lymph and plasma until be metabolized in the liver. Some POPs, such as 4-HNE and MDA, can be conjugated with reduced glutathione (GSH) and excreted via renal, which decreases the concentration of GSH, potentially contributing to increase the oxidative stress (Staprans *et al.*, 1994; Staprans *et al.*, 1996; Staprans *et al.*, 2000). Actually, the excretion of POPs follows the

same physiological mechanism involved in the intoxication caused by drugs such as paracetamol (Zhao and Pickering, 2011). α,β-unsaturated aldehydes have different mechanisms to regulate their concentration including the action of phase I and phase II metabolic mechanisms. Phase I metabolism of aldehydes involves aldehyde dehydrogenases (ALDHs) that generate carboxylic acids by oxidative reactions. Aldoketo reductases (AKR) can also be involved in the metabolism of aldehydes, generating alcohols through reduction reactions instead of carboxylic acids (**Figure 7**). Phase II metabolism of reactive aldehydes is carried out by enzymatic glutathionylation by glutathione-S- transferases (GSTs), and the product of this last reaction also undergoes to reaction with ALDHs or AKRs (Moghe *et al.*, 2015; Rodríguez-Zavala *et al.*, 2019)(**Figure 7**).

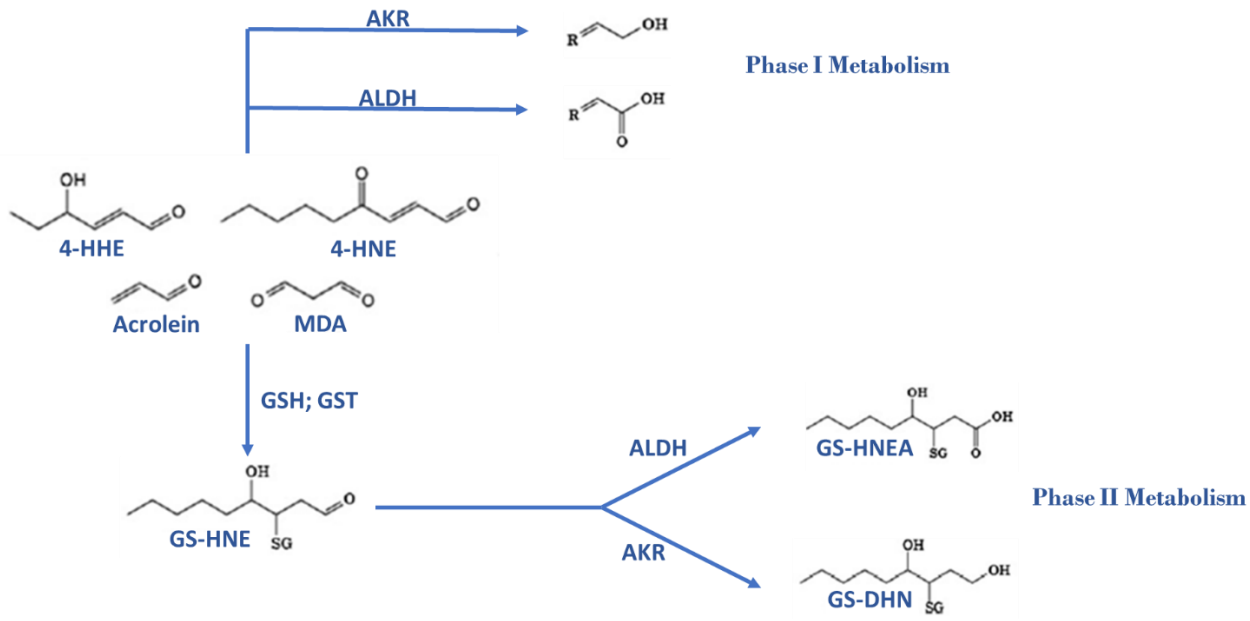

Figure 7: Aldehydes metabolization. Abbreviations: ALDH, aldehyde dehydrogenases; AKR, aldoketo reductases; GST, glutathione-S- transferases; GSH, glutathione. Adapted from Hauck and Bernlohr (2016).

Aldehydes that do not react with GSH or that were not metabolized by ALDH are incorporated into VLDL. The particles of VLDL containing POPs in their composition are converted to LDL. Taking part of LDL, 4-HNE and MDA may react with lysine in ApoB, modifying their structure and generating modified LDL, also called oxidized LDL (oxLDL) (Brown and Goldstein, 1983; Staprans *et al.*, 1996). The oxLDL is not recognized by the LDL receptors but binds to scavenger receptors present in the

macrophages surface, being phagocytized producing foam cells, that contribute to the atherosclerotic process (Levitan *et al.*, 2010; Itabe, 2012; Iwata and Nagai, 2012).

Unsaturation of a fatty acid chain is a major determinant of the fluidity of biological membranes that present a bilayer of phospholipids (PLs). Glycerophospholipids comprise an abundant class of lipids consisting of a glycerol backbone, phosphate-containing polar head group and two fatty acid residues (Bochkov *et al.*, 2010). Phosphatidylcholine (PC) is the main phospholipid in all mammalian cells (40–50%) and thus, most oxidized phospholipids detected in mammalian tissues (Fruhwirth *et al.*, 2007). The phospholipids usually present a saturated fatty acid in the sn-1 position that is either linked to an acyl residue via an ester bond or an alkyl residue via an ether bond, whereas the sn-2 position almost exclusively contains acyl residues, where the highly oxidizable (n-3 and n-6 FA) are preferably bound. Thus, most of the oxidized phospholipids are modified at this position (Catalá, 2009).

The formation of oxidized phospholipids is initiated either by enzymes, such as LOX, or by ROS. The oxidation of sn-2 position leads to formation of hundreds of structurally diverse PL species, with several biological activities depending on the structure and type of cell or tissue. During pathophysiological conditions, such as atherosclerosis, inflammation and cancer, oxidized phospholipids (OxPL) has been found in higher concentration (Leitinger, 2003; Catalá, 2009; Khandelia and Mouritsen, 2009). OxPL are known to act directly on activation of platelet aggregation, monocyte adhesion and inflammatory signaling as cytokine generation and neutrophil superoxide release. The oxidation of the fatty acid in sn-2 position can also alter membrane structure and physical properties, including bilayer packing and phase separation (O'Donnell, 2011) . When the polyunsaturated sn-2 residue is oxidized, the volatile and soluble fragments, such as MDA, 4-HNE and acrolein are separated from the fatty acid while the proximal oxidation fragment is retained as a new sn-2 residue (Mcintyre *et al.*, 1999).

The four most abundant species of phospholipids that contain PUFA are 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine (PLPC), 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine (SLPC), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidyllcholine (PAPC) and 1-stearoyl-2-arachidonoyl-sn-glycero-3-

phosphatidylcholine (SAPC) (Milne *et al.*, 2005). The oxidation of PL occurs by several manners, forming fragmented or non-fragmented OxPL. The fragmentation of OxPL sn-2 arachidonic acid or linoleic acid occurs via mechanisms including β-scission, Hock rearrangement or by cyclization of alkoxy radical produced from hydroperoxide. The fragment bound in the PL is classified as truncated unsaturated or saturated OxPLs (Bochkov *et al.*, 2010) (**Figure 8**).

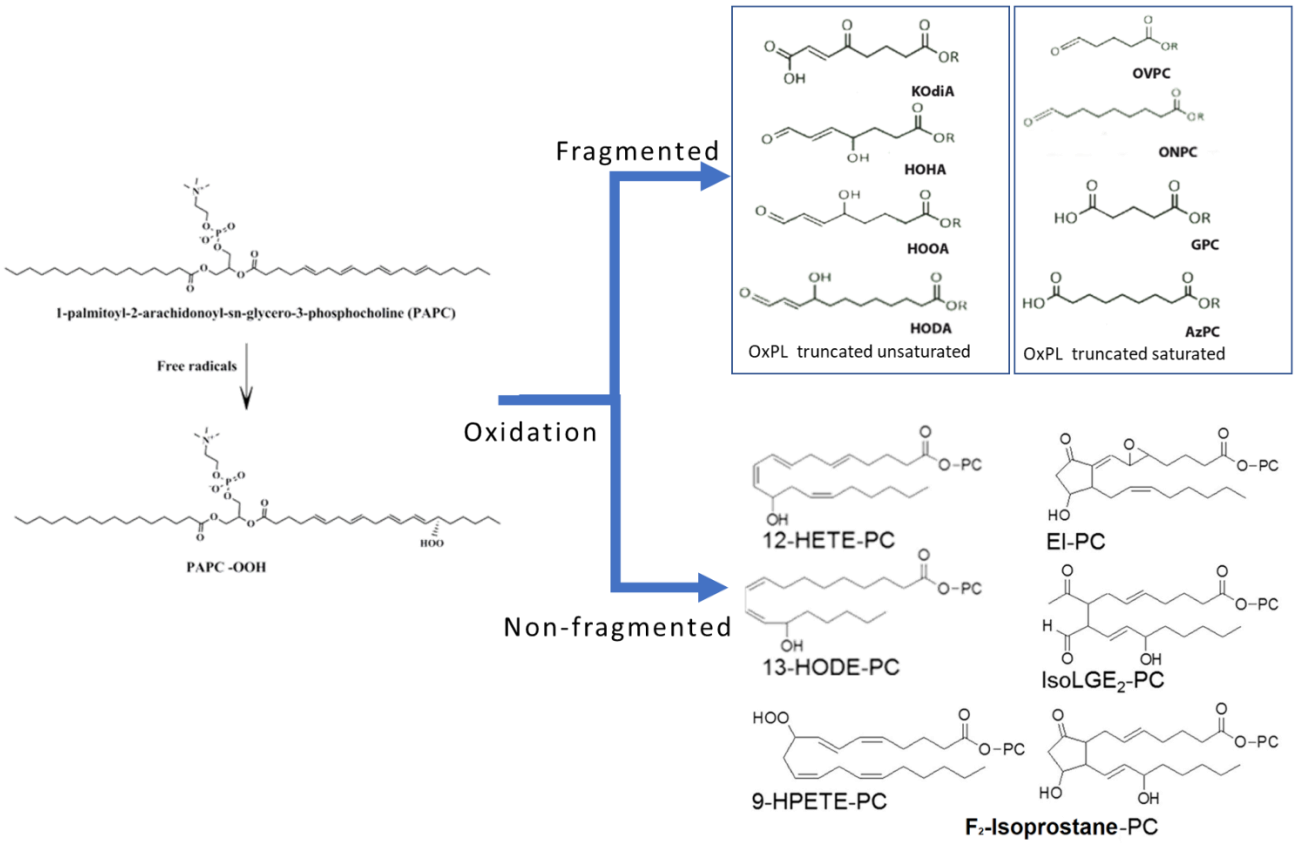

Figure 8: Formation of oxidized phospholipids. Adapted from Hasanally *et al.* (2014).

The principal truncated unsaturated OxPLs are the 9-hydroxy-12-oxododec-10-enoyl-PC (HODA-PC), 5-hydroxy-8-oxooct-6-enoyl-PC (HOOA-PC), 4-hydroxy-7-oxohept-5-enoyl-PC (HOHA-PC) and 7-carboxy-5-oxo-hept-6E-enoyl-PC (KOdiA-PC) (Gao *et al.*, 2006; Bochkov *et al.*, 2010). They are characterized for presenting a similar structure to 4-HNE. Based on this similar structure, the hydroxy alkenal-PC forms covalent adducts with cysteine and lysine residues of proteins, which alters the amino acids function. The hydroxy alkenal-PC are known to induce chemokines and monocyte binding and to have a high affinity to the scavenger receptor CD36 (Furnkranz and

Leitinger, 2004). The oxidative fragmentation also produces truncated saturated species containing terminal carbonyl groups. Most common species are oxononanoate (PONPC or SONPC) and azelaoate (PAzPC or SAzPC) formed from linoleic acid, oxovaleroate (POVPC or SOVPC), and glutaroate (PGPC or SGPC) generated from arachidonic acid (Bochkov *et al.*, 2010) (**Figure 8**) . POVPC is one of the most bioactive OxPL. It was reported that POVPC can switch the phenotype of smooth muscle cells (SMCs) *in vivo* to an inflammatory state by enhancing the rate of cellular proliferation and increasing the synthesis of extracellular matrix proteins (Deigner and Hermetter, 2008). POVPC and PGPC cause suppression of smooth muscle actin and myosin heavy chain expression and increase the expression of cytokines MCP-1 and MCP-3. POVPC and PGPC also induce apoptosis and inhibition of vascular SMCs growth while PAz-PC seems to exert a direct effect on mitochondria, causing a rapid swelling of these organelles, leading to apoptosome formation (Pidkovka Nataliya *et al.*, 2007).

The non-fragmented OxPL are formed when hydroperoxides derived from fatty acids with three or more double bound on the sn-2 position undergo cyclization, followed by rearrangements yielding bicyclic endoperoxide, or further oxidation with additional formation of noncyclic or cyclic peroxide groups. The bicyclic endoperoxide is a precursor of IsoPs, isothromboxanes and isolevuglandins (IsoLG), whereas rearrangement and further oxidation of molecules containing one cyclic and one noncyclic peroxide groups produces isofurans (IsoFs) (Bochkov *et al.*, 2010). IsoLG are produced mainly in PL-esterfied form, and rapidly form covalent complexes with proteins. The adducts formed are found in oxidized LDL and inflamed tissues and they are suggested as integral markers of oxidative stress due to their long half-life in circulation (Bochkov *et al.*, 2010). Bicyclic endoperoxides spontaneously converts to isothromboxane A2, which undergo further transformation to IsoTxB2, that are strongly elevated during oxidative stress (Bochkov *et al.*, 2010).

The first discovered class of IsoPs was analogous to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). For this reason they were named F_2-IsoPs (Morrow and Roberts, 1991). The F_2-IsoPs are prostaglandin (PG) like compounds formed from arachidonic acid peroxidation and have been shown to be a reliable biomarker of endogenous lipid peroxidation, since they are chemically stable in biological samples such as plasma, urine and tissues (Milne *et al.*, 2005).

Four F_2-IsoP regioisomers are formed from the oxidation of arachidonic acid (**Figure 9**). Each one is composed of 8 racemic diastereomers, in a total of 64 compounds (Milne *et al.*, 2015). The nomenclature of the four classes is based on the carbon number on which the side chain hydroxyl group is attached, considering the carboxyl carbon as the first one (Taber *et al.*, 1997).

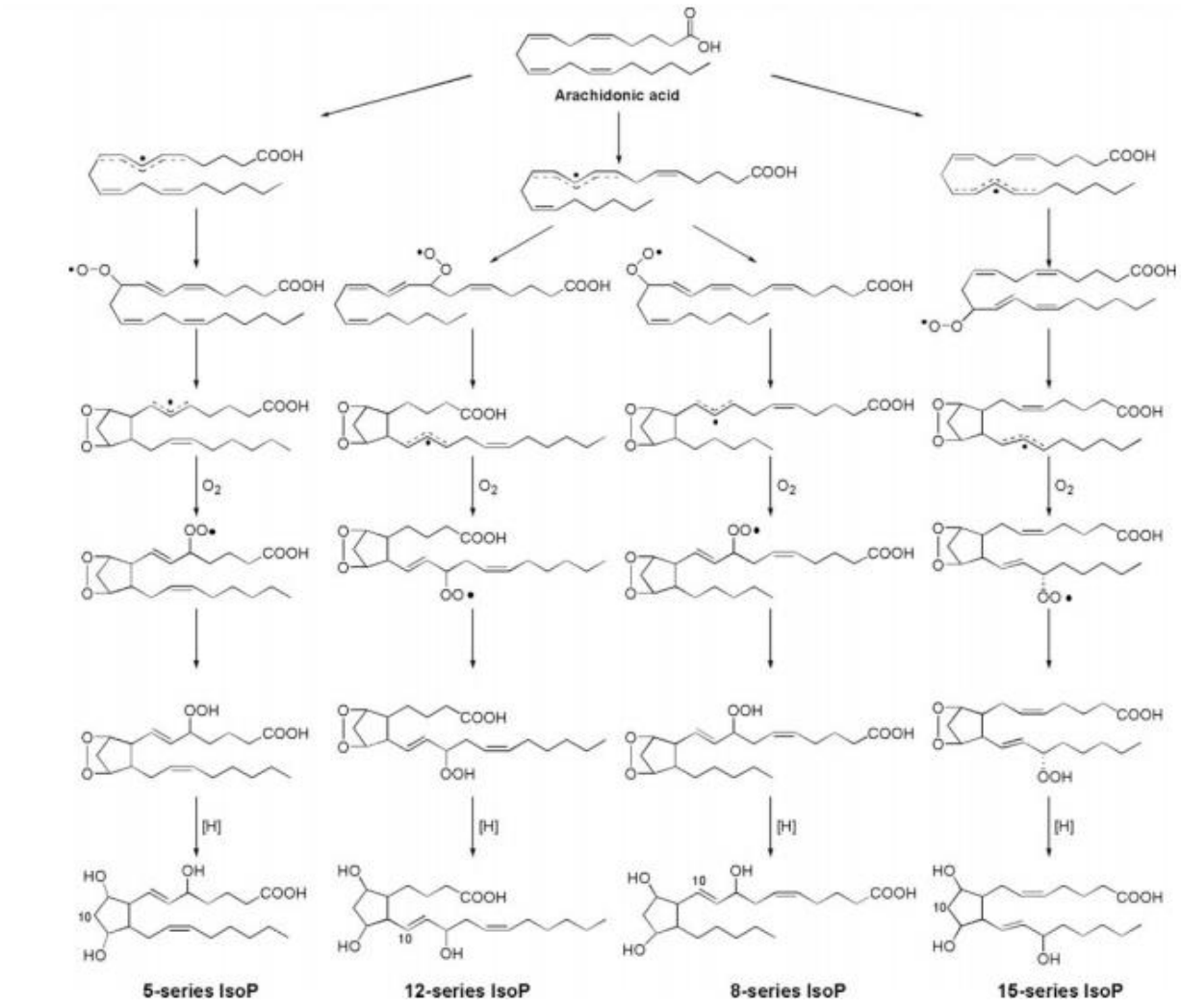

Figure 9. Mechanism of formation of F_2-IsoPs from the peroxidation of arachidonic acid. Adapted from Milne et al. (2015).

The 5- and 15-series regioisomers accumulate in biological fluids and tissues more than the 8- and 12-series regioisomers, due to the fact that 5- and 15-series F_2-IsoPs cannot undergo further oxidation (Fruhwirth *et al.*, 2007). In fact, the most commonly measured is 15-F_{2t}-IsoP. 15-F_{2t}-IsoP is commonly referred as 8-iso-$PGF_{2\alpha}$ because it only differs from $PGF_{2\alpha}$ in the inversion of the upper side chain stereochemistry at the C-8 position (Milne *et al.*, 2005). The biological activity of 8-iso-

$PGF_{2\alpha}$ has been more studied than any other F_2-IsoP isomer because it is a potent vasoconstrictor, induces endothelin release and activates proliferation of vascular SMCs (Milne *et al.*, 2008). Importantly, some studies reported that 8-iso-$PGF_{2\alpha}$ can be also formed from enzymatic oxidation of arachidonic acid via the cyclooxygenase (COX) (Van't Erve *et al.*, 2016).

The measurement of F_2-IsoPs to assess oxidant stress is unquestionable (Roberts and Fessel, 2004); however, as O_2 tension increases, the formation of IsoPs is disfavored whereas the formation of IsoFs is favored. IsoFs, that have a substituted tetrahydrofuran ring, are formed when O_2 reacts with the carbon radical in the pathway of IsoP formation. There are eight different regioisomers of IsoFs and each one can exist as 16 racemic diastereomers. Thus, 256 enantiomerically IsoFs could be formed (Fessel *et al.*, 2002). IsoF formation is associated with pathophysiological states of oxidative stress and mitochondrial dysfunction (Patel *et al.*, 2008).

PUFA oxidation products (POPs) and Obesity

Obesity is defined as a disease in which excess body fat has accumulated such that health may be affected (Kopelman, 2000). Obesity is accompanied by molecular changes in adipose tissue that includes the increase of macrophage infiltration, increased stress in the endoplasmic reticulum and mitochondrial dysfunction. The increased oxidative stress results in elevated protein carbonylation, promoting the development of insulin resistance (Long *et al.*, 2013).

White adipose tissue (WAT) provides a long-term fuel reserve, which can be activated during food deprivation. The size of adipose tissue stores increases in periods when the amount of calories intake is higher than the energy expenditure and declines when energy expenditure is in excess of intake (Trayhurn and Beattie, 2001). Different from the WAT, the brown adipose tissue (BAT) is the most studied mediator of the non-shivering thermogenesis involving uncoupling protein-1 (UCP1) (Nedergaard *et al.*, 2001; Kozak and Anunciado-Koza, 2009). UCP1 is located in the inner mitochondrial membrane (Pedersen *et al.*, 2001) and increases the conductance that makes mitochondria generate heat rather than ATP (Fedorenko *et al.*, 2012). There is almost no expression of UCP1 in WAT, however it has been observed the expression of UCP1

in some WAT in response to cold exposure or β3-adrenergic receptor agonist, such as epinephrine, norepinephrine and dopamine (Kozak, 2010; Knudsen *et al.*, 2014). More recently was found that hormones, enzymes and lipid oxidation products can also stimulate the UCP1 expression in WAT, suggesting that expression of BAT genes in WAT may shift WAT metabolism from an energy storage into an energy-disposal site, "browning" some WAT depots (Carrière *et al.*, 2014). Browning is associated with a protective effect against obesity, as the depletion of UCP1 induced obesity in a high-fat model (Feldmann *et al.*, 2009).

Echtay *et al.* (2005) demonstrated that 4-HNE and other compounds with similar molecular structures such as trans-2-nonenal, trans-2-nonenoic acid, trans-retinoic acid and trans-retinal were able to induce the uncouple proteins UCP1, UCP2 and UCP3 expression (**Figure 10**).

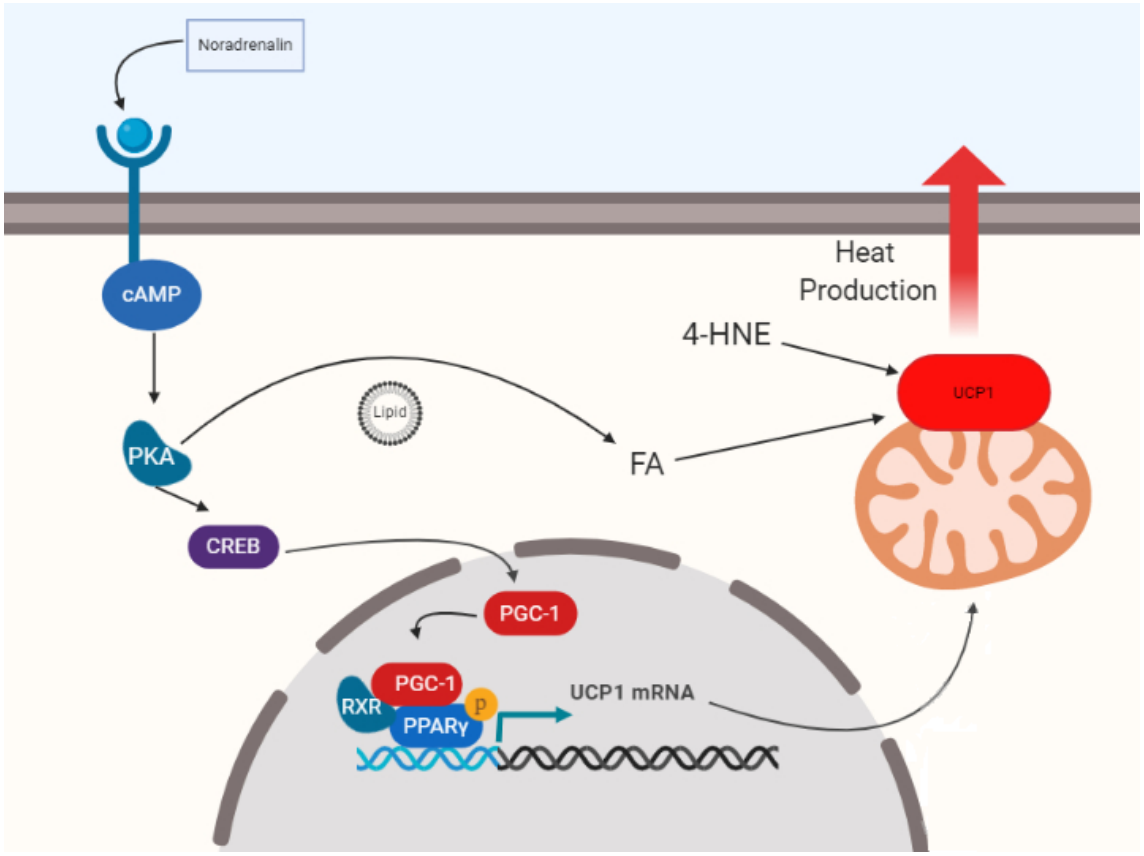

Figure 10: During thermogenesis, UCP1 synthesis is stimulated by transcription factors of the PKA pathways. These pathways also induce lipolysis, which generates fatty acid (FA) ligands that activate UCP1. 4-HNE can also directly activate UCP1. Abbreviations: CREB, cAMP response element binding; FA, fatty acid; PPAR, peroxisome proliferator-activated receptor; 4-HNE, 4-hydroxynonenal. Adapted from Azzu and Brand (2010)

Papa and Skulachev (1997) suggested that 4-HNE exerts a physiologic signal, decreasing mitochondrial production of ROS. In addition, 4-HNE accumulation in adipose tissues contributes to obesity-related lipolytic activation. The role of 4- HNE on impairment of adipocyte differentiation was shown in 3T3L1 murine preadipocytes, where short-term as well as repeated exposure of these cells to physiological concentrations of 4-HNE, promoted subsequent oxidative stress and impaired adipogenesis. 4-HNE also incresed the expression of adipokines, lipolytic gene expression and free fatty acids release. These effects were suggested to be mediated by a decrease in aldehyde dehydrogenase 2 activity (Dasuri *et al.*, 2013).

PUFA oxidation products (POPs) and atherosclerosis

The intake of POPs and its effects in atherosclerosis are still a controversial topic. Some authors have shown that the consumption of POPs has an anti-atherosclerotic effect (Chao *et al.*, 2001; Sülzle *et al.*, 2004; Ringseis *et al.*, 2007; Kämmerer *et al.*, 2011), while others reported an atherosclerotic effect (Staprans *et al.*, 1993; Staprans *et al.*, 1994; Staprans *et al.*, 1998; Awada *et al.*, 2012).

Yin *et al.* (2007) reported that the same POPs generated *in vitro* can be detected *in vivo*. Esterbauer (1993) demonstrated that heavily oxidized oils given orally are not acutely toxic, because di- and polymeric- POPs are not well absorbed in the intestine, and therefore do not reach the bloodstream. Some studies have shown a reduction in the absorption of lipids and cholesterol after eating oxidized fatty acids (Chao *et al.*, 2001; Sülzle *et al.*, 2004; Ringseis *et al.*, 2007; Kämmerer *et al.*, 2011). These studies showed that POPs, especially hydroxides and hydroperoxides, trigger peroxisome proliferator-activated receptors alpha (PPAR-α) in the rat liver (Chao *et al.*, 2001; Sülzle *et al.*, 2004; Ringseis *et al.*, 2007; Kämmerer *et al.*, 2011). PPAR-α are a nuclear receptor that regulates physiological functions such as β-oxidation of fatty acids, ketogenesis and gluconeogenesis. In the liver, its function is related to protection against hepatic steatosis, by favoring the β-oxidation (Peyrou *et al.*, 2012). Garelnabi *et al.* (2008) reported that intake of POPs results in atherosclerosis when the animals are fed a high cholesterol diet, but when fed in the absence of cholesterol, mice appeared to have lower triglyceride levels.

Other studies have shown that diets containing POPs can cause oxidative damage in pancreatic islets and impairment of insulin secretion (Chiang *et al.*, 2011). Huang *et al.* (2014) showed that during pregnancy, maternal ingestion of POPs, modulates PPARα, altering the metabolism of retinoic acid, resulting in teratogenesis. Most of these studies was performed using oils with high levels of oxidation (Staprans *et al.*, 1998; Chao *et al.*, 2001; Sülzle *et al.*, 2004; Ringseis *et al.*, 2007; Kämmerer *et al.*, 2011; Awada *et al.*, 2012). In addition, the administration was usually carried out via intragastric, which cannot simulate the human oral intake.

In summary, PUFA are being applied as a healthy alternative to replace saturated and trans fatty acids in the oils and foods. However, PUFA are very susceptible to oxidation forming products potentially atherogenic. High level of oxidation is not a problem because the unpleasant odor of the secondary products of oxidation usually inhibits the oil intake. If consumed, the POPs absorption rate in the intestine is low. But there is no agreement about the long-term intake of partially n-3 or n-6 FA POPs on atherosclerosis process. Based on growing consumption of PUFA and consequently POPs, it is important to investigate if there is a difference between n-6 and n-3 FA in terms of oxidative stress and its consequences towards atherosclerosis. Thus, based on the more pro-inflammatory characteristics of POPs derived from n-6 FA than n-3 FA, our hypothesis is that moderate oxidized n-6 but not n-3 FA would lead to a pro-atherogenic condition, because some of the benefic effects attributed to n-3 FA involves their enzymatic and non-enzymatic oxidation.

Hypothesis

The hypothesis of this study is schematized in **Figure 11.**

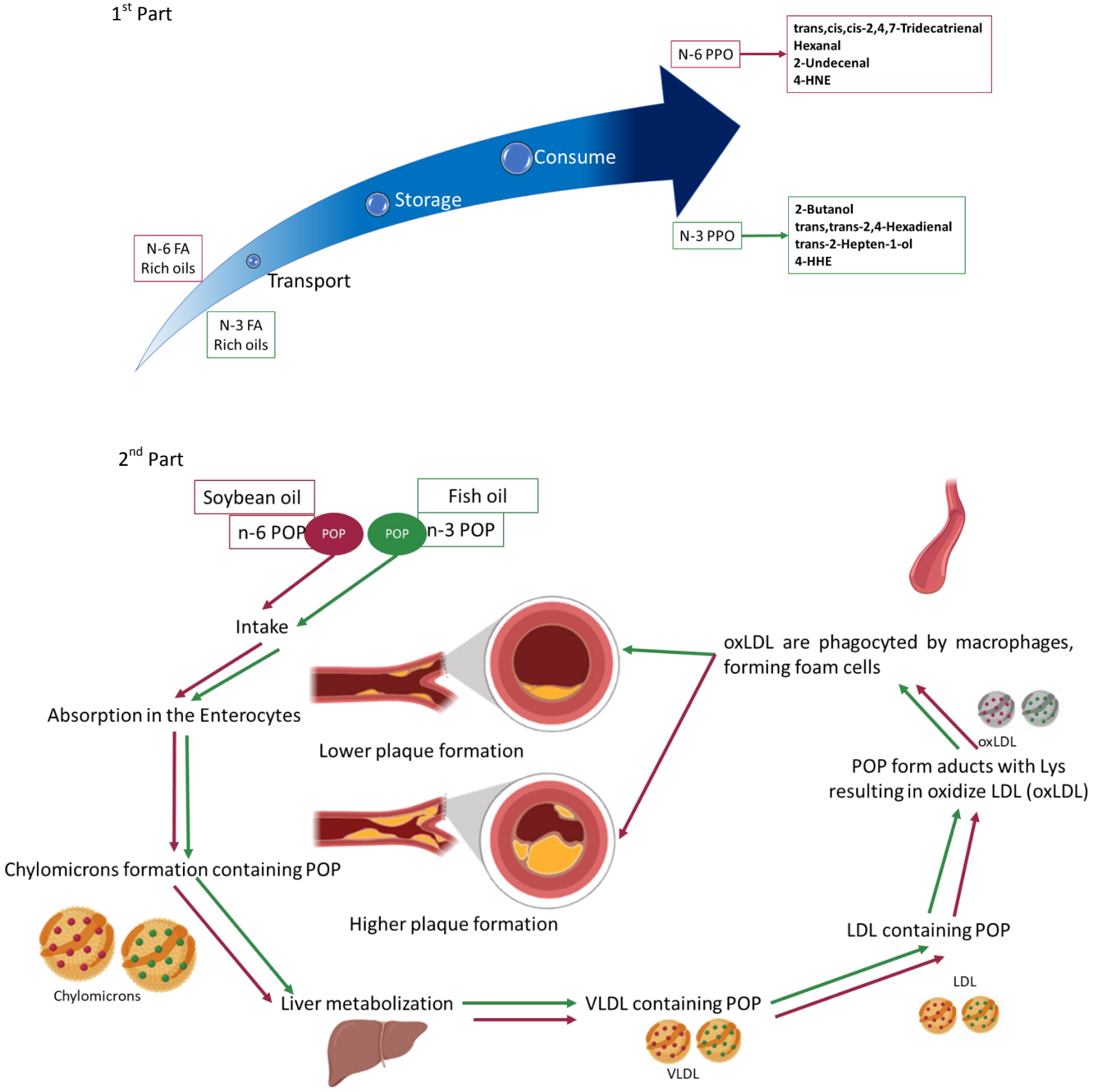

Figure 11: Hypothesis of the study.

Objective

The main objective of this study was to evaluate the effect of long-term consumption of partially oxidized n-6 and n-3 FA on atherosclerosis in LDLr$^{(-/-)}$ mice.

Description of chapters

In order to answer the main aim of this thesis, the study was divided into three parts with specific aims and the manuscripts of each part was presented as chapters. The first one was already published, the second one was submitted and the last one is still being discussed.

As it was already commented in the review section, PUFA is susceptible to oxidation and the main source of PUFA are the vegetable oils. The information about the oxidative stability of edible oils during their transport, storage and domestic consumption is scarce. Therefore, it was essential to analyze the oxidative stability of oils since their extraction until their consume and identify more specific oxidative markers for each type of oil. Thus, in the first chapter, six edible oils with different FA profile were selected, flaxseed oil (ALA rich oil), echium oil (SDA rich oil), fish oil (EPA rich oil) and algae oil (DHA rich oil) were selected as n-3 FA rich oils and soybean oil and grapeseed oil (LNA rich oils) were selected as n-6 rich oils in this project. The six oils were submitted to an experimental design that mimicked the process that oils undergo during transport (48 hours, 50°C, in the dark) storage (30 days, 25°C, in the dark) and finally the consume stage (30 days, 25°C, in the light and opened bottles). The results were compared with the standard oxidation oil (15 days at 60°C in the light and opened bottles). The results were published in the LWT- Food Science and Technology, entitled “Oxidation products from omega-3 and omega-6 fatty acids during a simulated shelf life of edible oils” (Nogueira *et al.*, 2019).

After the analyzis of primary (hydroperoxides) and secondary (TBARS, MDA, p-Anisidine and volatile compounds) products of oxidation and tocopherol concentration in each stage of the process, it was possible to conclude that all oil samples showed increased oxidation since the first step (transport). In most of the oils, the oxidative markers exceeded recommended limits at the “consume” condition. Oxidation

products identified were specific to the oil and affected by minor components. The profile of oxidation products identified in the mildly oxidized conditions of consumption differed from fresh samples, as well as highly oxidized reference standards.

In addition to the published results, a “fried condition” was also included for all oils. The “fried condition” was chosen as a high level of lipid oxidation consumed by the population. The oil was produced in a model of “reused oil”, where fresh oil was heated to 180°C for one hour. After this time, frozen French fries were fried for 5 minutes with a 25 minutes interval, reestablishing the temperature to 180°C (Bansal *et al.*, 2010). The 5/25 cycle was repeated 4 more times, in a total of 5 cycles per day for 4 days. The oxidative markers after fried condition are shown at S**upplement 1** of this thesis. Volatiles and tocopherol standard curves besides other details are described in **Supplement 2** and **3** respectively.

Based on the results observed in Chapter One, soybean oil and fish oil were the oils rich in n-6 and n-3 FA, respectively, that showed the highest level of oxidation in the “consume condition” and were chosen as the oils to be used in the next steps of our study. In order to test the hypothesis of this thesis, a comparison between n-6 and n-3 in fresh (low oxidation level), consume (medium level of oxidation) and fried (high level of oxidation) conditions should be carried out. However, mice in the groups that received the atherogenic diet made with 20% of fish oil refused to eat, causing the interruption of the study for those groups, according to the Ethics Committee recommendation. Therefore, the second part of the project continued only with the groups that received the soybean oil.

In this new context, described in the second chapter, the aim was to evaluate the effect of long-term ingestion of different levels of partially oxidized soybean oil on atherosclerosis. Thus, 3 months old male LDL receptor $^{(-/-)}$ mice were fed for 6 months with an atherogenic diet containing 20% oil, 8.75% lard and 1.25% cholesterol. The oils that composed the diet were: fresh (LOW), consume (MED) or fried (HIGH) soybean oil, that were oxidized as described in Chapter One. A Control group that received a standard diet (4% fresh soybean oil) was also included in the study. The results are presented as a manuscript entitled “*Chronic intake of PUFA oxidation products alters body composition but has no effect on mice atherosclerosis.*” submitted to publication.

After 24 weeks of treatment, the group fed with fried oil (HIGH) showed an alteration in the body composition, presenting a low percentage of fat, similar to the percentage observed in the Control group. The fried group showed less lipid deposited in the adipose tissue and, consequently, the highest level of total cholesterol and all the lipoproteins cholesterol fractions in plasma. It would be expected that high level of cholesterol in plasma would cause a larger deposit of lipids in other tissues such as liver. However, no difference was observed in the steatosis score in the group that received the diet containing fried soybean oil when compared with the other groups that received an atherogenic diet. The Inflammatory biomarkers analyzed in the liver suggested that the inflammation seemed to be associated with higher body weight. Although groups received diets containing different levels of oil oxidation, no difference on the biomarkers of oxidative stress was observed among the groups. The absence of body weight gain observed in the fried group was investigated. It was observed a higher expression of UCP-1 in the WAT. As explained in the review, WAT only express UCP-1 when the tissue changes function and starts to present characteristics of BAT. In relation to the aortic plaque lesions, it was not observed any difference between the groups.

During the biological experiment, one more group designed as “positive control” was analyzed. This group received an atherogenic diet with the same composition of the other groups, with corn oil oxidized at 60°C for 15 days applied in the diet formulation, replacing the soybean oil. Even though this group also used an n-6 rich oil, the FA composition was quite different, which became difficult to include these data in the discussion. For this reason, data of the “positive control” was not include in the manuscript but, is presented in **Supplement 4.** In addition, other additional analyzes that were not included in the manuscript are presented in **Supplement 5.**

Due to the unexpected result occurred with the animals under fish oil supplementation, another protocol was carried out, to test the hypothesis that oxidized n-6 FA but not n-3 FA intake contributes to increase fatty streak lesion. For that, 3 months old male LDLr $^{(-/-)}$ mice received a high-fat high-cholesterol diet (20.75% lard, 1.25% cholesterol and 8.0% fresh soybean oil) for 12 weeks. Mice were split into 4 groups that received 20 µL of fresh or oxidized oil (consume condition from Chapter

One) by gavage once a day, 5 days/week. Two groups received fresh or oxidized oil soybean oil, while the other two groups received fresh or oxidized fish oil. The results are presented as a previous manuscript temporarily entitled ‘‘Interaction between the type of fatty acid and oxidation on biomarkers of atherosclerosis in LDLr$^{(-/-)}$ mice” to be submit to publication.

The results obtained in the Chapter One was not compared among the oils but, just in the same oil at different conditions. However, it is important to highlight that oxidized fish oil presented 1.5 times more hydroperoxides, 38 times more p-Anisidine, 13 times more MDA and 11 times more volatiles than oxidized soybean oil in the same condition.

After 3 months of treatment it was observed no difference in the body weight gain and in the body composition between the groups. However, the groups that received a supplementation with oxidized oil showed a higher retroperitoneal adipose tissue size, probably caused by an inflammatory signalization induced by the products of oxidation. The combination of products of oxidation and fish oil promoted a bigger liver and a higher glucose level in the plasma, associated with a higher β-oxidation activity. More activated mitochondria oxidize more fatty acids, consuming oxygen in this process, which leads to a lower oxygen tension in the tissue. This condition favored the formation of IsoP in the groups supplemented with fish oil. Fresh fish oil presented a higher concentration of IL-6 and SONPC, both related to hepatoprotective signalization, but these biomarkers were lower when fish oil was oxidized. As expected, mice supplemented with soybean oil showed higher levels of eicosanoids derived from arachidonic acid. When the aortas were analyzed, the histological results showed that products of oxidation of soybean or fish oil were not responsible to promote accelerate atherosclerotic lesion. In fact, the atherosclerotic plaques in the animals that received fish oil (with or without oxidation) were higher than those observed in the animals that received soybean oil.

During the biological experiment one more group was analyzed. This additional group received the same high-fat/high-cholesterol diet and 20 µL of medium chain triglycerides (MCT) as isocaloric control. MCT was selected as an oxidative control. However, as data were statistically treated by Factorial ANOVA (2^2), results from this

group were not included in this third manuscript, but they are presented in **Supplement 6**.

Conclusion

The efforts to replace trans and saturated FA to PUFA as a healthier source of fat, is increasing the consumption of lipids highly susceptible to oxidation. We demonstrated that levels of POPs found in the oils at the moment of their consumption were higher than the values recommended by the agencies. Population is been exposed to a small concentration of a number of potentially toxic POPs. This exposition is chronic and unavoidable. In an animal model of atherosclerosis, it was demonstrated that POPs and the type of FA oxidized alters biomarkers of inflammation, oxidative stress and can even modulate body composition. However, they were not able to alter atherosclerotic process, measured by the lesion area in the mice aorta.

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Chapter I

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