UNIVERSITY OF SÃO PAULO

Faculty of Pharmaceutical Sciences

Department of Food and Experimental Nutrition

LADAF – LABORATORY OF FUNCIONAL FOODS DEVELOPMENT

CLINICAL RELEVANCE OF OXIDATIVE STRESS BIOMARKERS IN CARDIOVASCULAR PREVENTION

Mariana Vieira de Mello Barros Pimentel

São Paulo

2022

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“Science progresses best when observations force us to change our preconceived ideas”

Vera Rubin

RESUMO

Pimentel, M.V.M.B. Relevância clínica de biomarcadores de estresse oxidativo na prevenção cardiovascular. 2022. 83f. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2022.

As doenças cardiovasculares envolvem hiperlipidemia, inflamação e estresse oxidativo. Embora essa relação esteja bem estabelecida, apenas biomarcadores associados à hiperlipidemia e inflamação são atuais na prática clínica para diagnóstico e avaliação do tratamento do paciente. Nossa hipótese é que biomarcadores de estresse oxidativo podem ser um fator de risco independente e podem auxiliar na estratificação de risco cardiovascular e contribuir para melhorar os escores atuais. Assim, o objetivo deste estudo foi investigar primeiramente quais são os biomarcadores e metodologias utilizados nos estudos clínicos em humanos em diferentes condições de saúde. Com os resultados obtidos na primeira etapa, selecionamos os estudos conduzidos em indivíduos saudáveis e em prevenção cardiovascular primária e secundária a fim de avaliar os biomarcadores mais utilizados, os resultados obtidos conforme o perfil do indivíduo e a metodologia utilizada e finalmente correlacionar com as diferentes condições de saúde. Observamos que o malondialdeído (MDA) foi o biomarcador lipídico de estresse oxidativo mais frequente nos estudos, porém apresentou importante variabilidade nos resultados e fraca correlação com desfechos clínicos. O resultado desse estudo demonstra a importância da realização de um estudo multicentrico para validação dos valores de MDA nos diferentes perfis de indivíduos e a padronização metodológica baseada na cromatografia líquida de alta eficiência (HPLC).

Palavras-chave: estresse oxidativo, biomarcadores, estratificação de risco, aterosclerose.

ABSTRACT

Pimentel, M.V.M.B. Clinical relevance of oxidative stress biomarkers in cardiovascular prevention. 2022. 83f. Dissertation (Master) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2022.

Cardiovascular diseases involve hyperlipidemia, inflammation and oxidative stress. Although this relationship is well established, only biomarkers associated with hyperlipidemia and inflammation are currently in clinical practice for diagnosis and evaluation of patient treatment. Our hypothesis is that oxidative stress biomarkers may be an independent risk factor and may assist in cardiovascular risk stratification and contribute to improving current scores. Thus, the objective of this study was to investigate which are the biomarkers and methodologies were used in clinical studies in humans with different health conditions. With the results obtained in the first part, we selected studies conducted in healthy individuals and in individuals under primary and secondary cardiovascular prevention in order to evaluate the most frequent biomarkers, the results obtained according to the individual's profile and the methodology used, and correlate with different health conditions. We observed that malondialdehyde (MDA) was the most frequent lipid biomarker of oxidative stress applied in the studies, but it presented significant variability in the results and a weak correlation with clinical outcomes. The result of this study demonstrates the importance of carrying out a multicentric study to validate the MDA values in individuals with different health conditions and the standardization of the methodology based on high performance liquid chromatographyy (HPLC).

Keywords: oxidative stress, biomarker, risk stratification, atherosclerosis

ABBREVIATIONS

AA – Arachidonic acid

ACAT – Acyl-coenzyme A cholesterol ester transferase

ACC - American College of Cardiology

AGES - Advanced glycation end products

AHA -American Heart Association

ALA - α-linolenic acid

ApoB - Apolipoprotein B

ASCVD- Atherosclerotic cardiovascular disease

BH4 - Tetrahydrobiopterin

BMI- Body mass index

CAD - Coronary artery disease

CAT - Catalase

CAVs - Calcific aortic valve stenosis

CHIP- Clonal aemotopoiesis of indeterminate potential

COX – Cyclooxygenase

CVD - Cardiovascular diseases

DAMP - Damage-associated molecular pattern

DHA – Docosahexanoic acid

DM - Diabetes mellitus

DNPH - Dinitrophenylhydrazine

DPA – Docosapentaenoic acid

eNOS - Endothelial nitric oxide synthase

EPA – Eicosapentaenoic acid

ETC – Eletron transpost chain

F2-IsoP – F2 -Isoprostanes

FRS - Framingham Risk Score

GC/MS - Gas chromatography/mass spectrometry

GLA - γ-linolenic acid

GPx - Glutathione peroxidase

GR – Glutathione redutase

GSH – Glutathiona

HDL – High density lipoprotein

HNE - 4-Hydroxynonenal

HPLC - High performance liquid chromatography

hsCRP - High sensitivity C- reactive protein

IsoPs - Isoprostanes

LC/MS- Liquid chromatography/mass spectrometry

LDL - Low-density lipoprotein

LNA – Linoleic acid

LOX -1 - Lectin-like oxLDL

LOX- Lipoxygenases

MDA – Malondialdehyde

MI - Myocardial infarction

MMPs - Metalloproteinase matrix

MPO – Myeloperoxidase

NADPH - Nicotinamide adenine dinucleotide phosphate oxidase

NCDs – Non- communicable diseases

NCEP- National Cholesterol Education Program

NICE- National Institute for Health and Care Excellence.

NIH – National Institute of Health

NO – Oxide Nitric

Nrf2 - Nuclear factor erythroid-2-related factor 2

oxLDL - Oxidized LDL

oxPLs – Oxidized phospholipids

PAF-AH – Platelet activating factor acetyl-hydrolase

PAMP - Pathogen associated molecular patterns

PC - Phosphocholine

PG- Prostaglandins

PLs- Phospholipids

PUFAs- Polyunsaturated fatty acids

PVD - Peripheral vascular disease

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

SBP - Systolic blood preassure

SCORE - Systematic Coronary Risk Evaluation

SMCs - Smooth muscle cells

SOD - Superoxide dismutase

TAC – Total Antioxidant Capacity

TBA - Thiobarbituric acid

TC - Total cholesterol

TG- Triacylglycerol

TLR4- Tool-like receptor 4

TxA2 - Thromboxane A2

WHO – World Health Organization

XO - Xanthine oxidase

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SUMMARY

1. LITERATURE REVIEW

Cardiovascular diseases (CVD) are the leading cause of mortality in the world, with their ischemic forms, such as myocardium infarction and stroke, being the most prevalent events (WHO, 2020). Atherosclerosis is a chronic inflammatory condition that begins early in life and progresses through adolescence and early adulthood, responsible for the ischemic forms of CVD (WHO, 2007). The beginning and progression of atherosclerosis are associated with the development of chronic non-communicable diseases (NCDs) such as hypertension, obesity, diabetes and dyslipidemia (WHO, 2007). Dyslipidemia is characterized by increased levels of plasmatic lipids, including an increased concentration of total cholesterol (TC), triglycerides (TG) and low-density lipoprotein (LDL) (BIBBINS-DOMINGO et al., 2016). It may occur due to inadequate lifestyle and diet or genetic origin (FALUDI et al., 2017).

The excess of LDL promotes increased permeability of blood vessels, allowing the internalization of these particles in the intima layer (HURTUBISE et al., 2016; HUANG et al., 2019). The LDL present in the subendothelial layer undergoes oxidation by reactive oxygen species (ROS) becoming oxidized LDL (oxLDL). The immune system cells recognize oxLDL as an antigen and releases cytokines and chemokines to recruit monocytes that differentiate into macrophages and phagocytize these oxLDL particles. Macrophages containing oxLDL become foam cells, triggering an immune-inflammatory process (HURTUBISE et al., 2016). Inflammatory cytokines and growth factors are secreted and smooth muscle cells proliferate and migrate to the intima layer, forming a fibrous plaque. This process generates more ROS, promoting cellular apoptosis, inflammation and oxidative stress, creating a vicious circle. Thus, atherosclerosis involves dyslipidemia, inflammation and oxidative stress condition (Figure 1) (HURTUBISE et al., 2016; BRITES et al., 2017).

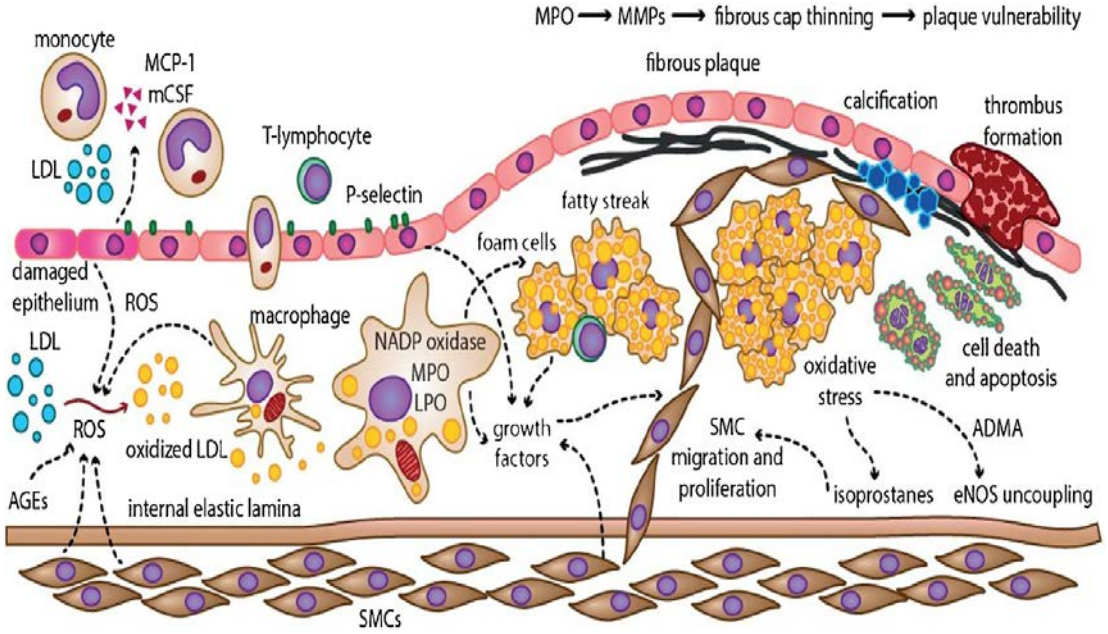

Figure 1: Development and progression of atherosclerosis. Adapted from TIBAUT et al. (2019). The internalization of LDL to the intimal layer of the artery promotes endothelial damage and activates the immune system. Reactive oxygen species and chemokines are released to attract monocytes that will differentiate into macrophages. Foam cells are formed, being inflammation and ROS generation intensifed. Growth factors are secreted to recruit vascular smooth muscle cells in order to contain the proliferation of foam cells, thus forming the fibrous cap, which, if ruptured, leads to thrombus formation. **Legend:** ADMA: asymmetric dimethylarginine; AGEs: advanced glycation end-products; eNOS: endothelial nitric oxide synthase; LDL: low-density lipoproteins; LPO: lipid peroxide; mCSF: macrophage colony-stimulating factor; MCP-1: monocyte chemoattractant protein-1, MMPs: metalloproteinases; MPO: myeloperoxidase; NADP: nicotinamide adenine dinucleotide phosphate; ROS: reactive oxygen species; SMCs: smooth muscle cells.

1.1 THE ROLE OF OXIDATIVE STRESS AND INFLAMMATION IN THE ATHEROGENESIS

Reactive species are molecules that contain one or more unpaired electrons. The main physiological sources of reactive species are the respiratory chain and the immune system. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in an organism during physiological or physiopathological conditions (YANG et al., 2008; TIBAUT et al., 2019). The imbalance between the lower antioxidant capacity of the organism and the higher oxidizing capacity of ROS and RNS was defined by SIES (1991) as "*a disturbance in the prooxidant – antioxidant balance in favor of the former, leading to potential damage*" (SIES, 1997; YANG et al., 2017). This status leads to the disruption of ROS and RNS signaling as biological messengers, resulting in irreversible cellular changes and damage in macromolecules such as DNA, protein and lipids (MÜNZEL et al., 2017; YANG et al., 2017; NIKI, 2018). Oxidative stress is involved in the development and progression of several disfunctions such as neurological, some types of cancer, metabolic and cardiovascular diseases (FRIJHOFF et al., 2015). In addition, oxidative stress influences all stages of atherosclerosis,

since the beginning of arterial injury until the plaque rupture (HURTUBISE et al., 2016; KATTOOR et al., 2017).

The role of oxidative stress on atherosclerosis was investigated by SACKS et al. (1978), who verified *in vitro* model that ROS produced by immune system cells promoted endothelial damage (SACKS et al., 1978; HEISTAD, 2006). Initially, it was only thought that immune cells were sources of ROS in the endothelium (HEISTAD, 2006). However, with the progress of research, it was discovered that the vascular environment had important enzymatic sources of ROS such the uncoupled endothelial synthase (eNOS) present in blood vessels, besides immune system enzymes as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, lipoxygenases and myeloperoxidases (NIKI, 2014; KATTOOR et al., 2017)

ROS also regulate multiple functions of vascular cells (MÜNZEL et al., 2017), including vasoconstriction, that is associated to the increase of blood pressure and initial damage in the arterial walls (HEISTAD, 2006; KATTOOR et al., 2017). Oxidative stress is also involved in the immune response, endothelial damage, oxidation of LDL and induces the release of the metalloproteinases (MMPs) that degrades the fibrous wall of the atheroma plaque, resulting in plaque disruption in the final stage of the ischemic diseases (Figure 2) (HURTUBISE et al., 2016; KATTOOR et al., 2017).

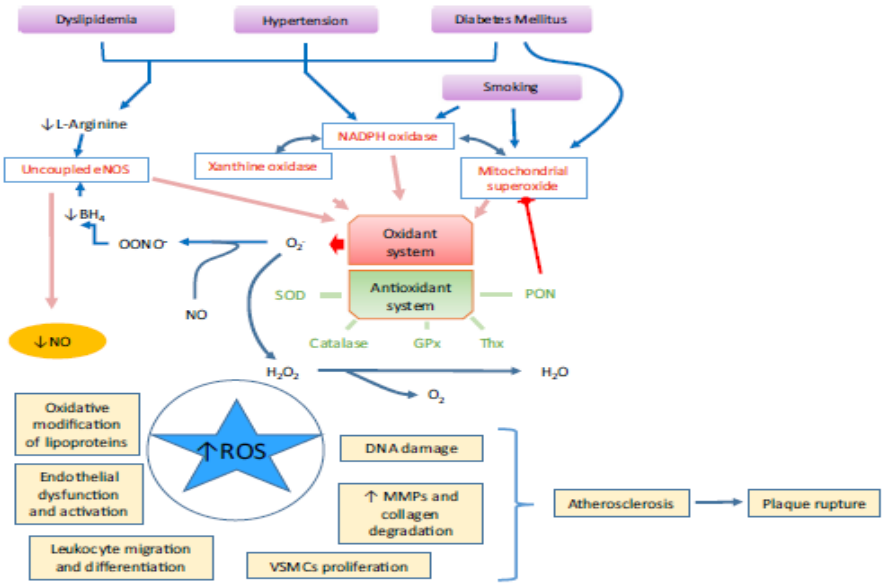

Figure 2: Different cardiovascular risk factors affect the generation of ROS in various stages of atherosclerosis. Adpated from KATTOOR et al. (2017). Legend: PON: paraoxonases, BH4: tetrahydrobiopterin; SOD: superoxido dismutase; GPx: gluthathione peroxidase; $OONO^-$: peroxynitrite; NO: oxide nitric; $O_2^{\bullet-}$: superoxide anion; H_2O_2: hydrogen peroxide

Inflammation is equally present in all stages of atherogenesis. This relation became more evident after studies conducted by Poole and Florey in 1958 in hypercholesterolemic rabbits, showing the mechanism of cell adhesion in the endothelium, mediated by inflammatory chemokines, during the formation of atherosclerotic plaque (POOLE & FLOREY, 1958; LIBBY, 2012).

Studies about inflammation in the atherosclerotic process have evolved over the years, showing the relevance of using biomarkers, such as high sensitivity C- reactive protein (hsCRP) (LIBBY, 2012). The Cantos study showed how reducing inflammation could be effective in preventing cardiac events. RIDKER et al. (2008) used an inflammatory biomarker, high sensitivity Creactive protein (hsCRP), to screening patients without hyperlipidemia and found that the reduction of inflammation was associated with a decrease in the incidence of cardiovascular events.

These findings demonstrate that inflammation is an independent risk factor for atherosclerosis (LIBBY, 2012; SHAH; LECIS, 2019), leading the inclusion of hsCRP as part of the risk stratification scores for cardiovascular prevention (FALUDI et al., 2017; ARNETT et al., 2019).

1.2 CARDIOVASCULAR PREVENTION AND GUIDELINES.

Cardiovascular prevention can be divided into primary or secondary in order to identify groups of indivuduals with greater or less cardiovascular risk (WHO, 2007). Primary prevention corresponds to individuals, who have cardiovascular risk fators and have risk of atherosclerotic cardiovascular disease (CVD) of 10 years, estimated by algorithmic tools recommended in guidelines (ARNETT et al., 2019). Secondary prevention is for individuals with clinical cardiovascular disease manifestation, as coronary heart disease and others atherosclerotic diseases, such as vascular diseases, including peripheral artery disease, atherosclerotic aortic disease, and carotid artery disease, who must control risk factor to increase lifespan, reduce the chance of recurrent cardiovascular events and need of interventions (SMITH et al., 2007; ARNETT et al., 2019).

Guidelines for prevention and treatment of cardiovascular disease are constantly updated due the specificity of the population in each country (GARG et al., 2017). Recommendations vary according to cardiovascular risk, which can be calculated using distinct algorithms such as Framingham Risk Score, CVD risk score, Reynolds risk scores, SCORE

(Systematic Coronary Risk Evaluation) and others **(Table 1)** (ARNETT et al., 2019; FALUDI et al. 2017).

Table 1: Cardiovascular Risk Scores Systems and Guidelines

MODELS	VARIABLES	GUIDELINES
Framingham Risk Score and its updates .	Age, gender, TC, HDL, SBP, DM, hypertension medication, smoking and vascular disease (CAD, PVD, Stroke)	Brazilian guideline on dyslipidemias and prevention of aerosclerosis - 2017; ACC/AHA Guideline 2019; NCEP guidelines, Canadian CV guidelines
SCORE	Age, gender, TC, TC/HDL, SBP and smoking. Versions avaible for use in high and low-risk countries.	ACC/AHA Guideline 2019; Brazilian Guideline 2017; European guideline on CVD Prevention
UK National Health Service (QRISK2; QRISK3)	Age, gender, TC/HDL ratio, SBP, BMI race, smoking, DM, family history, chronic kidney disease, atrial fibrilation, migraines, rheumatoid arthritis, lupus, mental disease and medication use (antipsychotic, steroid and erectile disfunction).	NICE guidelines on lipid modification QRISK Lifetime recommended by JBS3 guidelines
Reynold´s score	Age, gender, TC, HDL, SBP, smoking, hsCRP and family history.	ACC/AHA Guideline 2019
Joint British Societie (JBS3)	Age, gender, race, smoking, DM, family history, atrial fibrilation, rheumatoid arthritis, Cholesterol/HDL ratio, SBP and BMI.	ACC/AHA Guideline 2019
Pooled Cohort Equations CV Risk calculator ACC/AHA	Age, gender, race, TC, HDL, SBP, anthypertensive and lipid lowering treatment, diabetes and smoking.	ACC/AHA Guideline 2019

Legend: TC= total cholesterol; HDL= high-density lipoprotein cholesterol; SBP= Systolic blood preassure; DM= diabetes mellitus; CAD= coronary artery disease; PVD: peripheral vascular disease; BMI= body mass index; ACC= American College of Cardiology; AHA= American Heart Association; NCEP= National Cholesterol Education Program; NICE= National Institute for Health and Care Excellence. Adapted from: European Guidelines on cardiovascular disease prevention in clinical practice 2016; Prevention Guideline ACC/AHA 2019; Brazilian guideline on dyslipidemias and prevention of aerosclerosis - 2017.

These algorithms use predictive criteria as variables for estimating cardiovascular risk in 10 years. Given the variety, the guidelines define which is the best procedure according to the patient's profile (FALUDI et al., 2017; ARNETT et al., 2019, ECKARDSTEIN, 2016). After of applying the algorithm, the patient is classified as low, intermediate, high and very high risk (WHO, 2007; FALUDI et al., 2017; ARNETT et al., 2019).

However, depending on age and gender, these criteria have different impact on cardiovascular risk, except for LDL and HDL levels, that have the tendency of increasing the impact over the time (LIND et al.; 2018; MANFRINI; CENKO; BUGIARDINI, 2020). In

some cases, the ACC/AHA suggests complementary tests such as hsCRP, lipoprotein A and apolipoprotein B to enhance risk stratification (ARNETT et al., 2019).

Depending on risk classification, interventions may occur toward lifestyle, such as diet, physical activity and smoking or prescripting antihypertensive, hypoglycemic and hypolipidemic drugs (WHO, 2007; FALUDI et al., 2017). The relation between cardiovascular risk and lipid profile was strongly evidenced by the classical 4S Study in 1994, which showed a 30% reduction in the overall mortality of patients with coronary heart disease after statin prescription through a multicenter study (SCANDINAVIAN SIMVASTATIN SURVIVAL STUDY GROUP, 1994). Statins have been the drugs of choice for the treatment of hypercholesterolemia in primary and secondary prevention (FALUDI et al., 2017; ARNETT et al., 2019).

Despite the effectiveness of statins, some patients still have cardiovascular events. Studies conducted with statins reported that the treatment can reduce about 30% relative risk, leaving a residual risk above 60% (LINTON et al., 2019; KONES, 2013).

1.3 RESIDUAL RISK AND BIOMARKERS

Although scores and guidelines are very useful tools, it is not uncommon for treated patients to suffer a sudden attack with serious consequences (KONES, 2013). This condition has been attributed to a “residual risk” that is difficult to predict by the current scores (KONES, 2013; DHINDSA et al., 2020).

The classic components of CVD risk are dyslipidemia, hypertension and diabetes. However, it has been known that inflammatory, prothrombotic and metabolic pathways contribute to cardiovascular events (PRADHAN et al., 2018; DHINDSA et al., 2020). Among the multiple pathways, oxidative stress plays an important role (FUENTES et al., 2019; MARCHIO et al., 2019). ROS mediate platelet aggregation by inhibiting nitric oxide synthesis, promote lipoperoxidation that activates the cyclooxygenase-1 (COX1) pathway and, consequently, the formation of TxA2. This relationship between ROS and platelet activation may explain why some patients do not respond to treatments with antiplatelet agents like acetyl salicylic acid (BECATTI et al. 2013, MARCHIO et al. 2019; FUENTES et al. 2019). In this sense, biomarkers involved in lipid peroxidation such as isoprostanes (Isop) and malondialdehyde (MDA) could be considered risk factors for residual risk (TSIKAS, 2017; FUENTES et al., 2019).

In addition, other biomarkers, such as hsCRP, have been included as routine analysis (ARNETT et al., 2019). Nonetheless, the high level of cardiovascular events and the evidence that other metabolic pathways are involved in CVD implies the investigation of other biomarkers (DHINDSA et al., 2020), in special, in the early stages of the disease, when more options of interventions are still viable for the patients, like diet changes and antioxidant therapies (SENONER; DICHTL, 2019).

Based on the well-established association among lipid oxidation, inflammation, immune system, lipid profile and atherosclerosis, oxidative stress biomarkers could be strong candidates to improve the predictive capacity of the current scores for risk stratification (FRIJHOFF et al., 2015). However, several factors have contributed to delay this investigation, including the lack of reference values determined in different populations, the large variation of the values even when determined using similar methods, and the use of non-validated methodologies based on different designs (NIKI, 2014; FRIJHOFF et al., 2015).

Despite oxidative stress being physiologically associated with the development and progression of atherosclerosis, none of the evaluated guidelines recommends oxidative stress biomarkers evaluation to improve patient risk stratification.

1.4 OXIDATIVE STRESS BIOMARKERS

Several studies on oxidative stress biomarkers and redox signaling have been published aiming to better understand the oxi-reduction physiological processes (NIKI, 2014; FRIJHOFF et al., 2015). Even though MDA and oxLDL have been recognized as key elements in the initiation and progression of atherosclerosis, and potential markers of cardiovascular disease severity (KANNER et al., 2017), there is so far no consensus about the "best" biomarker for evaluation of oxidative stress and its relation to the prevention or progression of CVD (NIKI, 2014).

Lipid oxidation can occur by enzymatic or non-enzymatic mechanisms, resulting in the formation of a variety of reactive metabolites (NIKI, 2014; LINTON et al.,2019). For example, MDA and 4-Hydroxynonenal (HNE) are products of lipid peroxidation (Figure 3).

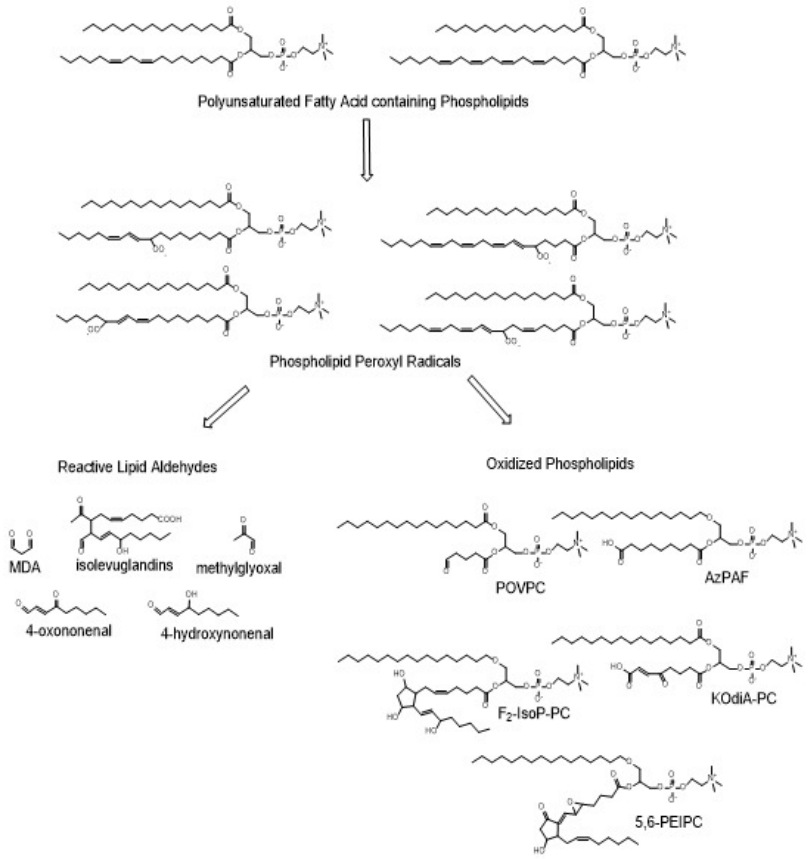

Figure 3: Oxidation of PUFA containing phospholipids. Adapted from LINTON et al. (2019). **Legend:** MDA: malondialdehyde; IsoLG: isolevuglandins; MGO: methyglyoxal; ONE: 4-oxononenal; HNE: 4-hydroxynonenal; POVPC: Oxidized phospholipids include 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine; azPAF: 1-O-alkyl-2-azelaoyl-sn-glycero-3-phophorylcholine; KOdiA-PC: 1-(Palmitoyl)-2-(5-keto-6-octene-dioyl)phosphatidylcholine; F2IsoP-PC : 1-palmitoyl-2-F2-isoprostane-sn-glycero-3-phosphocholine; PEIPC: 1-palmitoyl-2-(5,6)-epoxyisoprostane E2-sn-glycero-3-phosphocholine

HNE is formed through the enzymatic and non-enzymatic oxidation of linoleic acid. HNE can be up to 80 times lesser than MDA, but is considered more reactive and toxic due to its ability to form adducts with proteins (CASTRO et al., 2017). Isoprostanes are another type of secondary product of fatty acid non-enzymatic oxidation (Figure 3) (VIGOR et al., 2014; TIBAUT et al., 2019). According to VIGOR et al. (2014), they are transported in the bloodstream predominantly in HDL and excreted in the urine. They are present in the body in different isoforms, being the F_2-Isoprostane considered as “gold standard” for oxidative stress due to its chemical stability (NIKI, 2014).

MDA and other oxidative stress metabolites have been investigated and related to cardiovascular outcomes (FRIJHOFF et al., 2015). SZCZEPAŃSKA-SZEREJ et al. (2011) evaluated the levels of isoprostanes in patients with acute ischemic stroke. The authors observed a significant association between the oxidative stress in patients with cerebral ischemia

compared to healthy individuals. Already TSAI et al. (2014), assessed MDA levels in its prospective study, and reported that higher levels of MDA were associated with poor cardiovascular outcomes, after 3 months of acute phase stroke.

In the same way that oxidation occurs, the antioxidant defense can act through enzymatic and non-enzymatic pathways. The enzymatic system mainly involves the enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), while the non-enzymatic system consists in endogenous or dietary substances, such as glutathione (GSH), ascorbic acid and tocopherol (NIKI, 2014).

Glutathione is an important molecule that plays a role in the biological antioxidant system. It is a cofactor for several detoxifying enzymes and can reduce ascorbic acid and tocopherol (ASHFAQ et al., 2006; BIRBEN et al., 2012). Its oxidized structure (GSSG) is formed from the reaction of GSH with oxidants or by GPx catalysis (ASHFAQ et al., 2006). Therefore, high concentrations of GSSG are related to oxidative stress and the evaluation of the redox state (GSH/GSSG) has been considered as an independent predictor of the risk for atherosclerosis (ASHFAQ et al., 2006; PATEL et al., 2016). The GSH/GSSG ratio was associated with mortality in patients with coronary artery disease, independent of inflammatory status in a prospective study, corroborating the importance of oxidative stress biomarkers in the clinical practice (PATEL et al., 2016).

Although the association between oxidative stress and atherosclerosis is well-established, there is not any scientific agreement in terms of biomarkers and methodologies (NIKI, 2014; FRIJHOFF et al., 2015). In addition, according to the World Health Organization, a biomarker only can be valiated if the results are reproducible, have a prognostic value and is related to the disease steps. These conditions allow that the effectiveness of the treatment can be assessed using the valiated biomarker (WHO, 2001).

This dissertation presents two chapters. In the first one was done on extensive research in published studies that evaluated the oxidative stress biomarkers associated with the lipid profile of individuals with different conditions, aiming to identify which were the most used methodology in the clinical researchs.

Then, the second chapter presents the most relevant lipid oxidative stress biomarkers evaluated in healthy individuals and patients undergoing primary and secondary cardiovascular prevention. Data were obtained from clinical studies selected in the first part of the work according to the individual's profile. The chapter presents a discussion of the variability of biomarkers and the correlation with clinical outcomes.

CHAPTER 01: OXIDATIVE STRESS BIOMARKERS: WHICH AND HOW ARE THEY APPLIED IN CLINICAL TRIALS?

1. HYPOTHESIS

Oxidative stress lipid biomarkers can be independent of cardiovascular risk factors, improving the risk stratification of individuals in primary and secondary prevention.

2. OBJECTIVE

The objective of this study was to discuss the importance and limitations of some biomarkers of lipid oxidative stress as risk factor for cardiovascular disease.

3. METHODS

3.1 Studies selection

Studies were searched in the Pubmed database from 2008 until 2020. **Figure 4** shows the criteria and flow adopted to select the studies. From 355 original studies, 116 were selected to compose this analysis.

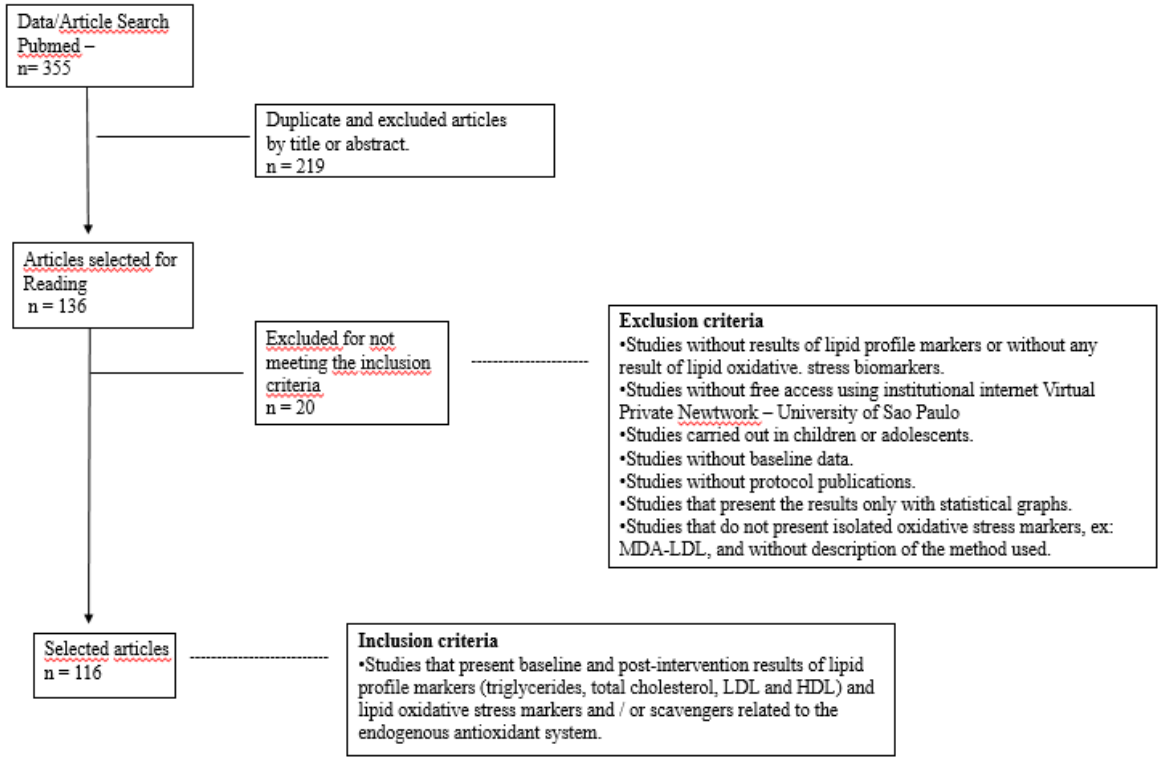

Figure 4: Criteria and flow adopted to select the articles

4. RESULTS

One hundred and sixteen (116) clinical trials describing 244 treatments were selected to compose this analysis. All studies evaluated the individual's lipid profile by analyzing total cholesterol, LDL, HDL and triglycerides. However, it was identified only 03 biomarkers of lipid oxidative stress: Malondialdehyde (64.75%), F_2-isoprostanes (21.31%) and oxidized LDL (17.21%). The evaluation of oxidative stress also occurred through the analysis of biomarkers of the antioxidant system. It was found 5 biomarkers including superoxide dismutase, catalase, glutathione, glutathione peroxide and GSH/GSSG ratio. In addition, different markers that assess antioxidant capacity and status were present, such as TAC (total antioxidant capacity), ORAC (oxygen radical absorbing capacity, FRAP (iron-reducing antioxidant power), BAP (biological antioxidant potential) and d-ROMS (derived from reactive oxygen metabolites). Biomarkers of inflammation were also detected in the studies that included high-sensitivity C-reactive protein (hsCRP), tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10).

The methodologies, instruments and type of biological sample used to evaluate the biomarkers associated with oxidative stress varied as shown in **Table 2**. The lack of consensus

in the choice of sample and method used in clinical studies makes it difficult to define reference values for certain population groups.

Table 2: Biomarkers, samples, methods and instruments ound in the clinical trials.

Biomarker	Sample Method and/or Equipment	References
MDA	plasma, TBARS, Spectrophotometer	Bumrungpert et al. 2018; Rahmani et al. 2017; Giolo et al. 2018; Karamali et al. 2018; Raygan et al. 2016; Nasri et al. 2018; COSTA E SILVA et al. 2020; Jamilian et al. 2016; Yen et al. 2018; Mameghani et al. 2016; Lima et al. 2017; Mazloom et al. 2011; Tamtaji et al. 2019; Bahls et al. 2011; Chandra et al. 2020; Li et al. 2010; Stanek et al. 2018; SOFI et al. 2018; ALIPOOR et al. 2012; Abdel-Zaher et al. 2014; Abdel-Zaher et al. 2012; Kozirog et al. 2011; Gulati et al. 2014; Asemi et al. 2015; Garcı´a-Alonso et al. 2012; Samimi et al. 2016; Kamali et al. 2019; Zhang et al. 2011; Arani et al. 2019; Bermejo et al. 2014; Babadi et al. 2019; Farrokhian et al. 2017; Colica et al. 2017; Mohamadi et al. 2020; Bento et al. 2014; Chacaroun et al. 2020; Dorkova et al. 2008; Kooshki et al. 2020; Ling et al. 2011; Jamilian et al. 2016; Daud et al. 2013; Whittaker et al. 2015; Vahedpoor et al. 2017; Whittaker et al. 2017; Asemi et al. 2016; Mesdaghinia et al. 2017; Derosa et al. 2016.
	plasma, TBARS + HPLC	Kouchaki et al. 2018; Zade et al. 2016; Fayh et al. 2018; Bobeuf et al. 2011; Campolo et al. 2017; Jamilian et al. 2015; Flammer et al. 2009; Sarriá et al. 2014;
	serum. TBARS, specotrophotometer	Usharani et al. 2019; KOOSHK et al. 2019; BOLDAJI et al. 2019; Lee et al. 2008; Faghihi et al 2014; Mahdavi et al. 2015; GORDON et al. 2008; CHEN et al. 2018; El-Aal et al. 2018; Helli et al. 2016; Baraibar et al. 2014;
	plasma, ELISA	Javid et al. 2018; CHOI et al. 2011; Takaki et al. 2011; Liao et al. 2019; Hosseini et al. 2016;
	serum, spectrofluorimeter	Barzegari et al. 2019; Khabbazi et al. 2012; Gheflati et al 2019;
Isoprostanes	serum, ELISA	Kostapanos et al. 2011;
	serum, GC/MS	Gupta et al. 2015;
	plasma, ELISA	CHOI et al. 2011; Stockler-Pinto et al. 2014; Fayh et al. 2018; Mattos et al. 2017; TONUCCI et al. 2017; Zhang et al. 2011; Bermejo et al. 2014; Braith et al. 2008;
	plasma, GC/MS	Wu et al. 2009; Rivara et al. 2015; RYAN et al. 2008; Groussard et al. 2015; Kam et al. 2016;
	urine, ELISA	DEVARAJ et al. 2008; Cedron et al. 2019; Takaki et al. 2011; Albuquerque et al., 2008; Bobeuf et al. 2011; Ciancarelli et al. 2011; Gladine et al. 2013
	urine, LC-MS/MS	Davinelli et al. 2015; Hongu et al. 2014;

	serum, ELISA	Kostapanos et al. 2011; Yen et al. 2018; Schiffl et al. 2010; Park et al. 2011;
oxLDL	plasma, ELISA	Bumrungpert et al. 2018 ; Davinelli et al. 2015; Burak et al. 2019; Paquette et al. 2017; Cedron et al. 2019; Verhoeven et al. 2015; Stanek et al. 2018; MESA et al. 2016; Groussard et al. 2015; Zhang et al. 2011; Colica et al. 2017; Ciancarelli et al. 2011; Kullisaar et al. 2016; Flammer et al. 2009; Kanellos et al. 2014; Gladine et al. 2013
	plasma, spectrophotometer	DEVARAJ et al. 2008;
GSH	plasma, spectrophotometer	Usharani et al. 2019; Rahmani et al. 2017; Karamali et al. 2018; Raygan et al. 2016; Nasri et al. 2018; Aghadavod et al. 2018; Kouchaki et al. 2018; Zade et al. 2016; Jamilian et al. 2016; Sepehrmanesh et al., 2016; Boaventura et al. 2012; Tamtaji et al. 2019; Chandra et al. 2020; ASEMI et al., 2014; El-Aal et al. 2018; Asemi et al. 2013; Asemi et al. 2015; Kamali et al. 2019; Samimi et al. 2016; Arani et al. 2019; Babadi et al. 2019; Farrokhian et al. 2017; Jamilian et al. 2016; Asemi et al. 2014; Jamilian et al. 2015; Vahedpoor et al. 2017; Asemi et al. 2016
	plasma, HPLC	Campolo et al. 2017;
	erythrocytes, HPLC	Ling et al. 2011
GPx	erythrocytes, spectrophotometer	Derosa et al. 2016; Yen et al. 2018; Li et al. 2010; Stanek et al. 2018; ALIPOOR et al. 2012; Kozirog et al. 2011; Groussard et al. 2015; Dorkova et al. 2008; Ling et al. 2011;
	plasma, spectrophotometer	Stockler-Pinto et al. 2014; Barzegari et al. 2019; El-Aal et al. 2018; Chacaroun et al. 2020; Baraibar et al. 2014;
GSH/GSSG	plasma, spectrophotometer	Groussard et al. 2015;
SOD	erythrocytes , spectrophotometer	Derosa et al. 2016; Yen et al. 2018; Takaki et al. 2011; Li et al. 2010; GORDON et al. 2008; ALIPOOR et al. 2012; Kozirog et al. 2011; Groussard et al. 2015; Bento et al. 2014; Ling et al. 2011;
	erythrocytes , spectrophotometer	Giolo et al. 2018; Barzegari et al. 2019; Fayh et al. 2018; Chandra et al. 2020; Stanek et al. 2018; CHEN et al. 2018;
	plasma, ELISA	CHOI et al. 2011; Chacaroun et al. 2020; Ciancarelli et al. 2011;
CAT	serum, spectrophotometer	Barzegari et al. 2019;
	erythrocytes , spectrophotometer	Yen et al. 2018; Chandra et al. 2020; GORDON et al. 2008; Stanek et al. 2018; Kozirog et al. 2011; Ling et al. 2011;

Legend: MDA: malondialdehyde, TBARS: thiobarbituric reactive acid; HPLC: High Performance Liquid Chromatography; ELISA: enzyme immunoassay; Gas chromatography/MS: Gas chromatography–mass spectrometry; LC-MS/MS: liquid chromatography - mass spectrometry; oxLDL: oxidized LDL, GSH: glutathione, GPx: glutathione peroxide, GSH: reduced glutathione; GSSG: oxidized gluthathione; SOD: superoxide dismutase, CAT: catalase, TAC: total antioxidant capacity, ORAC: oxygen radical absorbing capacity, FRAP: iron-reducing antioxidant power, BAP: biological antioxidant potential, d-ROMS: derived from reactive oxygen metabolites.

Concerning the interventions used in the clinical trials, it was noted that only 14.28% were performed with drugs, being the others performed with different bioactive compounds, food supplements, probiotics and dietary interventions.

Regarding the individual´s health profile, it was identified 32 profiles. The majority being healthy individuals (29%), type II diabetics (18%), dyslipidemics (6.55%) and individuals with metabolic syndrome (6.55%). Individuals with cardiovascular disease corresponded to only 4% of these. The general biomarkers related to the health condition of the individuals is present im Table 3.

Table 3: General biomarkers related to the health condition of the individuals included in the selected studies.

Characteristics	**unit**	**N**	**Range**	**Reference values**
Individuals	N	488	6 - 104	
Male	%	44		
Body Weight (BW)	Kg	298	45.94 – 95.30	
Body Mass Index (BMI)	kg/m^2	422	21.20 – 37.30	18.5 to <25***
Waist Circumference **(**WC)	cm	74	76.78 – 123.00	Men ≥ 102 **** Women ≥ 88
Body Fat (BF)	%	66	17.21 – 46.71	
Age	years	310	21.40 – 79.00	
Blood preassure systolic	mmHG	178	97.14 – 191.10	<120****
Blood preassure diastolic	mmHG	178	61.60 – 108.00	<80****
Glucose	mg/dL	286	69.79 – 219.00	< 100**
Insulin	µIU/ml	184	5.1 – 32.10	
Glycated Hemoglobin **(HbA1c) *[1]**	%	74	3.99 – 8.83	< 5.7**
Homeostasis Model Assessment estimated b cell function (HOMA-B)	index	76	21.21 – 100.00	
Homeostasis Model Assessment Insulin Resistance (HOMA-IR)	index	168	1.02 – 9.34	

Quantitative insulin sensitivity check index (QUICKI) *	index	92	0.30 – 0.39	
Alanine aminotransferase (ALT)	U/L	60	9.44 – 62.00	
Aspartate aminotransferase (AST**)**	U/L	54	12.70 – 49.00	
Total cholesterol[1]	mg/dL	487	119.70 – 258.90	< 190*
Triglycerides	mg/dL	488	52.86 – 295.30	< 150*
High density lipoprotein (HDL)	mg/dL	488	29.00 – 79.43	> 40*
Low desity lipoprotein (LDL) [1]	mg/dL	487	51.43 – 174.00	< 100*

[1] Some values that were completely out of the normal range were excluded.
**Reference values of the Brazilian Dyslipidemias and Atherosclerosis Prevention Directive - 2017*
***Reference Values of the Brazilian Diabetes Society 2017-2018*
****Centers for disease control and prevention (CDC)*
*****National Heart. Lung and Blood Institute (NIH)*

5. DISCUSSION

It was observed that the evaluation of the antioxidant capacity was evaluated in more than 50% of the studies. However, the markers and methodologies used in the studies only assess the ability to scavenge free radicals that does not reflect *in vivo* the antioxidant capacity (NIKI, 2010).

Several reasons may explain the qualitative variation found in oxidative stress biomarkers applied in the studies. For example, costs, storage conditions, methodology and applied instruments to run the analysis (NIKI, 2014; FRIJHOFF et al., 2015; TSIKAS, 2017).

However, the variability of biological samples used in the studies is noteworthy, as seen in Table 2. Different sample matrices were used to evaluate the same biomarker such as plasma, serum, erythrocytes and urine. According to OLIVEIRA et. al (2018), serum and plasma samples are not interchangeable. The difference between serum and plasma is the presence of fibrinogen in the plasma, but this difference can lead to divergent results for the same biomarker

depending on the sample matrix that was used to run the analysis, because the coagulation can increase the concentration of some lipid metabolites (OLIVEIRA et al., 2018).

This relationship between the sample matrix and the results obtained with lipid metabolites may explain the difference in MDA concentration found in some studies. Despite the individual metabolic differences, LEE et al (2008) found a baseline MDA value of 14 µmol/L in healthy postmenopausal women in serum by reaction with reactive thiobarbituric acid (TBARs) and quantified by spectrophotometry. On the other hand, LIMA et al. (2017) evaluated the MDA of overweight/obese women in plasma with the same method used by LEE et al. (2008), and the baseline value found was 3.45 µmol/L.

These differences can be attributed mainly to the metabolic profile of these women. However, in addition, other variables in the MDA analysis must also be considered, such as the method of derivatization of the molecule. For the formation and stabilization of MDA in the sample, different molecules can be used such as TEP (1,1,3,3-tetraethoxypropane), TMP (1,1,3,3-tetramethoxypropane) to promote the polymerization of MDA (GUTTERIDGE. 1975) and $NaBH_4$ (sodium borohydride) to reduce and stabilize the adducts of MDA with amino acids (REQUENA et. al. 1997). The sample preparation method will interfere will affect the type of molecule found. While derivatization through TEP results in MDA molecules with different polarities and molecular weights during polymerization processes (GUTTERIDGE. 1975), the method with specific adduct/MDA reaction forms more stable and specif molecules (REQUENA et. al. 1997). Furthermore, unlike free MDA, MDA adducts are related to LDL oxidation and the development of atherosclerosis (REQUENA et. al. 1997).

In this regarding, the method and sample matrix can be an important variable and should be considered for standardization of methods and definition of reference values for biomarkers of oxidative stress. However, this variable is little discussed in the studies evaluated.

In our literature review, it became evident that MDA was the main biomarker applied to evaluate oxidative stress. Diverse methods have been proposed for MDA quantification, from commercial kits to more sensitive methods with more accurate instruments (NIKI, 2014; FRIJHOFF et al., 2015; TSIKAS, 2017). The most used method is based on the reaction with thiobarbituric reactive acid (TBARs), which is quantified by spectrophotometry or high-performance liquid chromatography (HPLC). Although less specific, because it quantifies other molecules that also react with TBA, the spectrophotometer is the widest instrument applied to MDA analysis (TSIKAS, 2017). The heterogeneity of analytical methods available for MDA analysis becomes more difficult to identify the reference values and correlate them with different health conditions (FRIJHOFF et al., 2015).

In vivo oxidative stress analysis is a challenge. The oxidation of different molecules is influenced by several other mechanisms that are not related to atherosclerosis, confounding the interpretations of some results. For example, the consumption of regular and successive hyperlipidemic meals keeps the organism in a constant postprandial state and this condition increases the levels of free fatty acids in tissues, increasing glycolysis, β-oxidation and oxidative phosphorylation, resulting in oxidative processes (KRÜGER et al., 2015; DIMAURO; SCHON, 2003). In addition to the postprandial bias, an increase in oxidative stress can occur after acute inflammatory events, intense physical exercise, exposition to some toxic compounds and pollutants, psychological stress and other conditions that have no direct relation to atherosclerosis and CVD (NIEMMAN et al., 2017; PINGITORI et al., 2015).

Nowadays, the main criteria considered to calculate cardiovascular risk stratification scores are age, sex, blood pressure, smoking, lipid profile, diabetes and family history (WHO, 2007). To decrease the risk score, the patient must achieve the therapeutic targets that accompany the reference values for healthy individuals. Therefore, interventions are necessary through drugs or not. Statins, for example, reduce LDL levels by 30 to 50% (NCEP, 2001; FALUDI et al., 2017). In the 4S study, the use of simvastatin promoted a 25% reduction in TC, 35% LDL, and increased HDL by 8%. These results can explain a 30% reduction in total mortality (SCANDINAVIAN SIMVASTATIN SURVIVAL STUDY GROUP, 1994).

To enhance risk stratification, other tests can be done such as hsCRP, lipoprotein A and apolipoprotein B (ARNETT et al., 2019). hsCRP was included in some cardiovascular prevention guidelines and risk scores after the recognition of inflammation as an independent cardiovascular risk factor (QUISPE et al., 2020). The relationship between inflammation and CVD endpoint became evident after numerous clinical trials showing that higher levels of hsCRP were associated with increased relative risk and increased cardiovascular events (DHINDSA et al., 2020). However, there is still no consensus about the best moment to start inflammation monitoring.

The American College of Cardiology (ACC)/American Heart Association (AHA) Primary Prevention (2019), for example, reports that hsCRP can be evaluated in some individuals as a risk enhancer (ARNETT et al., 2019). The Brazilian guideline (2017) recognizes the measurement of hsCRP as a risk stratification tool for individuals at intermediate risk (FALUDI et al., 2017). Besides, the benefit of anti-inflammatory therapies has still been investigated, as well as the type of patient who would benefit from this type of therapy (EVERETT et al., 2020; QUISPE et al., 2020).

Another criterion to calculate CVD risk is smoking. It is widely known the relationship between smoking and oxidative stress (NIEMANN et al., 2017). Several studies have reported that smoking is associated with CVD and high levels of oxidative stress biomarkers, such as MDA and isoprostanes (NIEMANN et al., 2017). Smoking is one of the risk factors that modify LDL goals (NCEP, 2001). However, despite all evidence about the role of oxidative stress in atherosclerosis progression, the inclusion of oxidative biomarkers as part of the CVD scores calculation seems to be more complex than inflammatory biomarkers and lipoprotein A, a biomarker recently included in ACC/AHA Guideline (ARNETT et al., 2019).

It was observed that there is no consensus about a sample matrix, biomarker and methodological standard for conducting clinical studies that aim to assess oxidative stress. The relationship of oxidative stress with different pathologies has been studied, but is necessary to standardize the studies in relation to the biomarkers, samples, methodologies and instruments.

As consequence of this lack of standardization, there are not enough studies that show the relation between oxidative stress, antioxidant therapies and clinical outcomes in cardiovascular events or risk of mortality (SENONER; DICHTL, 2019). After the standardization, it will be possible to get reference values for access the individuals and correlate the results with clinical outcomes.

As highlighted by the World Health Organization (2007), risk stratification is a resource that helps to save the largest number of lives at the lowest cost. Despite these limitations, the potential use of oxidative biomarkers to improve the current scores for risk stratification is high and more investigations are necessary. Increasing the accuracy of risk stratification in primary prevention offers more options for interventions such as lifestyle changes, diet, physical activity and non-drug therapies.

6. CONCLUSION

It is necessary to standardize biomarkers, samples, methodologies and reported values for individuals of different health conditions. After, it is also necessary to clarify the clinical relevance of these markers, identifying correlations between reference values and clinical end-points.

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CHAPTER 02: COULD A LIPID OXIDATIVE BIOMARKER BE APPLIED TO IMPROVE RISK STRATIFICATION IN PREVENTION FOR CARDIOVASCULAR DISEASE?

COULD A LIPID OXIDATIVE BIOMARKER BE APPLIED TO IMPROVE RISK STRATIFICATION IN PREVENTION FOR CARDIOVASCULAR DISEASE?

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Abstract

There is significant evidence demonstrating the influence of oxidative stress on atherosclerosis progression and consequently cardiovascular diseases (CVD). However, different from dyslipidemia and inflammation biomarkers, non-oxidative biomarkers have been applied routinely to analyze the primary or secondary prevention treatment of these at-risk patients. Many factors can explain this paradox, including the higher complexity of the methods applied to quantify oxidative markers, the high variability observed among the studies, lack of reference values and weak correlation with clinical endpoints. In this review, data from 116 treatments in 55 studies that evaluated oxidative stress markers under the atherosclerotic context, showed that antioxidant capacity measured as Ferric Reducing Antioxidant Power (FRAP), Superoxide Dismutase (SOD), Glutathione (GSH), Malondialdehyde (MDA), oxidized LDL (oxLDL) and Isoprostanes (F_2-IsoP) were the oxidative markers present in a higher part of the studies. From them, MDA, IsoPs and oxLDL are directly formed from lipid oxidation, while FRAP, SOD and GSH have their values associated to general oxidative conditions. Among the lipid oxidative markers, MDA had the highest proportion among the treatments. A higher concentration of MDA ($p=0.041$) in patients with CVD (17.05 ± 37.24 µmol/L, $n=51$) was found than in healthy individuals (5.07 ± 7.54 µmol/L, $n=21$), despite the very high general variability (235.84%). Multivariate analysis suggested that MDA was an independent factor compared with some traditional markers used in the algorithms to stratify the patient's risk. However, the variability of MDA must be reduced by multicenter studies using a chromatographic validated methodology. In addition, it is also necessary to achieve a consensual reference value for patients under primary or secondary prevention paired with healthy individuals, and correlate MDA increase according to the disease's progression before including it in the algorithms applied to estimate CVD risk.

Key words: atherosclerosis, oxidative stress, risk score, MDA, prevention

Introduction

Cardiovascular diseases (CVD) are the leading cause of death globally, with their ischemic forms, such as heart attacks and strokes, being the most prevalent events [1-3]. These ischemic forms result from atherosclerosis, a chronic non resolving inflammatory condition that begins in childhood and progresses through adolescence and adulthood [2,4-5]. Actually, atherosclerosis may proceed in phases of relative quiescence punctuated by periods of rapid growth [3]. The atherosclerosis progression is associated with the development of non-communicable chronic diseases (NCDs), such as hypertension, obesity, diabetes and dyslipidemia, caused by an unhealthy diet, physical inactivity, tobacco use and harmful use of alcohol [6]. However, recent research has expanded on the traditional risk factors, now focusing on triacylglycerol-rich-lipoproteins besides low-density lipoprotein (LDL), disturbed sleep, the microbiome, air pollution and environmental stress, and clonal haemotopoiesis of indeterminate potential (CHIP) [3].

The excess of LDL promotes higher permeability of blood vessels, allowing the transcytosis of these particles in the intima layer, leading to their entrapment by extracellular matrix [2,3,7-9]. The subendothelial retention of lipoproteins is, in fact, an early step in atherogenesis [7]. Inside the LDL particle, the excess of free cholesterol undergoes re-esterification to cholesteryl fatty acids esters by Acyl-CoA:cholesterol acyltransferase (ACAT) [10], and migrates from the surface to the core of the particle. The polyunsaturated fatty acids (PUFAs) that are esterifying glycerol, cholesterol or phospholipids (PL), can be directly oxidized by reactive oxygen species (ROS) or indirectly by enzymes and metal ions, leading to the formation of oxidized LDL (oxLDL) [2,7,9.11]. The macrophages derived from recruited monocytes recognize and phagocyte these oxLDL as damage-associated molecular pattern (DAMP), becoming foam cells, triggering an immune-inflammatory response and forming the atherogenic plaques. In addition, smooth muscle cells suffer metaplasia becoming macrophages like cells, proliferate and migrate from the media to the intima layer, contributing to increase the plaques [3]. This process generates more ROS, promoting apoptosis followed by a secondary necrosis, inflammation and oxidative stress [2,3, 10,12]. The plaques, in turn, can suffer rupture, provoking ischemic insults such as myocardium infarction and stroke, besides thrombous formation in peripheral arteries caused by netosis of neutrophils [2]. Thus, atherosclerosis involves dyslipidemia, inflammation and oxidative stress as part of the immune response [13,14].

The prevention of CVD, occurs through the adoption of a healthy lifestyle that involves a balanced diet, physical activity and non-smoking, that must be practiced at all stages of the life [15,16]. The prevention of CVD can be divided in primary and secondary. Primary prevention aims to avoid a first heart attack or stroke in people who already have developed CVD risk factors by making healthy lifestyle changes and, if needed, taking medications. Secondary prevention, on the other side, aims to prevent a second heart attack or stroke, halt the progression of heart disease and prevent early death, involving more aggressive strategies that those adopted in the primary prevention [17]. Once a definitive diagnosis of atherosclerosis is established, physicians assess the patient and estimate the 10-years risk of atherosclerotic cardiovascular disease (ASCVD) using distinct algorithms models, such as American College of Cardiology/American Heart Association 13 (ACC/AHA13) score, Framingham Risk Score (FRS), the Systematic Coronary Risk Evaluation (SCORE) model and the Reynolds Risk Score [2, 16,18]. These scores apply predictive criteria as age, sex, smoking, inflammatory and biomarkers levels. Although risk estimation is imperfect and based on group averages, it is applied to classify individual patient as low, borderline, intermediate and high cardiovascular risk in 10 years [16].

Guidelines for the prevention and treatment of CVD generally recommend the use of several criteria and biomarkers to improve scores for risk stratification and are constantly updated, aiming to be more assertive in the risk stratification, due to the specificity of different populations [16,19]. However, around 50% of people having coronary artery disease are classified as low or intermediate risk [2,18], suggesting that there is a residual risk that has not been computed in the algorithms. For this reason, novel risk factors are continuously being searched in pathogenesis pathways of atherosclerosis [18]. For example, Ridker *et al*. [20] showed that healthy people, without hyperlipidemia but with elevated high-sensitivity C-reactive protein (hsCRP) levels, presented reduced incidence of major cardiovascular events after of receiving rosuvastatin treatment, suggesting the importance of hsCRP as an inflammatory biomarker to CVD. However, even though some results are promising, risk scores and guidelines use to be very conservatives, being common to apply always the same biomarkers due to technical and economic reasons. According to the World Health Organization [21], a biomarker only can be validated if the results are reproducible, have a prognostic value and be related to the disease steps. These conditions allow that the effectiveness of the treatment can be assessed [21]. Thus, although inflammation and oxidative stress are deeply involved in the development and progression of atherosclerosis and can predict

risk of CVD [2], except for hsCRP, that is a biomarker associated with acute inflammation, no others have been adopted as factors to complement the current scores [16].

Several studies have discussed how traditional risk factors translate into oxidative stress and contribute to atherosclerosis, and the importance of following the oxidative status of individuals aiming to reduce CVD risks [13,22]. Some of them have also suggested that oxidative stress biomarkers could be useful in the early stages of CVD, when individuals have lower risk and there are more options for their treatment than in the advanced stages [14,18,23. However, despite all these advantages and the scientific knowledge achieved about the role of oxidative stress as a biomarker, none have been included up to date to improve the predictive capacity of the scores [18,22]. This fact can be due to many reasons, including the absence of reference values in different populations, more complex and expensive methodologies to determine them in biological samples and absence of the direct association between oxidative biomarkers and CVD endpoints. Furthermore, all biochemical markers applied to follow the atherosclerosis progression are usually systemic, quantified in non-invasive samples of the patients, being thus susceptible to the influence of other pathologies than atherosclerosis. Anyway, the scientific basis of the oxidative modification hypothesis relevance to human disease is strong [9], justifying more studies in this area.

Thus, the objective of this revision was to discuss the importance and limitations of some biomarkers of oxidative stress, mainly those related to lipid oxidation, and suggest some strategies to include them in the algorithms to improve risk stratification scores.

1 Oxidative stress in vascular cells and atherosclerosis

The term “oxidative stress” was firstly introduced by Helmut Sies in 1991 and defined as “*a disturbance in the prooxidant – antioxidant balance in favor of the former, leading to potential damage*” [24]. Under controlled conditions, some basal levels of oxidative reactions are crucial for proper cell signaling, being necessary to stem cell differentiation, response to endoplasmic reticulum stress and control of inflammation [25]. Although essential for vascular homeostasis, uncontrolled production of pro-oxidants is implicated in vascular injury [13]. In the biological systems, the principal reactive species that are able to react with proteins, lipids and nucleic acids, can be mainly represented by “Reactive Oxygen Species (ROS)”. Among ROS, the anion superoxide ($O_2^{\bullet -}$) and the hydroxyl radical ($HO^{\bullet}$) as well nonradical molecules

such as hydrogen peroxide (H_2O_2) are involved in the most of oxidative processes that cause cell damage associated to the chronic diseases [8,25,26].

Oxidative stress associated to atherosclerosis can occur either locally in the vessel wall or in the systemic via [27] and involves multiple cell types, including endothelial cells, smooth muscle cells (SMCs), immune cells and stem/progenitor cells [25]. The cellular sources of ROS in vascular cells are the nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) oxidases, mitochondrial electron transport in the respiratory chain, xanthine oxidase, transition metals, lipoxygenase, myeloperoxidase and uncoupled nitric oxide synthase [13,25,26,28,29]. Hypertension, diabetes, smoking, and dyslipidemia activate the NADPH oxidase system resulting in excess generation of superoxide anions, that lead to L-arginine and tetrahydrobiopterin (BH4) deficiency causing uncoupling of eNOS, endothelial dysfunction, and further ROS generation [13]. Hyperglycemia stimulates superoxide anion generation from the mitochondrial electron transport chain, leading to production of advanced glycation end products (AGEs) which can activate NADPH oxidases, inhibit eNOS, and activate LOX-1 (lectin-like oxLDL) [30,31]. In fact, the link between hyperglycemia and oxidative stress also involves polyol pathway and activation of protein kinase C [30]. External factors like smoking are also a potent activator of NADPH oxidase at the endothelial level and directly oxidizes LDL. Oscillatory shear stress on vessel walls decreases eNOS expression and promotes ROS generation via NADPH oxidase activation. The ROS and NO regulate LDL uptake in arterial walls, leading to oxidation of various phospholipids and generation of ox-LDL [13].

On the other side, antioxidant defense can be characterized by a complex system, mainly represented by superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S–transferase, thioredoxins, peroxiredoxins and ubiquinol-10, as well as endogenous and exogenous molecules, such as glutathione (GSH), ascorbate, phenolic compounds and others. The nuclear factor erythroid-2-related factor 2 (Nrf2) is also an important antioxidant pathway, but its pro- or antiatherogenic properties still deserves more investigation [25]. **Figure 1** summarizes some mechanisms by which pro and anti-oxidants act towards early steps of atherosclerosis.

The important role of oxidative stress in cardiovascular pathophysiology could be followed by ROS quantification. However, this has proven to be a complex challenge given the evanescent nature of ROS [35]. Thus, other oxidative markers than ROS have been analyzed in the clinical trials. Biomarkers of oxidative stress can be classified as molecules that are modified by interactions with ROS in the microenvironment or molecules of the antioxidant system that change in response to increased redox stress. Under normal conditions, the

antioxidant defense is enough to keep the cell homeostasis, but when the reactive species are strongly increased as response of immune cells activation for example, those that are not neutralized by the antioxidants will oxidize lipids, proteins, carbohydrates and nucleic acids [35].

Regarding to the lipids, reactive species can mainly oxidize PUFAs, free or esterifying other molecules, leading to the formation of oxysterols, oxidized phospholipids, hydroperoxides and several other secondary compounds. Some of these products of lipid oxidation have been considered potentially cytotoxic and are involved in the progression of several diseases, including the atherosclerosis.

2 The role of secondary products of fatty acids oxidation on atherosclerosis

Anichkov proposed the first experimental model of atherosclerosis in 1912, but only in 1973, Michael Brown and Joseph Goldstein identified the cholesterol receptors and received the Nobel prize in 1985 due to this important contribution, that confirmed LDL as the main risk factor to CVD. This confirmation was corroborated by the classical 4S Study, that showed 4 % reduction of CVD mortality due to 35% reduction of LDL after 4 years median follow-up period [36,37]. At this time, the studies carried out by Tora Henriksen and Daniel Steinberg about the role of oxidative modifications of the LDL particle, configured as the main contribution to better understand the interaction between the innate immune cells and atherosclerosis [38-40]. In summary, it was identified that oxidative changes were essential for converting LDL to the cytotoxic form and that macrophages were able to phagocyte native LDL particles very slowly, while those that showed some chemical modification, such as acetylation, succinylation or oxidation were avidly phagocyted [8,9].

ApoB-LDL particles are made mainly in liver and intestinal cells and consist of a core of cholesteryl fatty acyl esters and triacylglycerol, surrounded by a monolayer of phospholipids and protein, having about 22.3% of phospholipids, 9.6% free and 42.3% esterified cholesterol and 22% apolipoprotein B (ApoB), besides some α-tocopherol and ubiquinol ($CoQ10H_2$) [8,41]. Briefly, it was proposed a sequence of chemical events that started with the reaction of transition metals, superoxide anion and hydroxyl radical with polyunsaturated fatty acids esterifying the phospholipids in the native LDL particle, as summarized in **Figure 1** [42,43]. These polyunsaturated fatty acids, part of the phospholipids, or after of being liberated from these molecules by the action of different lipases, are prone to be oxidized, inducing biological

effects to different types of pathologies [11,34,44]. Actually, 12/15 lipoxygenases (LOX) and cytochrome c are capable of direct oxidize PL-esterified PUFAs, producing hydroperoxides of correspondent phospholipids (PLs), while several other enzymes oxidize free fatty acids forming hydroperoxy- (LOXs), endoperoxy- (COXs) or epoxy- (cytP450) PUFA [11]. It has been proposed that products of polyunsaturated fatty acids oxidation, such as malondialdehyde (MDA) and 4-Hydroxynonenal (4-HNE), are strong electrophiles and cause post-translational modification of cysteine, histidine and lysine residues, resulting in the formation of a stable protein-lipid Michael adduct, and Schiff base between the ε-amine of lysine and lipid [34]. These adducts are recognized by receptor scavengers such as such as CD36, scavenger receptor A (SRA) and toll-like receptor 4 (TLR4) present in the macrophage's membrane [8,10, 41,42,45]. Thus, the major modification of the Apo B100 lipoprotein is the aminoacid charge change [46]. Actually, the LDL oxidation depends on the action of ROS with fatty acids that are esterifying phospholipids on surface of the particle. In this context, oxidized phospholipids play an important role in the atherosclerosis, because they are CD36 ligands, being considered a separate risk factor for coronary events [47]. For this reason, lipoprotein (a) [Lp(a)], that is the major lipoprotein carrier of phosphocholine-containing oxPLs, has been considered also a risk for CVD [48]. Independent of the adduct formation, MDA appears to be the most mutagenic product of lipid oxidation, whereas 4-HNE is the most toxic [44].

From the seminal studies reported by Henriksen and Steinberg, many others were carried out clarifying the mechanisms involving between oxidative LDL particle (oxLDL), foam cells and plaque formation in the endothelial intima [46], growing awareness of the importance of immune response in the pathogenesis of atherosclerosis [9]. In addition to the oxidation of LDL, ROS is involved with the release of the metalloproteinase matrix (MMPs) that degrades the fibrous wall of the atheroma plaque, resulting in plaque disruption in the final stage of the ischemic diseases [13]. Thus, the oxidative stress influences all stages of atherosclerosis, from the beginning of arterial injury and initiation of fatty streak, until the plaque rupture [28].

Although there is a strong consensus about the mechanisms summarized in **Figure 1**, and a significant number of clinical trials which has measured oxidative biomarkers in, no advances have been observed toward the inclusion of these biomarkers as clinical routine. This fact can be partially explained by the limitation and high complexity of some methodologies, low specificity of biomarkers of oxidative stress in human diseases, the weak correlation between oxidative markers and the clinical endpoints and the high variability of these

biomarkers measured in healthy individuals and patients under primary or secondary prevention.

3 The variability of oxidative stress biomarkers associated to atherosclerosis.

In order to exemplify the variability of the major lipid oxidative stress biomarkers evaluated in the clinical trials, 116 treatments, including 44 placebo and 72 treatment groups were selected from 55 clinical trials at the beginning of the intervention according to **Supplementary Figure 1** criteria, and their general characteristics were summarized in **Table 1**. It was not included biomarkers measured in less than 10 from the 116 treatments. As observed in **Table 1**, seven oxidative stress biomarkers were evaluated: FRAP (mmol/L), SOD expressed as U/g Hb and also as SOD U/ml, GSH (µmol/L), MDA (µmol/L), oxLDL (U/L), F_2IsoP in urine (pg/mg creatinine) and F_2IsoP in plasma (pg/mL). From them, MDA, F_2-IsoPs and oxLDL are directly formed from lipid oxidation, while FRAP, SOD and GSH have their values indirectly associated to lipid oxidation.

Among the oxidative stress markers, MDA (µmol/L) was the most evaluated (n=76), and for this reason it was selected in our review, aiming to check its correlation with other biomarkers usually applied in the algorithm models to obtain scores for risk stratification. MDA results reported by 2 studies (**Table 1**) were excluded due to their expressive difference when compared with the majority of reported values. Thus, MDA concentration from 72 treatments were submitted to a Principal Component Analysis (**Figure 2A)** and cluster analysis (**Figure 2B**). These active variables were selected in order of not reducing the number of treatments applied in the multivariate analysis. As observed in **Figure 2A**, BMI, age and triacylglycerol (TG), positively contributed to Factor 1, while HDL (high density lipoprotein) negatively contributed to Factor 1, that represented 41.43 % of variation. Total cholesterol and LDL negatively contributed to Factor 2 (not shown). On the other side, isolated MDA positively contributed to Factor 3 (+0.83), suggesting the independence of this biomarker for cardiovascular risk considering all 72 treatments. The three first PC explained 82.11 % of total variation. Cluster analysis, using Ward`s method and Euclidean distances (**Figure 2B**) also showed that MDA seems to be an independent biomarker, when other classical biomarkers for risk score calculation were included.

The patients who took part of the 116 treatments were classified into 2 groups: Group 1 – healthy individuals and Group 2 – individuals under primary or secondary prevention. **Table**

2 presents the major characteristics and biomarkers obtained from the 116 treatments according to the two groups. It is important to highlight that all values reported in our analysis were obtained at the beginning of the trials, thus without the intervention effect. However, some patients in the Group 2 (under primary or secondary prevention) were being prescribed with drugs before their inclusion in the respective trials. These drugs could influence the values observed to their biomarkers even at the beginning. Major drugs reported to these patients were hypoglycemic agents (insulin, sulphonylurea, biguanides, glitazone, acarboze, gliptin), antihypertensive (angiotensin II, receptor blocker, calcium channel blocker, beta blocker, diuretic), and lipid lowering (statins and fibrate).

Regarding to these results and taking into account the number of observations, it can be realized that the variability of oxidative markers: MDA (235.85%), oxLDL (40,40%) or F2-IsoPs in plasma (226,16 %), was much higher compared with other classical biomarkers to CVD: LDL (22,14%), HDL (17.99%) or TG (26.68%). This fact could be partially due to the higher methodological complexity and the influence of other physiological condition besides atherosclerosis on these values. Alajbeg *et al*. [55] evaluated the variability of 6 oxidative stress markers in saliva of 15 healthy subjects over 3 days at 2 time intervals. The authors observed a wide between-subject variability for all markers (CV% 30.08 to 85.70%) with MDA showing the largest intra-individual difference, which suggest the need of validation. In another study, MDA analyzed in plasma of 213 healthy individuals ranged from 0.36 to 1.24 µmol/L, being independent of gender, but dependent of age, smoking and weekly alcohol consumption. It was also observed a high within-subject (5.9 to 30%) and dairy variability (6.5%) [56]. Mas-Bargues *et al*., [57] reported in their review values changing from 0.85 to 2.54 µmol/L of MDA to healthy and young individuals, while values from 0.55 to 7.40 µmol/L were found in individuals with pulmonary disease or Alzheimer. In this case, variation coefficients about 90% were also observed, confirming the high variability of MDA determinations. This high level of variation can be due to many pre- and post-analytical factors. For example, Tsikas *et al*. [58] pointed out to pre-analytical pitfalls including sampling, sample storage, and arte-factual formation of MDA during the analysis. With regard to blood sampling, coagulation and hemolysis can considerably contribute to artificial formation of MDA [58]. Nielsen *et al*. [56] recommended EDTA instead of heparin as anticoagulant in order to provide chelation of iron in the TBA assay. Sample storage can also modify the MDA analysis, arising the question of the most proper time of sample analysis for MDA and other lipid peroxidation biomarkers such as 4-HNE and F_2-IsoPs [58]. Although pre-analytical factors should be observed, the post-analytical

ones, involving methodologies, seem to be more relevant in order to compare results from different studies.

4 Methodologies applied to measure lipid peroxidation in biological samples

Malondialdehyde (MDA), 4-hydroxy-nonenal (4-HNE) and F_2-IsoPs are clinically relevant secondary products of PUFAs oxidation (**Figure 3**). Major PUFAs that esterifies phospholipids, cholesterol ester and triacylglycerol are: linoleic (LNA), arachidonic (AA), docosahexaenoic (DHA), eicosapentaenoic (EPA), α-linolenic (ALA), docosapentaenoic (DPA) and γ-linolenic (GLA) acids [59]. F_2-IsoPs are derived mainly from AA, while MDA is a product of oxidation from fatty acids containing two or more double bounds [57] and 4-HNE is formed only from omega 6 PUFAs. Thus, the number of PUFAs that may contribute to MDA is much higher than to 4-HNE and F_2-IsoPs [44,57,58].

MDA and 4-HNE can be quantified as free molecules or binding to amino acids, using chromatography or immune histology techniques [22]. Both can be generated by decomposition of PUFAs, through enzymatic or non-enzymatic process [44]. In more than 98% of times**,** MDA is determined by the reaction with thiobarbituric acid (TBA), in which one molecule of MDA reacts with two molecules of TBA to form an adduct visible at 532 nm and fluorescent at 515 nm (excitation) and 553 nm (emission) [57]. Formation of MDA-TBA2 adduct occurs by a nucleophilic attack involving carbon-5 of TBA and carbon-1 of MDA, followed by dehydration and similar reaction with a second molecule of TBA, producing a red pigment according to the lipid peroxidation extension [60]. MDA levels can also be measured using an MDA adduct competitive ELISA kit, that give the capacity of MDA to bind proteins, forming stable adducts [55]. However, the spectrophotometric and spectrofluorimetric TBA-based methods are the most common assays, although they lack specificity, since other compounds can react with TBA. Several methods for MDA determination, such as gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/ mass spectrometry (LC-MS), including different derivatization strategies have been developed during the last decade [44]. These methods based on visible or fluorescence detection or mass spectrometry, providing reliable and reproducible results [57].

4-HNE is the major α,β-unsaturated aldehyde product of omega 6 fatty acids. Due to its electrophilic characteristic, 4-HNE is highly reactive towards nucleophilic thiol and amino groups [45] and has an important role in oxidative stress. Millimolar concentrations of 4-HNE cause depletion of glutathione, inhibition of DNA, RNA and protein synthesis being acutely

cytotoxic [60]. Like MDA, 4-HNE can be quantified in biological samples using different derivatization methods, the most used is the reaction with dinitrophenylhydrazine (DNPH) measured by spectrophotometry with absorbance maximum up to 360 – 390 nm. Chromatographic and immunohistochemical analyzes with antibodies against 4-HNE protein adducts can also be applied [61].

Isoprostanes (IsoPs) are prostaglandins (PG)-like compounds formed directly from arachidonic acid by non-enzymatic reaction with reactive oxidative species (ROS) resulting in four F_2-IsoPs regioisomers: 5-, 12-, 8- or 15-series, depending on the carbon atom to which the side chain hydroxyl is attached, and 64 stereoisomers of F_2-IsoPs, that become the analysis not trivial [18,27,62]. IsoPs are generated independent of cyclooxygenase (COX) by free radical-induced peroxidation of AA and other PUFAs. The first IsoPs species were isomeric to prostaglandin $F_{2\alpha}$ and, for this reason, termed F_2-IsoPs [62,63]. F_2-IsoPs differs from prostaglandins because they are formed *in situ* esterified to phospholipids and contain side chains predominantly oriented *cis* to the prostane ring, while prostaglandins are generated from free AA and present only *trans* side chains [27]. After its formation, F_2-IsoPs can be hydrolyzed to the free acid form by platelet activating factor acetyl-hydrolase (PAF-AH) [64]. Variety of analytical procedures has been applied to measure F_2-IsoPs, including liquid chromatography/mass spectrometry (LC/MS), gas chromatography/mass spectrometry (GC/MS) and immunological approaches [27,62,65].

Besides MDA, 4-HNE and F_2-IsoPs, oxLDL (**Figure 3**) has been also applied to determine oxidative stress associated to CVD. Modified LDL acts in all stages of atherosclerosis, being present in the arterial wall and in the circulation. This chemical modification can be result of oxidation, glycosylation or carbamylation, generating electronegative LDL, that partially loss affinity to the LDL receptor [33]. The classic method for modified LDL quantification is based on the initial isolation by ultracentrifugation, followed by the separation of native LDL by anion-exchange chromatography, applying stepwise sodium chloride (NaCl) gradients [33]. However, this classic methodology is complex and time-consuming. Thus, immunoassays have been used to oxLDL determination [33], being now more frequently detected using specific monoclonal antibodies that directly recognize unique oxidations specific epitopes, since MDA or 4-HNE modified-type adducts [9]. EO6 is a natural immunoglobulin M murine monoclonal antibody that binds to the phosphocholine head group of oxidized but not native phospholipids (PL). MDA2 is a murine IgG monoclonal antibody that recognizes MDA-modified proteins and lipid adducts [48]. The OxLDL-E06 ELISA assay quantifies oxidized phospholipids on apoB-100 molecules. The LDL-DLH3 acts in reverse to

the OxLDL-E06 ELISA assay by quantifying apoB-100 on oxidized phospholipid molecules, but uses different monoclonal antibodies for detection. The OxLDL-4E6 sandwich ELISA assay detects MDA-LDL and copper oxidized-LDL epitopes, and is commercially available for experimental use [35].

In function of the oxPLs role in oxLDL, it has been suggested that own oxPLs are biomarkers of atherosclerosis [11]. Phosphocholine (PC) is both pathogen associated molecular patterns (PAMP) and DAMP, recognized by multiple pattern recognition receptors of innate immunity [9]. Oxidized phospholipids (oxPLs) can be classified as (1) non-fragmented (full) when present the same number of carbons of the precursor; or (2) fragmented when present shorter chains. Both have capacity to bind with amino acids through the formation of Schiff-bases or Michael adducts [11]. The oxPLs analysis can be done by antibody-methods using monoclonal antibodies specific for oxidized PLs (E06, DLH3 and 5O9), being E06 able to recognize also PL-protein adducts and quantify PC-containg oxPLs on human oxLDL [11]. However, antibody methods present some limitation that have been overcome by liquid chromatography (LC) and mass spectrometry (MS), after high-resolution separation techniques, that become the analysis more complex [11].

5 The weakness correlation between main lipid oxidative markers and clinical endpoints.

According to the “National Institutes of Health (NIH)” a biomarker must be objectively measured and its value characterizes normal biological and pathogenic processes, or pharmacological responses to a therapeutic intervention [66]. The classical “4S Study” is an example of the straight correlation between LDL concentration in plasma and atherosclerosis progression in clinical trials [36]. Based on this correlation, several drugs such as statins, ezetimibe and PCSK9 were assessed and approved to reduce CVD mortality [2]. However, although it has been observed higher concentrations of MDA, 4-HNE and F2-IsoPs in biological samples of patients with different diseases when compared with healthy individuals [22, 58] the same degree of association stablished to LDL and CVD has not been reported to oxidative stress biomarkers. It appears that the levels of oxidized lipids correlate weakly with lesion development, despite the fact that established lesions contain increased concentration of fatty acids oxidation products compared with healthy arteries [8]. In addition, oxidative stress takes part of a multiple types of pathological conditions, that can be simultaneously present in some individuals. This characteristic becomes difficult to establish a direct association between the oxidative biomarker and the progression or regression of the atherosclerosis. Nonetheless, it

does not mean that there is no correlation, it just means that this correlation remains unclear and that it is necessary to better investigate the association among these markers and clinical endpoints, applying, for example, longer clinical studies using validated methods and big data.

In general, clinical trials that applied antioxidants supplements to reduce CVD have generated controversial results. This fact contributed to weak the hypothesis about the influence of oxidative stress on atherosclerosis development. On the other side, some studies have also showed important correlation between other products from lipid oxidation and clinical end-points. For example, higher concentrations of circulating and/or urinary F_2-IsoPs have been found in a wide of human clinical conditions [22,63], including atherosclerosis and CVD [27,64]. A systematic review investigated the relation between F_2-IsoP increase and cardiovascular disease, and identified a significant association in 20 studies out of 22 eligible [23]. F_2-IsoPs was also analyzed in 2828 subjects from Framningham Heart Study were positively associated with a history of cardiovascular disease [65]. However, Van't Eve *et al*. [67] classified the F2-IsoPs values reported in 242 publications and observed a low increase in CVD patients compared to healthy individuals. Thus, despite a number of studies on IsoPs, they are still not considered a clinically relevant biomarker [65]. In opposite, when MDA was determined in normo and hyperlipidemic subjects it was found a correlation ($r=0.61$, $p<0.05$) between MDA concentration (from 0.53 to 26.12 nmol/mL) and the atherogenic index calculated as "(Total cholesterol – HDL-cholestetol)/HDL-cholesterol" [68]. It was also observed in **Table 2**, that patients under primary and secondary prevention showed MDA concentrations about 3-fold higher than the values found in healthy individuals. 4-HNE concentration in human blood and serum was estimated to be around 0.05-0.15 µmol/L, but it can arise to more than 100 µmol/L in pathological situations, and it has been described in atherosclerotic lesions in both humans and animals [45]. Some studies showed that oxLDL could be generated *in vivo* in the artery wall of humans and was related to the extent of atherosclerosis in animal models and in some epidemiological studies [9], and according to Sánchez-Quesada *et al*. [33] oxLDL increases temporarily during the acute phase of myocardial infarction and could be a biomarker for the presence of unstable and ruptured plaque. Thus, although inconsistent results have been reported regarding the oxLDL connection with atherosclerosis, it could be an indicator of atherosclerosis and its severity if appropriate assays are developed [18].

However, it is important to consider that the increase of modified LDL can also be associate to the higher amount of LDL usually observed in patients with atherosclerosis [33]. For this reason, a ratio instead of the absolute values could be more useful as a biomarker. In

addition, oxPLs were also detected in plasma of patients with coronary artery disease [69]. In a study carried out with patients presenting calcific aortic valve stenosis (CAVs), it was found a progressive increase of oxPLs and MDA epitopes according to the CAVs severity grade [48]. Nonetheless, it has been suggested that native or aggregated LDL drives atherogenesis, beyond oxLDL [3], bringing more discussion about the oxidative hypothesis.

6 Next steps to include a lipid oxidative biomarker to compose the CVD risk scores algorithms.

Among the lipid oxidative biomarkers applied in clinical trials, F_2-IsoPs have been considered the most useful biomarker of oxidative damage *in vivo* in humans [62]. In addition, F_2-IsoPs determination is very precise in non-invasive biological samples (blood and urine), and reflects directly action of non-enzymatic pro-oxidants, avoiding the influence of other factors than the direct oxidation of fatty acids by ROS. However, as commented by Ho *et al*. [35], the clinical utility of a biomarker includes the ease and cost of measurement, its performance characteristics and evidence for guiding management and improving patient outcome. The analytical methods applied to determine F_2-IsoPs are costly, being limited to very trained technicians and demanding expensive chromatography analysis and standards. For these reasons, although F_2-IsoPs are relatively stable and has been consider the best option as a lipid marker [62], as discussed in our review, MDA quantification in plasma seems simpler than F2-IsoPs and could be a good option if its variability is reduced. Thus, it is essential to take care about the pre- and post-analytical factors that influence the results, standardizing the chromatographic methodologies and controlling all variables that could promoted some variability that has not relation with the disease.

Considering that MDA can be an adequate parameter to measure lipid oxidation in biological samples obtained from clinical trials, we suggest that the following sequence could contribute to include a lipid oxidative marker as part of the algorithms used to estimate the CVD risk:

1) Standardization of procedures to obtain and store the biological samples,
2) Validation of high-performance liquid chromatography (HPLC) methodology using fluorimetric or mass detector by various laboratories working with the same samples, considering factors associated to intra and inter variability,

3) Multicenter studies using validated methodology (HPLC) involving patients under primary (group 1) and secondary (group 2) prevention paired with healthy individuals (group 3), during a longer time interval,
4) Agreement about reference values according to these three groups.

After these four initial steps, it would be necessary an effort to include MDA as biomarker and follow the values at different steps of the disease. Using data obtained from different studies, it would be possible to include MDA as variable in the algorithms, and apply the statistical methods to evaluate the contribution of this oxidative marker to improve risk stratification. According to Milne [64], this is the coming age for the development of reliable biomarkers in redox biology. Clinical researchers need to team up to design studies that go beyond just validating the association of a marker with severity of disease [36].

7 Conclusions

Based on enough evidence of the oxidative stress contribution to the pathogenesis of atherosclerosis, it is necessary to include an oxidative biomarker as routine in the clinical trials. Among them, MDA seems to be viable and adequate marker of lipid oxidation. However, MDA variability must be reduced by multicenter studies using chromatographic validated methodology to analyze the samples. In addition, it is also necessary to achieve a consensual reference value for patients under primary or secondary prevention paired with healthy individuals. Finally, it would be necessary to include MDA in the risk score and evaluate the prediction improvement compared with the current scores.

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Declaration of competing interest

The authors have no conflict of interest to declare.

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Table 1: The general characteristics of 116 treatments (44 placebo and 72 treatment groups) from 55 studies selected according to criteria showed in **Supp. Figure 1**. It was included parameters present in at least 10 of 116 treatments.

Characteristics	unit	Treatments (n)	Mean ± SD	Range	CV(%)
Individuals	n	116	26.90 ± 17.17	6.00 – 84.00	63.83
Body Weight (BW)	Kg	56	72.74 ± 6.31	57.20 – 83.60	8.67
Body Mass Index (BMI)	kg/m^2	98	27.04 ± 2.31	22.30 – 33.86	8.56
Waist Circumference **(**WC)	cm	30	93.90 ± 8.87	76.78 – 109.13	9.45
Body Fat (BF)	%	15	32.12 ± 3.57	25.60 -35.70	11.13
Age	years	79	52.81 ± 11.51	21.40 – 68.40	21.80
Blood pressure systolic (BPS)	mmHG	52	127.27 ± 15.86	97.14 – 191.10	12.46
Blood pressure diastolic (BPD)	mmHG	52	78.87 ± 9.92	63.57 – 108.00	12.58
Glucose	mg/dL	61	129.84 ± 37.13	79.86 – 219.00	28.59
Insulin	µIU/L	32	13.23 ± 4.43	6.26 – 22.80	33.50
Glycated Hemoglobin **(**HbA1c)[1]	%	24	7.18 ± 1.02	5.35 – 8.83	14.17
Homeostasis Model Assessment Insulin Resistance (HOMA-IR)	index	30	4.31 ± 1.68	1.47 – 7.94	39.03
Quantitative insulin sensitivity check index (QUICKI)	index	12	0.32 ± 0.01	0.30 – 0.34	4.41
Alanine aminotransferase (ALT)	U/L	17	23.50 ± 8.37	10.86 – 41.80	35.62
Aspartate aminotransferase (AST**)**	U/L	12	21.61 ± 3.52	17.36 – 28.50	16.29
Creatinine	mg/dL	18	0.88 ± 0.23	0.74 – 1.60	16.29
Total cholesterol (TC)	mg/dL	116	194.53 ± 29.49	119.70 – 258.50	15.16
Triacylglycerol (TG)	mg/dL	116	133.75 ± 38.36	53.00 – 238.27	28.68
High density lipoprotein (HDL)	mg/dL	116	49.63 ± 8.93	32.00 – 76.00	17.99
Low density lipoprotein (LDL)	mg/dL	116	117.34 ± 25.98	64.20 – 174.00	22.14
Non-HDL	mg/dL	11	162.24 ± 32.27	105.15 – 197.00	19.89
Very Low density lipoprotein VLDL	mg/dL	25	33.76 ± 8.98	22.60 – 47.49	26.61
Frap[2]	mmol/L	34	367.32 ± 467.73	0.07 – 1,118.30	127.34
Superoxide dismutase (SOD)	U/g Hb	13	17,170.85 ± 25,409.67	1.90 – 88,900.00	147.98
Superoxide dismutase (SOD)[3]	U/ml	11	22.23 ± 36.42	0.16 – 96.20	163.82
Glutathione (GSH)	µmol/L	24	335.49 ± 274.90	5.30 – 763.95	81.94
Malondialdehyde (MDA**)** [4]	µmol/L	72	13.56 ± 31.98	0.002 – 173.77	235.85
Oxidized low-density lipoprotein(oxLDL)	U/L	18	59.20 ± 23.92	31.00 – 108.00	40.40
F_2-IsoPs (urine)	pg/mg creatinine	14	3,844.29 ± 8,694.11	62.99 – 26,600.00	226.16
F_2-IsoPs (plasma)[5]	pg/ml	11	846.99 ± 795.41	239.00 – 3,150.00	93.91
High-sensitivity C-reactive protein hs-(CRP)	mg/L	39	5.63 ± 7.87	0.57 – 33.60	139.94
TNFα	pg/ml	10	12.56 ± 16.49	1.00 – 44.22	131.32
Interleukin 6 (IL-6)	pg/ml	13	2.25 ± 1.14	0.58 – 4.30	50.92

[1] *Glycated Hemoglobin: it was excluded the value reported by Whittaker et al. [49]: 56.0 and 57.3%*

[2] *Frap: it was excluded the value reported by Bento et al. [50]: 701.8 and 719.6 mmol TE/mL*

[3] *SOD (U/mL): it was excluded the value reported by Choi et al. [51]: 1,016.0 and 996 U/ml*

[4] *MDA: it was excluded the value reported by Bermejo et al. [52]:66.8 and 62.3 (µmol MDAeq/mL) and Barzegari et al. [53]: 1.4 and 1.5 mmol/L*

[5] *F_2-IsoPs (plasma): it was excluded the value reported by Fayh et al. [54]: 325.9 and 284.5 ng/mL*

Table 2: Comparison of the general characteristics according to the groups represented by (1) healthy individuals and (2) patients under primary or secondary prevention. It was included parameters present in at least 10 treatments on at least one group.

		Groups		
Characteristics	**unit**	**(1)**	**(2)**	***p*[1]**
Individuals	n	22.92 ± 12.64 (n=38)	28.83 ± 18.76 (n=78)	*0.134*
Body Weight (BW)	Kg	69.47 ± 6.38 (n=23)	75.02 ± 5.23 (n=33)	*0.001*
Body Mass Index (BMI)	kg/m^2	25.34 ± 1.76 (n=29)	27.75 ± 2.15 (n=69)	*<0.001*
Waist Circumference **(**WC)	cm	87.83 ± 10.57 (n=8)	96.11 ± 7.23 (n=22)	*0.021*
Age	years	46. 08 ± 18.09 (n=21)	52.25 ± 6.62 (n=58)	*0.274*
Blood pressure systolic (BPS)	mmHG	117.84 ± 12.54 (n=18)	132.27 ± 15.28 (n=34)	*0.002*
Blood pressure diastolic (BPD)	mmHG	73.44 ± 7.02 (n=18)	81.75 ± 10.11 (n=34)	*0.011*
Glucose	mg/dL	84.80 ± 6.61 (n=10)	138.67 ± 34.07 (n=51)	*<0.001*
Insulin	µIU/L	9.94 ± 4.12 (n=4)	13.70 ± 4.34 (n=28)	*0.114*
Glycated Hemoglobin **(**HbA1c)	%	5.65 ± 0.18 (n=3)	7.40 ± 0.89 (n=21)	*0.003*
Homeostasis Model Assessment Insulin Resistance (HOMA-IR)	index	2.54 ± 0.80 (n=4)	4.58 ± 1.62 (n=26)	*0.011*
Quantitative insulin sensitivity check index (QUICKI)	index	-	0.32 ± 0.01 (n=12)	-
Alanine aminotransferase (ALT)	U/L	16.40 ± 5.91 (n=4)	25.69 ± 7.92 (n=13)	*0.062*
Aspartate aminotransferase (AST**)**	U/L	22.50 ± 0.71 (n=2)	21.43 ± 3.86 (n=10)	*0.713*
Creatinine	mg/dL	0.77 ± 0.03 (n=4)	0.91 ± 0.25 (n=14)	*0.089*
Total cholesterol (TC)	mg/dL	193,54 ± 22.82 (n=38)	195.01 ± 32.38 (n=78)	*0.995*
Triacylglycerol (TG)	mg/dL	102.44 ± 27.61 (n=38)	149.01 ± 33.35 (n=78)	*<0.001*
High density lipoprotein (HDL)	mg/dL	56.39 ± 6.92 (n=38)	46.34 ± 7.90 (n=78)	*<0.001*
Low density lipoprotein (LDL)	mg/dL	117. 08 ± 19.27 (n=38)	117.47 ± 28.80 (n=78)	*0.869*
Very Low density lipoprotein VLDL	mg/dL	23.70 ± 1.41 (n=2)	34.63 ± 8.83 (n=23)	*0.064*
Frap	mmol/L	0.43 ± 0.31 (n=13)	594.44 ± 468.32 (n=21)	*0.002*
Superoxide dismutase (SOD)	U/g Hb	183.00 ± 9.90 (n=2)	20,259.55 ± 26,581.39 (n=11)	*0.199*
Superoxide dismutase (SOD)	U/ml	-	22.23 ± 36.42 (n=11)	-
Malondialdehyde (MDA**)**	µmol/L	5.07 ± 7.54 (n=21)	17.05 ± 37.24 (n=51)	*0.041*
Oxidized low-density lipoprotein(oxLDL)	U/L	54.38 ± 19.72 (n=10)	62.23 ± 28.54 (n=8)	*0.354*
High-sensitivity C-reactive protein hs-(CRP)	mg/L	1.87 ± 0.79 (n=9)	6.75 ± 8.68 (n=30)	*0.010*

[1]*P value obtained by T test or Mann-Whitney test for independent groups*.

Figure Caption

Figure 1: Pro- and antioxidants involved in the early steps of atherosclerosis. PUFAs, that are esterifying phospholipids in the LDL surface, can be oxidized forming secondary products, including F2-IsoPs, MDA, 4-HNE and others. These products of PUFAs oxidation, in turn, can reduce the glutathione concentration (GSH) in their metabolism, leading to the reduction of antioxidant defense of the cell. Moreover, the activation of NADPH oxidase, COX, LOX, GO, XO and the uncoupled NOS, caused by different stimulus, promote the increase of superoxide anion ($O_2^{-\bullet}$), that can be partially neutralized by the antioxidant enzymes, such as SOD, GPx and catalase. However, the H_2O_2 formed during this neutralization processes and also by ETC in the mitochondria, can also react with transition metals forming the hydroxyl radical ($^{\bullet}OH$), able to oxide more PUFAs. Furthermore, some secondary products of PUFAs oxidation, including 4-HNE and MDA, are able to form adducts with amino acids present in Apo B lipoprotein present in the LDL surface, characterizing the oxLDL. This adduct, formed by aldehydes and aminoacids, acts as a DAMP, and is recognized by scavenger receptors in the macrophages, that in turn, phagocyte the ox LDL, forming foam cells, generating fatty streaks and plaques [25,26,28,32-34]. Abbreviations: XO, xanthine oxidase; ETC, electron transport chain; COX, cyclooxygenase; LOX, lipoxygenase; GO, glucose oxidase; NOS, nitric oxide synthase; NOX, NAPPH oxidase; NO nitric oxide; ONNO-, peroxynitrite; PRDX, peroxiredoxins; MPO, myeloperoxidase.

Figure 2: Projection of variables on the fator-plane (F1 x F3) (**Fig.2A**) showing the factor loadings based on correlations (F1:F3), Tree Diagram for variables (**Fig.2B**) and Tree Diagram for cases (heatmap) (**Fig.2C**). Abbreviations: MDA, malondialdehyde; HDL, high-density-lipoprotein; LDL, low-density-lipoprotein; TC, total cholesterol, BMI, body mass index, AGE, TG, triacylglycerol. Principal Component Analysis was based on correlation and Cluster Analysis was carried out on standardized variables, adopting Ward's method and Euclidean distance, using Statistica v.13 (TIBCO Software Inc., Palo Alto, USA), Heatmap was based on the same parameters applied in the Cluster Analysis using R Studio software.

Figure 3: Some secondary products of PUFAs oxidation applied as a biomarker in clinical trials involving atherosclerosis [45,27,57,34].

Supplementary Figure Caption

Suppl.Figure 1: Criteria adopted to select and classify the articles applied in the multivariate analysis.

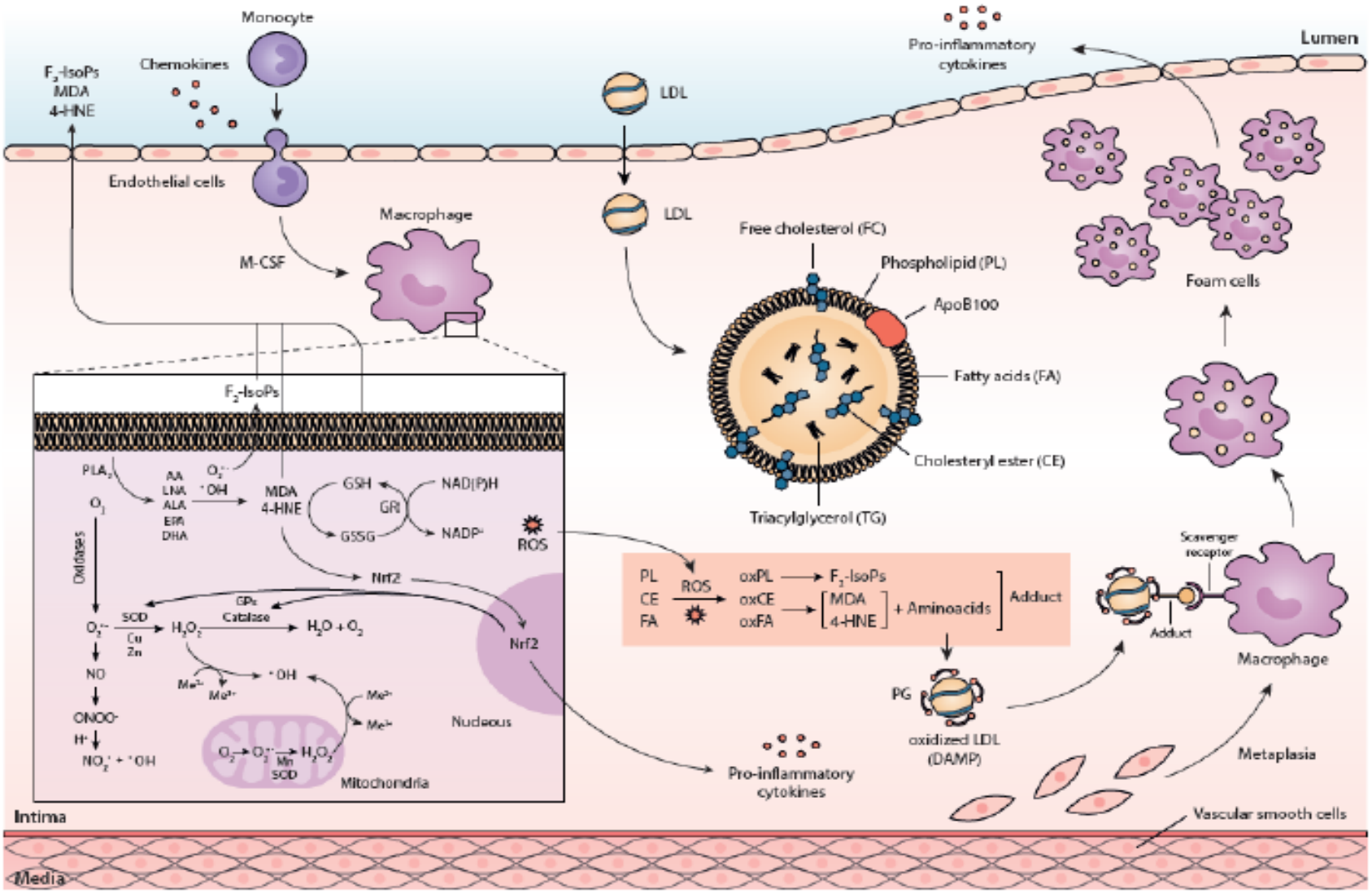
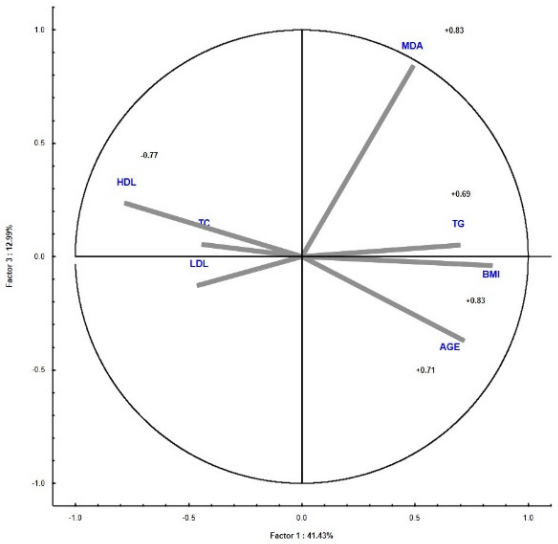
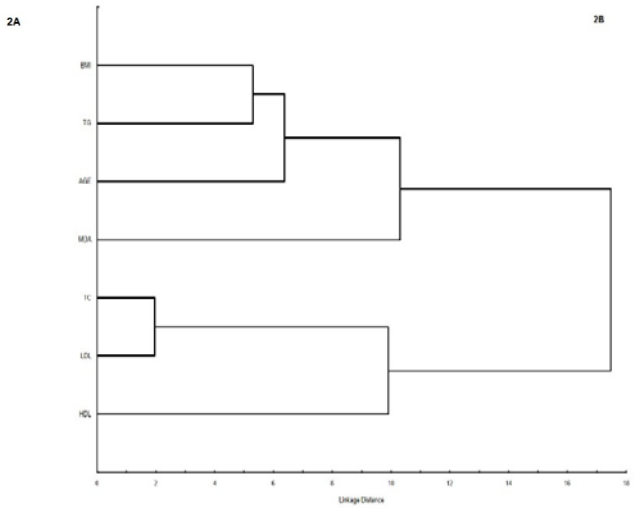

Monocyte
Chemokines
F2-IsoPs
MDA
4-HNE
Endothelial cells
LDL
LDL
Pro-inflammatory cytokines
Lumen
Macrophage
M-CSF
Free cholesterol (FC)
Phospholipid (PL)
ApoB100
Fatty acids (FA)
Cholesteryl ester (CE)
Triacylglycerol (TG)
Foam cells
F2-IsoPs
PLA2
AA
LNA
ALA
EPA
DHA
MDA
4-HNE
GSH
GSSG
GR
NAD(P)H
NADP+
ROS
Oxidases
Nrf2
SOD
Cu
Zn
GPx
Catalase
H2O + O2
NO
ONOO-
Nucleous
Mitochondria
Mn
SOD
PL
CE
FA
ROS
oxPL
oxCE
oxFA
F2-IsoPs
MDA
4-HNE
+ Aminoacids
Adduct
PG
oxidized LDL
(DAMP)
Scavenger receptor
Adduct
Macrophage
Metaplasia
Pro-inflammatory cytokines
Vascular smooth cells
Intima
Media

Figure 1


MDA
+0.83
HDL
-0.77
TC
LDL
TG
+0.69
BMI
+0.83
AGE
+0.71
Factor 1 : 41,43%
Factor 2 : 12,99%


2A
2B
BMI
TG
AGE
MDA
TC
LDL
HDL
Linkage Distance

2C

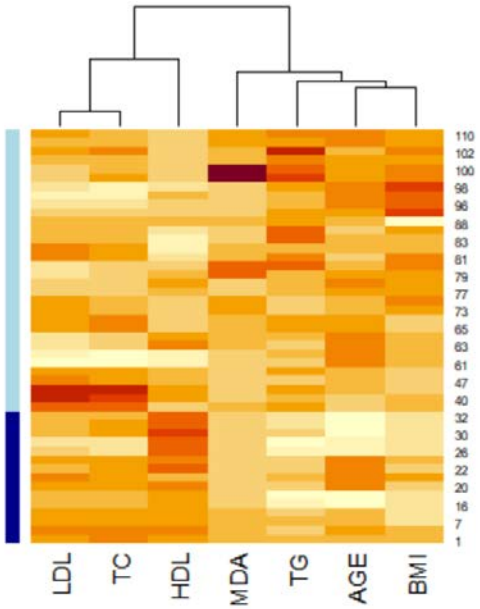

110
102
100
98
96
88
83
81
79
77
73
65
63
61
47
40
32
30
26
22
20
16
7
1
LDL
TC
HDL
MDA
TG
AGE
BMI

Figure 2

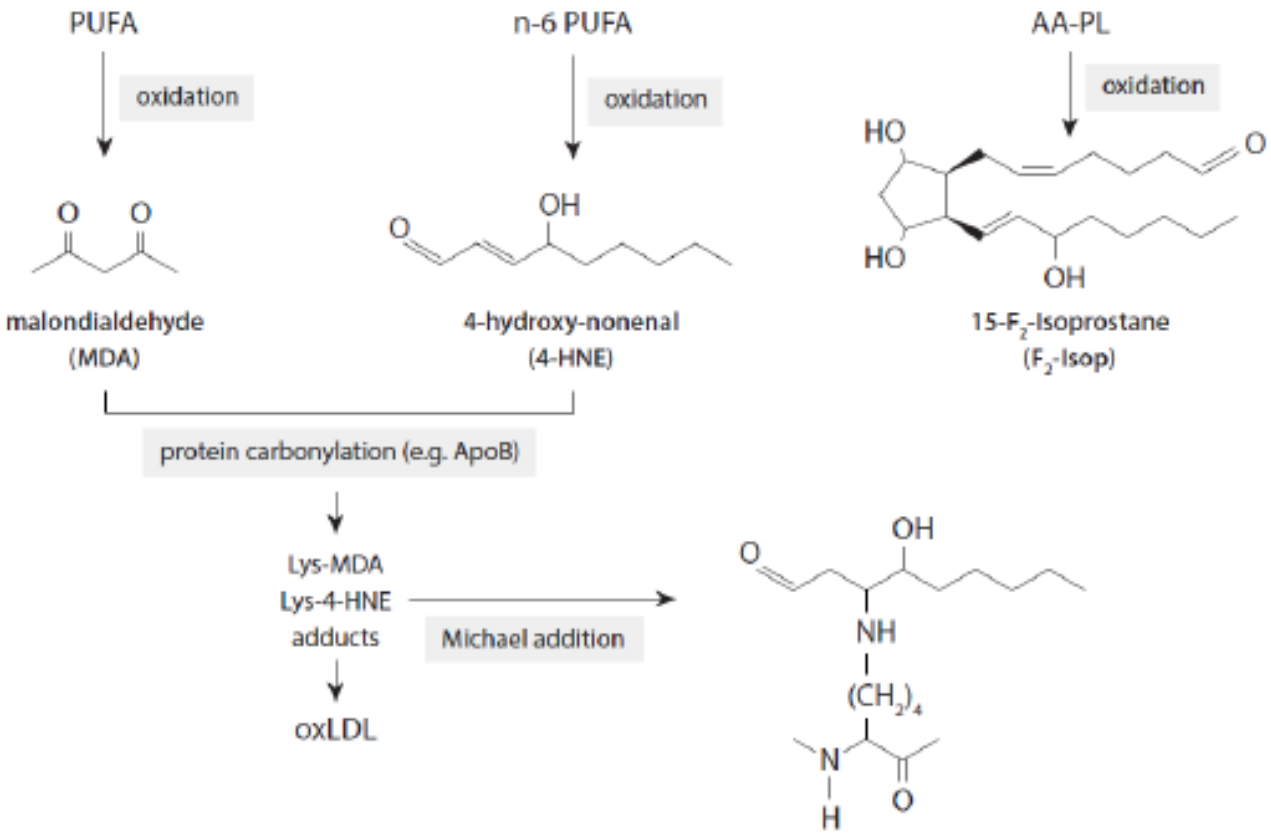
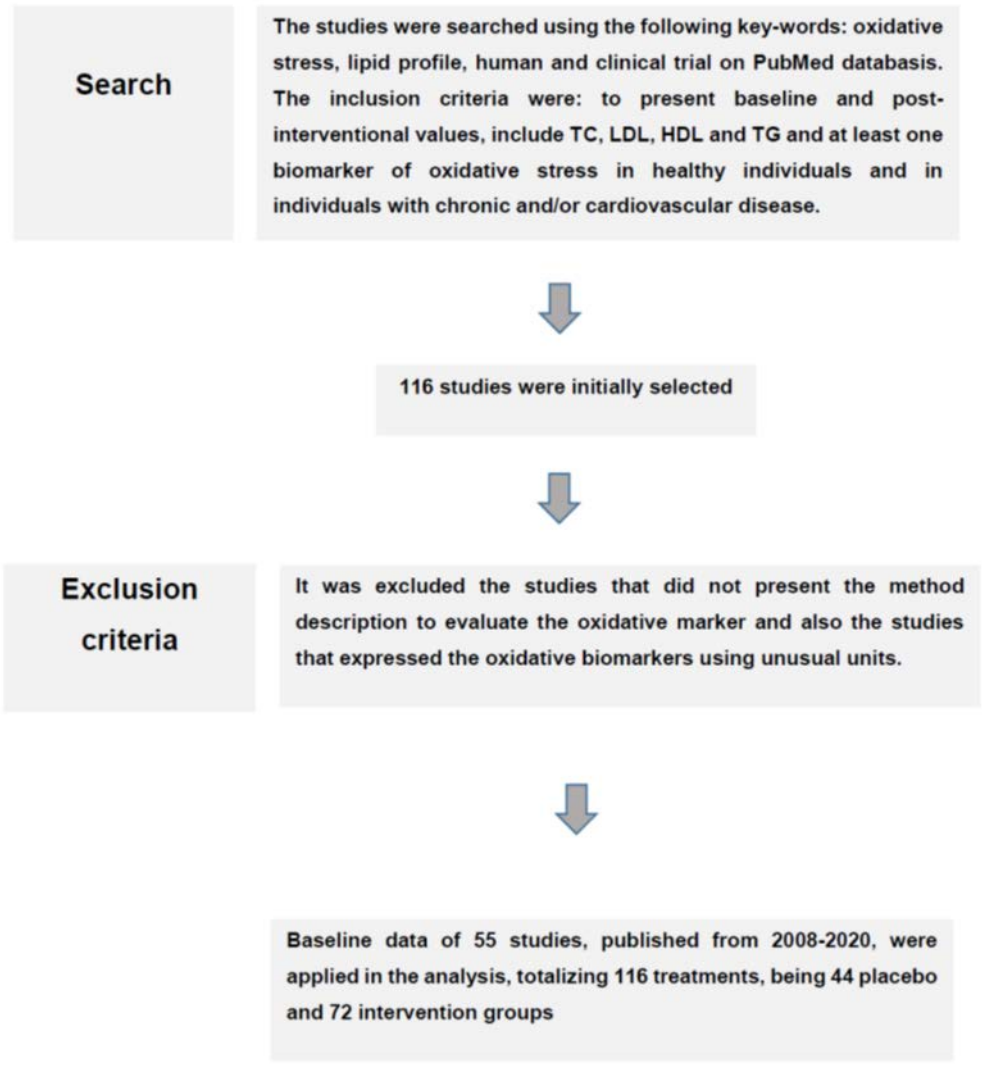

PUFA
oxidation
malondialdehyde
(MDA)
n-6 PUFA
oxidation
4-hydroxy-nonenal
(4-HNE)
AA-PL
oxidation
15-F2-Isoprostane
(F2-Isop)
protein carbonylation (e.g. ApoB)
Lys-MDA
Lys-4-HNE
adducts
Michael addition
oxLDL

Figure 3



Suppl.Figure 1: Criteria adopted to select and classify the articles applied in the multivariate analysis.

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FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

Our study demonstrated the wide variability of oxidative stress biomarker in biological sample through the survey of clinical studies and the use of statistical tools. Despite to the enormous variability of the results obtained with different methodologies, our results help to strengthen the relationship between oxidative stress and the atherosclerosis development. However, the available results are still not sufficient to include a biomarker of oxidative stress in the clinical practice. Among the numerous markers evaluated, MDA was the most frequent and the most accessible methodologies for large-scale use.

As pointed out throughout our study, it is necessary to standardize the biomarker, methodology and individual´s profile in order to obtain homogeneous results that justify the inclusion of a lipid biomarker as an independent risk factor for atherosclerosis.

The future perspectives are summarized in Figure 5. This knowledge will contribute to combine drugs and supplements, customizing the patient's treatment.

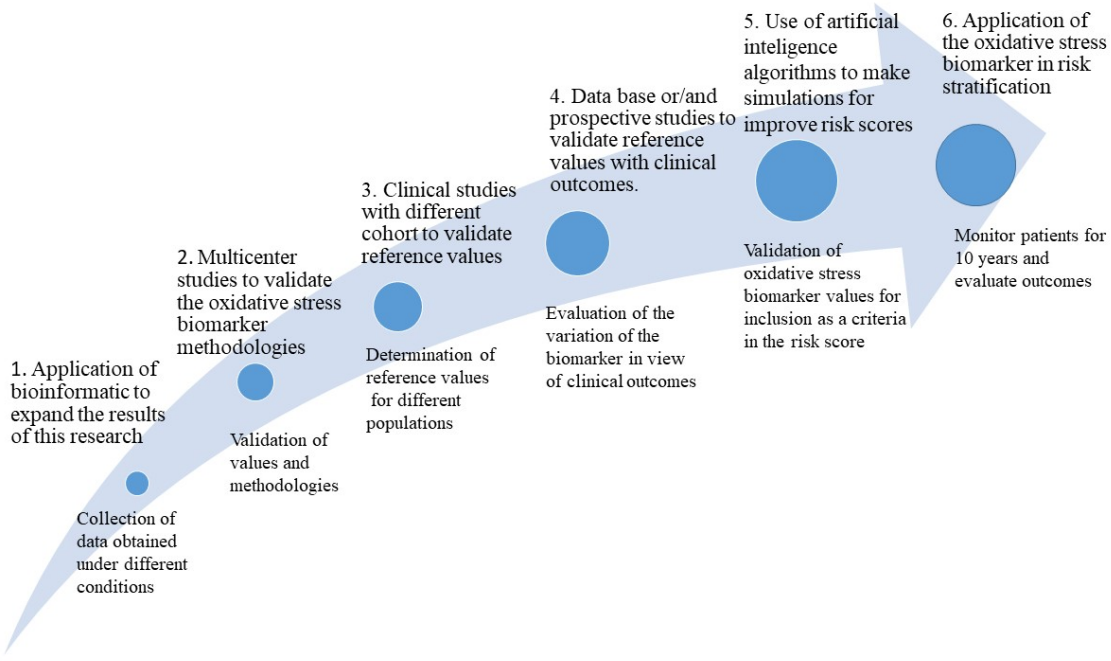

Figure 5: Suggestion of next steps for the validation of oxidative stress biomarkers