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In vivo and *in vitro* data supporting the involvement of oxidative stress in aging-related and metabolic diseases

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“The same thing that makes you
live can kill you in the end.”

Neil Young

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ABBREVIATIONS

8-OH-dg 8-hydroxy-2' -deoxyguanosine

AD Alzheimer's Disease

Akt protein kinase B

ANCOVA Analysis of covariance

ANN American Academy of Neurology

ANOVA Analysis of variance

AOPP Advanced Oxidation Protein Products

ApoE Apolipoprotein E

APP Amyloid Precursor Proteins

Aβ β-amyloid peptides

BAP Bone-specific Alkaline Phosphatase

BMD Bone Mass Density

BMI Body Mass Index

BSA Bovine Serum Albumin

CI Confidence Interval

CNS Central Nervous System

CSF Cerebrospinal Fluid

CT Computer Tomography

CTX-1 C-terminal telopeptide of type I collage

CU Carratelli Unit

CVD Cardiovascular Disease

DCF 5-(and-6)-carboxy-2,7 dichlorodihydrofluorescein diacetate

DXA Discovery dual energy X-ray Absorptiometry scanner

E_2 17-βestradiolo

EC (-)-Epicatechin

ECM (-)-Epicatechin Metabolites

EOA Early Onset Alzheimer

F_2-iso 8-isoprostane

FAD Familial Alzheimer's disease

FFAs Free Fatty Acids

FRAP Ferric Reduction Antioxidant Capacity

FSH Follicle-Stimulating Hormone

GDS Global Deterioration Scale

GLUT4 Glucose Transporter 4

GPx Glutathione Peroxidase

GSH Glutathione reduced

GSK3glycogen synthase kinase 3

GSSH Glutathione oxidized

H_2O_2 Hydrogen peroxide

HPOST Healthy Postmenopausal women

HRA Healthy Reproductive Age

HY Hydroperoxydes

IKK IκB kinase

IL-1 Interleukin 1

IL-6 Interleukin 6

IR Insulin Receptor

IRS Insulin Receptor Substrate

JNK c-Jun N-terminal kinase

LOAD Late Onset Alzheimer's Disease

MCI Mild Cognitive Impairment

MeS Metabolic Syndrome

MMSE Mini Mental State Examination

MRI Magnetic Resonance Imaging

NADPH oxidase Nicotinamide Adenine Dinucleotide Phosphate-Oxidase

NFT Neurofibrillary Tangles

NINCDS-ADRDA National Institute of Neurological and Communicative Disorders and Stroke the Alzheimer's Disease and Related Disorders Association

O_2 Molecular oxygen

$O_2^{\cdot -}$ superoxide anion

OPG Osteoprotegerin

OPOST Osteoporotic Postmenopausal women

OR Odds Ratio

OxS Oxidative Stress

Pal Palmitate

PDK1 phosphoinositide-depend kinase 1

PERI Perimenopausal women

PET Positron Emission Tomography

PI3-K phosphatidylinositol-3'-kinase

PIP_2 phosphatidylionositol-(4-5)-bisphosphate

PIP_3 phosphatidylionositol-(3,4,5)-trisphosphate

PO Postmenopausal Osteoporosis

POST Postmenopausal women

RANK Receptor Activator of Nuclear factor kappa-B

RANKL Receptor Activator of Nuclear factor kappa-B Ligand

RAP Residual Antioxidant Power

rCBF Regional Cerebral Blood Flow

ROS Reactive Oxygen Species

RT-PCR Real time Reverse Transcription Polymerase Chain Reaction

SD Standard deviation

SEM Standard error of the mean

SOD Superoxide dismutase

SREM Structurally Related (-)-Epicatechin Metabolites

T2DM Type 2 Diabetes Mellitus

TAP Total Antioxidant Power

TH Thiols

TNFα Tumor Necrosis Factor

UA Uric Acid

VaD Vascular Dementia

W/H Weight/Hip

τ tau protein

GENERAL INTRODUCTION

OXYGEN: A CLEAR EXAMPLE OF BIOLOGIC PARADOX

Oxygen plays a vital role in the breathing processes and in the metabolism of living organisms. In the human body, the oxygen is absorbed by the blood stream in the lungs, being then transported to the cells. Appropriate levels of oxygen are vital to support cell respiration. Human organism consumes almost 90% of oxygen to generate energy by mitochondrial oxidative phosphorylation, which is the culmination of a series of energy transformations, defined as cellular respiration. The remaining 10% of oxygen is used as an essential cofactor in the hydroxylation and oxidation reactions catalyzed by enzymes such as xantine oxidase and NADPH oxidase (Berg *et al*, 2002). All of these processes lead to the inevitable production of oxidant species that play an important role in aging and in the development of aging-related diseases.

1. OXIDANT SPECIES

2.1 Free Radicals

Oxidant agents are all those species that can subtract one or more electrons from other molecules (Figure 1). They are mostly represented by free radicals, i.e. molecules or molecular fragment containing one or more unpaired electrons in atomic or molecular orbitals. Free radicals are species very reactive and short-lived in a biological setting and they can react with any molecular types. Redox reactions include all chemical reactions in which atoms have their oxidation state changed; in general, redox reactions involve the transfer of electrons between species, thus, both oxidation and reduction.

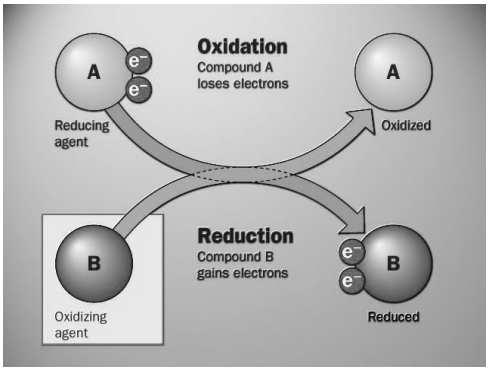


Figure 1. Oxidation-Reduction. Redox reactions include all chemical reactions in which atoms have their oxidation state changed; in general, redox reactions involve the transfer of electrons between species. (Taken from http://iws.collin.edu)

The radicals derived from oxygen are called reactive oxygen species (ROS) and are predominantly synthesized inside the cell. There are other reactive species, such as reactive nitrogen species (RNS) that can cause oxidative damage (Table 1). All of them are products of normal cellular metabolism.

Chemical species	Formula
Single oxygen	$^1O_2$
Hydrogen peroxide	H_2O_2
Superoxide anion	$O_2^{\cdot -}$
Hydroxyl radical	$HO^{\cdot}$
Nitric oxide	$NO^{\cdot}$
Nitric peroxide	$ONOO^-$
Peroxyl radical	$ROO^{\cdot}$
Hydroperoxides	ROOH

Table 1: Free radical species

2.2 Reactive Oxygen Species (ROS)

Molecular oxygen (O_2) has a unique electronic configuration and is itself a radical. It has two electrons unpaired on antibonding orbitals (Figure 2).

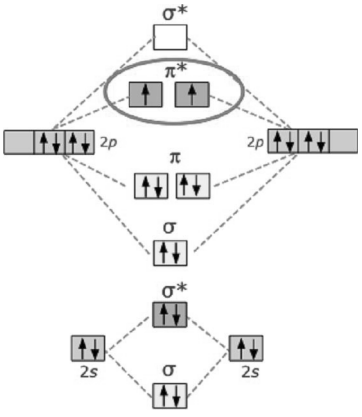


Figure 2. Orbital configuration of O_2. O_2 is a diatomic molecule, which contain 16 electrons. O_2 is a paramagnetic molecule due to presence of unpaired electron.

The high availability of oxygen in the organism makes reactive oxygen species (ROS) the most important oxidizing agents in the body; all ROS types have unpaired valence electrons or unstable bonds.

ROS can be neutral molecules (e.g. H_2O_2), ions (e.g. $O_2^{\cdot -}$) or radicals (e.g. $HO^{\cdot}$). Typically the initial reaction is a one electron transfer to O_2 to form superoxide, which then is converted (via

spontaneous or enzyme-catalyzed reactions) to H_2O_2. H_2O_2 can react with metal in reduced form, iron (Fe^{2+}) and copper (Cu^{2+}), to form hydroxyl radical and hydroxyl anion (OH^-) through the Fenton reaction (Fenton, 1894):

$$\mathbf{Fe^{2+} + H_2O_2 \rightarrow OH^- + Fe^{3+} + HO^{\cdot}}$$
$$\mathbf{Cu^{2+} + H_2O_2 \rightarrow OH^- + Cu^{3+} + HO^{\cdot}}$$

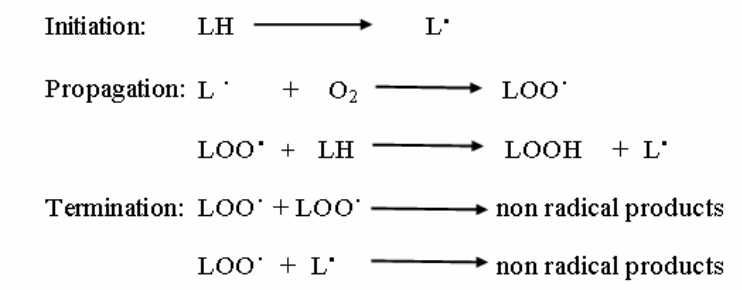
Although the major parts of free radicals are formed by Fenton reaction, the Haber-Weiss reaction (Koppenol, 2001) uses an iron ion catalyst to generate hydroxyl radicals from superoxide and hydrogen peroxide:

$$\mathbf{O_2^{\cdot -} + Fe^{3+} \rightarrow Fe^{2+} + O_2}$$
$$\mathbf{Fe^{2+} + H_2O_2 \rightarrow OH^- + Fe^{3+} + HO^{\cdot}}$$

$HO^{\cdot}$ is able to chemically modify the lipids (and all the other types of biomolecules) by triggering an autocatalytic process called oxidative peroxidation, which form new reactive species as hydroperoxides (ROOH), peroxyl radicals ($ROO^{\cdot}$) and alcossil radicals (RO □); these species increase the oxidative damage and are called reactive oxygen metabolites (ROM).

The oxidative peroxidation is a whole of chain reactions through which reactive species can be generated from generic organic substrates such as lipids, amino acids or carbohydrates. The radical chain reactions consist of three principal steps (Scheme 1):

1. ***Initiation:*** the radical is produced (L □) by attack of ROS (e.g. OH □) to generic organic substrate (LH) as lipids, proteins etc;
2. ***Propagation:*** the radical that was formed is not a stable molecule, so it reacts readily with molecular oxygen (O_2), thereby creating a peroxyl radical ($LOO^{\cdot}$). This radical is also an unstable species that reacts with another organic substrate, producing a different radical. This cycle continues, as the new radical reacts in the same way;
3. ***Termination:*** the radical chain reaction stops when two radicals react and produce a non-radical species. This happens when the concentration of radical species is high enough to have higher probability of collision of two of them. Furthermore living organisms have different molecules that speed up termination by catching free radicals and, therefore, protecting the cell membrane. These molecules are called antioxidant.



Scheme 1: Oxidative peroxydation. Consist in three phases: initiation (radical is produced by ROS attack), propagation (the radical formed reacts with O_2 to form another radical, LOO □ which in turn reacts with O_2 again) and termination (radical propagation is stopped or because two radicals react to each other or because of the presence of antioxidant specie).

2. SOURCES AND TARGETS OF ROS IN THE LIVING SYSTEM

Mitochondrial respiratory chain as well as enzymatic reactions by NADPH oxidase, xanthine oxidase, cyclooxygenases and lipoxygenase, are the most important sources of production of ROS.

2.1 NOX Family

A major source of ROS generation is the NOX family of NADPH oxidase. The NADPH oxidase complex was first described in neutrophils, where it is normally inactive (Lambeth et al, 2004). Upon exposure to bacteria and other pathogens, NADPH oxidase catalyzes the formation of $O_2^{\cdot-}$, which undergoes further modifications to generate a spectrum of ROS. However, NADPH oxidase is present in most cell types. NOX-derived ROS play an important physiological role in response to stimulation of various growth factors, cytokines and hormones, and have phatophysiological roles in endothelial dysfunction, inflammation, apoptosis, fibrosis, angiogenesis and important processes underlying diabetes and liver injury (Berdard and Krause, 2007). NADPH oxidase is a multi-subunit enzyme composed of membrane-bound components ($gp91^{phox}$ and $p22^{phox}$) and cytosolic components ($p47^{phox}$, $p67^{phox}$, and Rac1). In resting cells, this multi-component enzyme is inactive and its components are dispersed between cytosol and membrane. Under stressful conditions the cytosolic subunits translocate to the membrane upon activation (Figure 3).

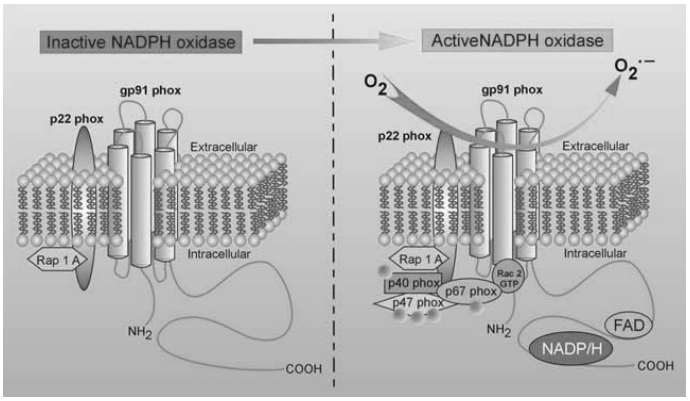


Figure 3. Activation of NADPH oxidase complex. In resting cells, this multicomponent enzyme system is inactive, and its components are dispersed between the cytosol and the membranes. The flavocytochrome b_{558} component, which is composed of two subunits, gp91^{phox} and p22^{phox}, is located in the plasma membrane and in specific granules. The other components of the NADPH complex (p47^{phox}, p67^{phox}, p40^{phox} and Rac1) are cytosol proteins. Stress conditions trigger the phosphorylation of the p47^{phox}, p67^{phox} and p40^{phox} cytosolic components and their translocation to the plasma membrane. This translocation leads to the activation of NADPH oxidase complex. (Taken from http://www.genkyotex.com_genkyotex-the nox company).

Once the multi-subunit complex is formed, $O_2^{\bullet-}$ production is catalyzed by the transfer of a single electron from NADPH to molecular oxygen by the following reaction:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet-}$$

The $O_2^{\bullet-}$ is quickly converted by superoxide dismutase (SOD) to produce H_2O_2. H_2O_2 is a mild oxidant that is a direct source of other ROS. The prototypal of NADPH oxidase is NOX2, which was discovered first in phagocytes. A new family of oxidase, the NOX family, has been defined on the basis of their homology with gp91^{phox}/ NOX2 catalytic subunits of phagocyte NADPH oxidase. In mammals, six NOX2 homologues have been identified: NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2 (Figure 4); these share many structural features with NOX2, but even if activation mechanism of NOX1 and NOX3 include the cytosolic subunits, similar to NOX2, NOX4 and NOX5 are different. NOX4 may be constitutively active and NOX5, with DUOX1 and 2 have a calcium-binding site and they are activated by Ca^{2+}. Most mammalian cell types, at least, express one NOX isoform, but many cells express many of them (Berdard and Krause, 2007).

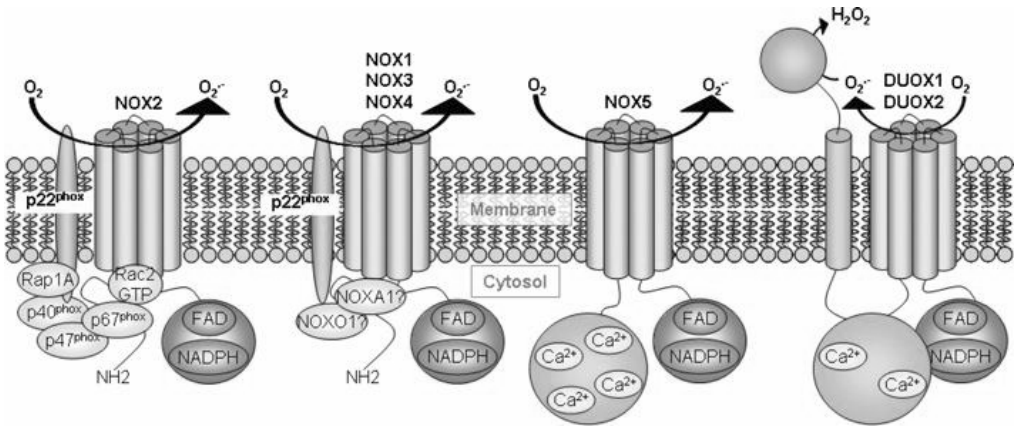


Figure 4. NADPH oxidase family. NOX family has been defined on the basis of their homology with the gp91^{phox}/NOX2 catalytic subunit of phagocyte NADPH oxidase. To date, six homologues (NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2) with levels of identity with NOX2 have been identified. (Taken from Berdad and Krause, 2007).

2.2 Oxidative Phosphorylation

During cellular respiration electrons are transferred from electron donors to electron acceptors. Oxidative phosphorylation is the process in which ATP is formed as a result of the transfer of electrons from NADH or $FADH_2$ to O_2 by a series of electron carriers (Figure 5). The flow of electrons from NADH or $FADH_2$ to O_2 through protein complexes located in the mitochondrial inner membrane leads to the pumping of protons out of the mitochondrial matrix. The resulting uneven distribution of protons generates a pH gradient and a transmembrane electrical potential that creates a proton-motive force. ATP is synthesized when protons flow back to the mitochondrial matrix through an enzyme complex. Thus, the oxidation of fuels and the phosphorylation of ADP are coupled by a proton gradient across the inner mitochondrial membrane (Berg *et al*, 2002).

This process is the major source of ATP in aerobic organisms, indeed oxidative phosphorylation generates 26 of the 30 molecules of ATP that are formed when glucose is completely oxidized to CO_2 and H_2O.

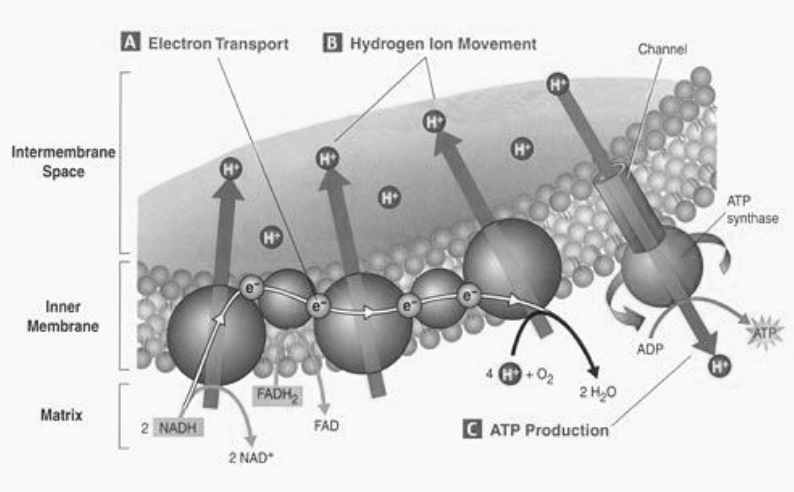


Figure 5. Electron transport chain. Series of compound that transfer electrons from electron donors to electron acceptors via redox reactions, and couples this electron transfer with the transfer of protons across a membrane.

ROS derive from an inevitable leaking of electron transport in the mitochondrial membrane. The electron binds diatomic oxygen (O_2) molecules to form $O_2^{\cdot -}$, which is released either inside the mitochondrial matrix or the inter-membrane space based on whether the superoxide is released from complex I or complex III of the electron transport chain (Figure 6). About 2% of oxygen in the mitochondria ends up as $O_2^{\cdot -}$.

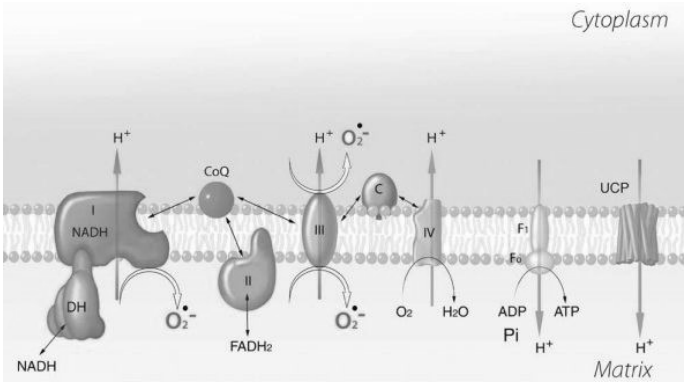


Figure 6. Mitochondrial electron transport chain. The electron binds O_2 to form $O_2^{\cdot -}$, which is released either inside the mitochondrial matrix or the inter-membrane space based on whether the superoxide is released from complex I or complex III of the electron transport chain. (Taken from www.wageningenur.nl)

2.3 Beneficial and harmful effects of ROS in the living system

Many physiological and pathological processes can increase level of oxidant species, in particular ROS. The overproduction of ROS is promoted by exogenous and endogenous stress-factors which act on the sources of free radicals described previously. The endogenous and exogenous factors include: cigarette smoke, asbestos, coal, diesel, drugs, xenobiotics, radiation and ozone, air

pollution, inflammation, metabolism etc. (Figure 7) (Venkataraman *et al*, 2013; Vallyathan and Shi, 1997).

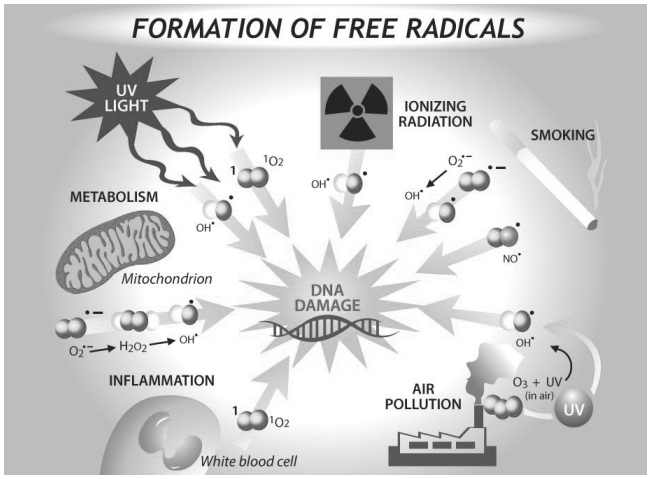


Figure 7. Endogenous and exogenous factors involved in the production of ROS. The overproduction of ROS is promoted by exogenous and endogenous stress-factors such as: UV light, smoke, inflammation, air pollution, metabolism etc.

ROS, as well as RNS, are recognized to have a dual role as both deleterious and beneficial species, since they can be both harmful and beneficial to living system (Valko *et al*, 2007).

Beneficial effects of ROS in physiological conditions are many; ROS are involved in cellular responses to noxia, as for example in defense against infectious agents and in the function of a number of cellular signaling systems. Moreover at low/moderate concentrations can induced a mitogenic response (Valko *et al*, 2006). In physiological signaling, ROS also can modify redox-sensitive amino acids in a variety of proteins including phosphatases, ion channel and transcription factors (Brieger *et al*, 2012). Because of their high reactivity, ROS have been identified as a major source of cellular damage in biological system. At high concentrations, ROS can be important mediators of damage to cells structures, nucleic acids, lipids and proteins (Brieger *et al*, 2012; Valko *et al*, 2006; Valko *et al*, 2007). DNA damage is prominent in presence of excess of oxidant species, and it is evidenced as a formation of adducts or single-stranded and double stranded breaks in nuclear DNA. In addition there is an increase in the formation of modified bases like 8-oxo-7,8-dihydrogunine (8-oxodG) (Venkataraman *et al*, 2013). Permanent modification of genetic material resulting from these oxidative damages may represent the first step involved in mutagenesis, carcinogenesis and ageing. Proteins are impaired by ROS both structurally and functionally, this damage occurs via formation of carbonyls in several cellular proteins in their amino acids, lysine, arginine, proline and threonine (Venkataraman *et al*, 2013; Jacob et al, 2013). Lipid peroxidation damage against LDL lead to the formation oxidized LDL (oxLDL) which is involved in the development of atherosclerosis by promotion the formation of foam cells from macrophages,

(Venkataraman *et al*, 2013; Giustarini *et al*, 2009; Orlandi *et al*, 2006). Oxidation of membrane system in cells results in altered fluidity, physiology and membrane damage, as seen in neurons, macrophages and red blood corpuscles (Venkataraman *et al*, 2013; De la Haba *et al*, 2013). For those reasons, ROS can induce irreversible functional alteration or even complete destruction of the cells (Figure 8). That harmful effect of free radicals on the cells is called **oxidative stress**.

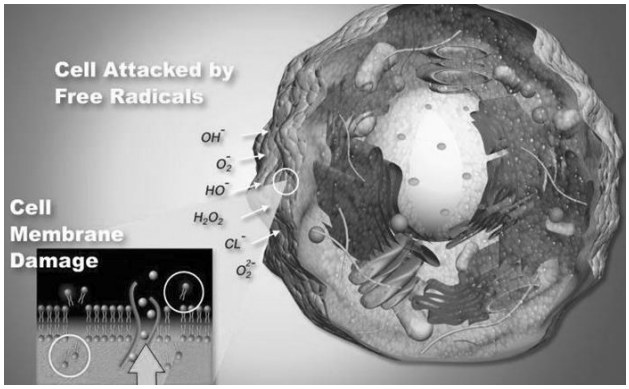


Figure 8. Cell damage by ROS. ROS are able to damage cells at different levels: lipids, proteins and DNA**.** (Taken from Gerasynchuk MR).

3. OXIDATIVE STRESS (OxS)

Oxidative stress has been defined as an **alteration in the balance between the production of free radicals, in particular, ROS, and the antioxidant defense system** in place to counter them (Jacob *et al*, 2013; Halliwell, 2004) (Figure 9). This physiopathological condition can occur whenever there is an excess of oxidant species or an insufficient levels, or inhibition of the antioxidant species.

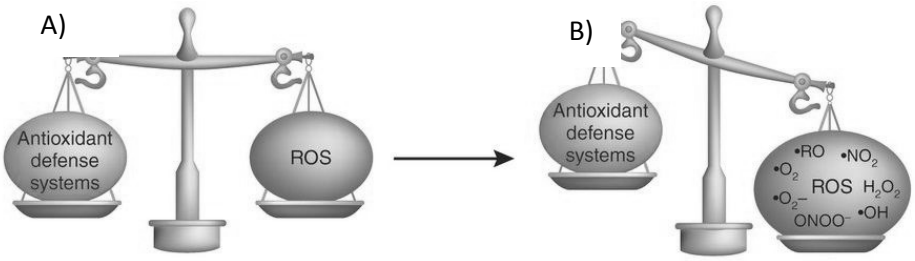


Figure 9. Condition of oxidative stress. Defined as an alteration in the balance between the production of free radicals, in particular, ROS, and the antioxidant defense system in place to counter them. A) Homeostasis condition; B) Oxidative stress. (Taken from Tomaselli and Barth, 2010).

4. ANTIOXIDANT SPECIES

Antioxidant has been definite as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell and Gutteridge, 2012) (Figure 10).

Exposure to free radicals from a variety of sources had led organism to develop a series of defence mechanisms. Defence mechanisms against ROS-induced OxS involve:

1. Preventive mechanisms;
2. Physical mechanisms;
3. Repair mechanisms;

The preventive mechanisms are the first defence against free radicals. Since prevention processes are not completely effective, products of the damage are continuously formed in low yields and may accumulate. This phenomenon causes DNA damage, which induces the activation of multiple enzymes involved in DNA repair capable to reactivate the regular functions. Antioxidants are molecules that inhibit the oxidation of other molecules; at cellular and molecular level they inactivate ROS and under specific low concentration inhibit or delay oxidative processes by interrupting the radical chain reaction. They do this by being oxidized themselves. Antioxidants can act in both aqueous and/or membrane domains and can also chelate the metal ions responsible for the generation of ROS.

Antioxidant defence includes compounds of a non-enzymatic as well as enzymatic nature. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health.

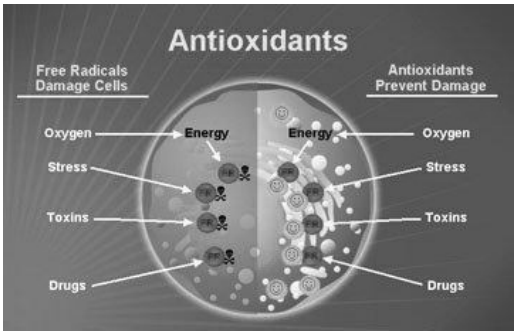


Figure 10. Antioxidant actions on cell. Antioxidant species are able to delays, prevents or removes oxidative damage to target molecules.

4.1 Enzymatic Antioxidants

All cells in eukaryotic organisms contain powerful antioxidant enzymes. The three major antioxidant enzymes are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx).

4.1.1 Superoxide Dismutase (SOD)

SOD is the most effective intracellular enzymatic antioxidants present in the living system. This enzyme catalyzes the dismutation of superoxide ($O_2^{\cdot -}$) into oxygen and hydrogen peroxide (Flora, 2009; Mc Cord and Fridovich, 1969) (Reaction 1).

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow{SOD} O_2 + H_2O \quad \textbf{(Reaction 1)}$$

4.1.2 Catalase

Catalase is an enzyme present in the cells of plants, animals and aerobic bacteria (Flora, 2009; Mates *et al*, 1999). Catalase is usually located in a cell organelle called peroxisome. The enzyme efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen (Reaction 2).

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \quad \textbf{(Reaction 2)}$$

4.1.3 Glutathione Peroxidases (GPx)

Glutathione metabolism is one of the most important antioxidant defence mechanisms in cells. In presence of GPx enzymes, tripeptide glutathione (GSH) is reduced in peroxides (Flora, 2009; Mates *et al*, 1999); then GPx decomposes peroxides to water (or alcohol) while simultaneously oxidizing GSH as is following:

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSH + 2H_2O$$

$$2GSH + ROOH \xrightarrow{GPx} GSSH + ROH + H_2O$$

The antioxidant properties of these enzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction.

4.2 Non-enzymatic Antioxidants

Non-enzymatic antioxidants are commonly classified into two broad divisions, in based on their solubility in water or in lipids: hydrophilic and lipophilic, respectively. In general, water-soluble antioxidants protect cell membranes from lipid peroxidation. The different antioxidants are present at a wide range of concentration in body fluids, like plasma, and tissues. Furthermore antioxidants can be synthesized *in vivo* (endogen), such as uric acid, glutathione and α-lipoic acid; or taken from the diet (exogen), such as acid ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids and flavonoids.

4.2.1 Uric Acid (UA)

Uric Acid (Figure 11) is the most concentrated antioxidant in human blood and provides over half, about 60% of the total antioxidant capacity of human serum. It doesn't react with some oxidants, like superoxide, but does act against peroxynitrite, peroxides and hypochlorous acid.

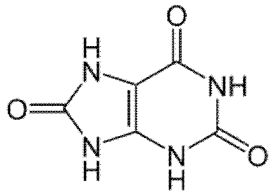


Figure 11. Chemical structure of uric acid

4.2.2 Glutathione (GSH)

Glutathione is the major tripeptide thiol antioxidant and redox buffer of the cell (Valko *et al*, 2007; Masella *et al*, 2005) (Figure 12).

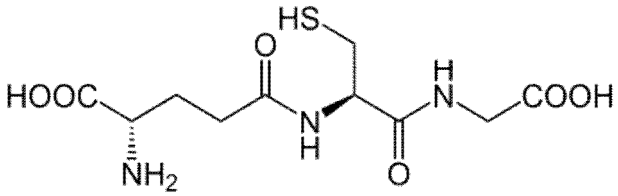


Figure 12. Chemical structure of GSH

GSH in the major antioxidant present in the cells and is present in high in the cytosol and nuclei. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and, in turn, reduces other metabolites and enzymes system. The main protective roles of glutathione against OxS are: i) to be a cofactor of several detoxifying enzyme against OxS, such as GPx, glutathione transferase etc.; ii) to be involved in amino acid transport through the plasma membrane; iii) to have scavenger action on hydroxyl radical and single oxygen directly; iv) to be able to regenerate ascorbic acid and α-tocoferol back to their active form.

4.2.3 α-Lipoic Acid (LA)

α-Lipoic Acid is a sulfur-containing antioxidant with metal-chelating and anti-glycation capabilities (Figure 13). It is readily absorbed from diet and is rapidly converted to dihydrolipoic acid (DHLA) by NADH or NADPH in most tissues.

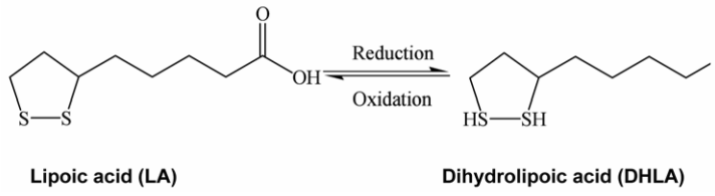


Figure 13. Chemical structure of LA

DHLA can neutralize free radicals and it is known that is able to regenerate Vitamin C and Vitamin E from their oxidized forms, even better than GSH and (Flora, 2009). α-Lipoic acid has been reported to be effective in reducing the amount of OH• generated by Fenton reaction and to be a good scavenger of peroxide and $O_2^{\cdot -}$ (Flora, 2009; Suh *et al*, 2004).

4.2.4 Ascorbic Acid (Vitamin C)

Vitamin C is a very important, and powerful, antioxidant that works in aqueous environments of the body and It has a number of well-defined biological functions, including the biosynthesis of collagen, catecholamine, and carnitine. Vitamin C also very effectively scavenges a wide array of ROS by a rapid electron transfer that inhibits lipid peroxidation. Ascorbic acid *in vivo* increases dose-dependently resistance to lipid peroxidation, even in presence of redox-active iron or copper and H_2O_2 (Flora, 2009; Suh *et al*, 2003).

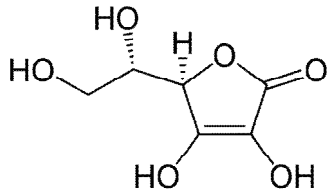


Figure 14. Chemical structure of ascorbic acid

Other studies indicate the ability of ascorbic acid to regulate factors that may influence gene expression, apoptosis and other cellular functions (You et al, 2000).

4.2.5 α-Tocopherol (Vitamin E)

Vitamin E is the collective name for a set of eight related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant proprieties. α-Tocopherol is the most active form of vitamin E in humans and is a powerful biological antioxidant and it is considered to be the major membrane bound antioxidant employed by the cell. Vitamin E performs a unique function by interrupting free radical chain reactions via capturing the free radical. The free hydroxyl group present on its aromatic ring is responsible for the antioxidant properties (Figure 15).

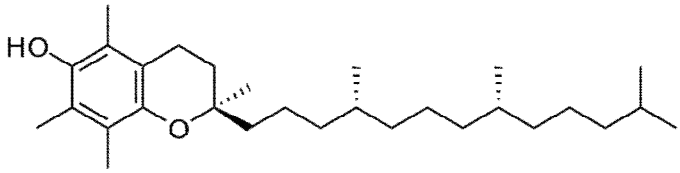


Figure 15. Chemical structure of α-tocopherol

During the antioxidant reaction, α-tocopherol is converted to α-tocopherol radical by the donation of labile hydrogen to a lipid or lipid peroxyl radical. This radical that is formed can be reduced to the original α-tocopherol form by ascorbic acid. Vitamin E can preserve cell membrane functions including ion transport and membrane fluidity, it also can prevents the release of Fe^{2+} and Mg^{2+} from their binding proteins, and can decreases the rate of lipid peroxidation (Flora, 2009).

4.2.6 Carotenoids

Carotenoids are pigments that are found in plants and microorganisms (Figure 16). In humans three carotenoids are present, beta-carotene; alpha-carotene and beta-cryptoxanthin that can be converted in retinol and can also act as antioxidants. They have effective antioxidant effects because they are able to remove free radicals from the system either by reacting with them to yield harmless products or by disrupting free radical chain reactions.

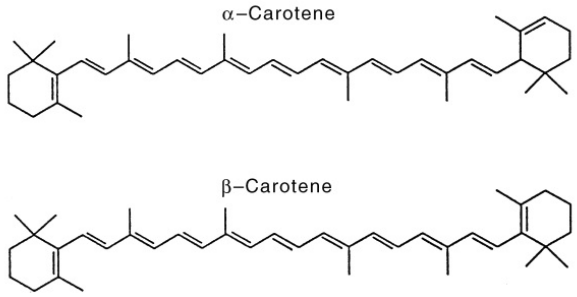


Figure 16. Chemical structure of carotenoids (α and β)

4.2.7 Flavonoids

Polyphenolic compounds constitute one of the most commonly occurring and ubiquitous groups of plant metabolites and represent an integral part of human (You et al, 2000; Flora, 2009). Their structure consist of two aromatic rings linked through three carbon atoms that together usually form an oxygenated heterocycle (Figure 17).

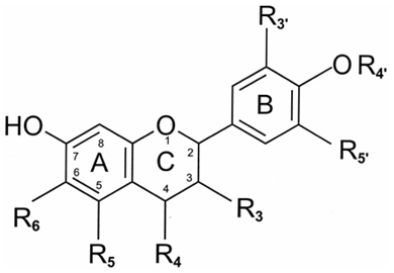


Figure 17. Chemical structure of flavonoids

Phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalyzing lipid peroxidation. The major abundant flavonoids in the fruit and vegetables is the (-)-epicatechin.

5. PERIPHERAL BIOMARKERS OF OXIDATIVE STRESS

Biomarkers are defined as "characteristics that can be objectively measured and evaluated as indicators of normal biological process, pathogenic processes, or pharmacological response to a therapeutic intervention" (Dalle-Donne *et al*, 2006). Several *in vitro* markers of OxS are available, but most of them have limited usefulness *in vivo* because they lack sensitivity and/or specificity. Furthermore, ROS are too reactive and/or have a half-life too short to allow to direct measurement in cells, tissues or in body fluids, such as serum, plasma and urine. Since molecular products formed from the reaction of ROS are more stable than ROS themselves, usually ROS are detected by measuring the concentration of their oxidation products (Dalle-Donne *et al*, 2006; Halliwell and Whiteman, 2004), including lipid peroxidation end-products and oxidized proteins. For these reasons the gold standard marker of OxS has not been identified yet, and, at present, the most reliable approach for quantifying it *in vivo*, is to measure a battery of different peripheral biomarkers, preferably reflecting lipid, protein and DNA oxidative damage as well as antioxidant (Kohen and Nyska, 2002). Oxidation of biological systems: OxS phenomena, antioxidants, redox reactions, and methods for their quantification (Kohen and Nyska, 2002). The peripheral biomarkers of OxS commonly measured *in vivo* to detect the redox status are:

- **Hydroperoxides**: produced from oxidation of various biomolecules (mainly lipids);
- **F_2-isoprostanes**: considered the best available biomarkers of lipid peroxidation;
- **8-Oxo-2'-deoxyguanosine (8-OH-dG)**: produced by oxidative damage of DNA by ROS;
- **Advance oxidation protein products (AOPP)**: originated under oxidative and carbonyl stress against protein;
- **Malodialdehyde (MDA)**: produced from lipid oxidation, it is the most common marker used to detect OxS;

- **Advanced glycation end products (AGEs)**: formed by glycation reaction, which refers to the addition of carbohydrate to a protein without the involvement of an enzyme;
- **Nitrotyrosine**: marker of nitrosative stress;
- **8-hydroxyguanosine (8-OHG)**: produced by oxidative damage of RNA by ROS.

6. OXIDATIVE STRESS: AGING AND DISEASES

The involvement of free radical associated damage in life process was first recognized by Harman (Harman, 1956) in the 1950s, who proposed the "free-radical theory of aging" which states that aging is the result of the accumulation of biomolecules damaged by free radicals produced during normal metabolism. He focused on mitochondria as both free radicals generators and targets of damage. Later, Miguel *et al* (Miguel *et al*, 1980) proposed a "mitochondrial theory of aging": progressive damage to mitochondrial DNA by ROS, leading to mitochondrial dysfunction that affects the whole cells.

In later years, these theories were expanded to include not only aging, but also age-related diseases. The process of aging is, in a large extent, due to the damaging consequences of free radical action, as lipid peroxidation, DNA damage and protein oxidation (Valko *et al*, 2007). OxS has been implicated in various pathological conditions, such as: cardiovascular diseases, cancer, neurological disorder (Alzheimer's and Parkinson's disease), diabetes, insulin resistance, ischemia/reperfusion, osteoporosis and other (Figure 18).

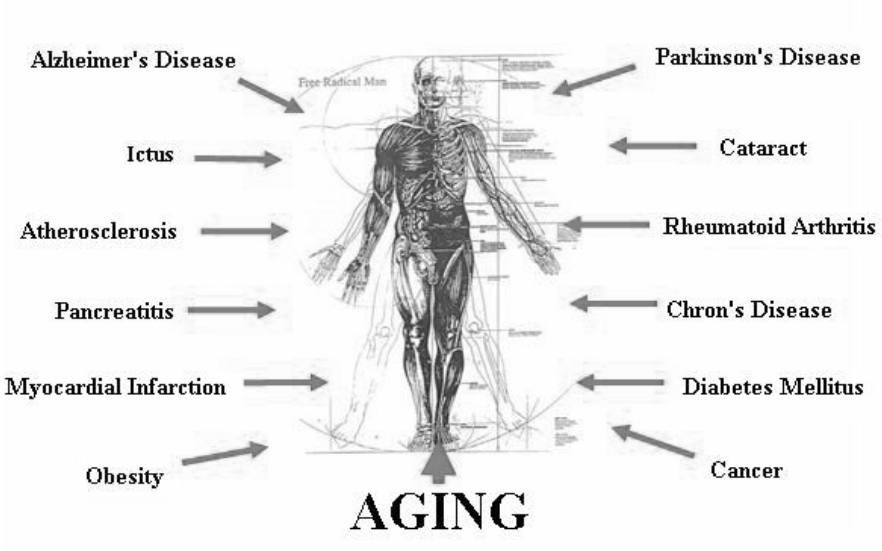


Figure 18. Oxidative stress and diseases. Oxidative stress is involved in aging process, accordingly in age-related diseases, such as ictus, Alzheimer and Parkinson's disease, diabetes etc.

All these diseases fall into two groups:

1. The first group involves diseases characterized by pro-oxidants;

2. The second group involves disease characterized by "inflammation oxidative conditions" and enhanced activity of either NADPH oxidase or xanthine oxidase-induced formation of ROS.

Inflammation is the reaction of the body to endogenous and exogenous harmful stimuli or the initial phase of the healing process. Inflammation can be classified into two types, acute and chronic. Chronic inflammation is a prolonged pathological condition characterized by mononuclear immune cell infiltration and tissue destruction. However, chronic inflammation exerts its cellular side effects mainly through excessive production of ROS, in the forms of superoxide, hydrogen peroxide, hydroxyl radical etc., and depletion of antioxidants (Jacob *et al*, 2013; Ghazavi et al, 2013; Shiri *et al*, 2006). For this reason, inflammation is regarded as one of the most important source of free radicals leading to OxS-related pathologies.

It is well-known that diet rich in fruit and vegetables is associated to a decrease of risk to develop ROS-associated diseases. These protective effects have been attributed, in large part, to the antioxidant content of these nutrients, such as ascorbic acid and β-carotene, but also plant phenolics such as flavonoids. However, the specific role of oxidative stress in the development of age-related diseases as well as the effectiveness of antioxidants in the prevention and care of these pathologies are still unclear.

7. OBJECTIVES OF THE THESIS

In the present thesis, I report and discuss the results of the main *in vivo* and *in vitro* researches that I have conducted in these last three years. The most important purposes of my studies were the following:

1. To evaluate a possible association between systemic markers of oxidative stress and the most common dementia-related diseases (Alzheimer's disease and vascular dementia). Besides we have further evaluated if that redox imbalance might be predictive of the clinical progression of this neurodegenerative disease.
2. To evaluate a possible involvement of oxidative stress in the development of postmenopausal osteoporosis.
3. To evaluate the effects of flavonoids, in particular (-)-epicatechin and its metabolites, on the regulation of expression and activity of NADPH oxidase in hepatic cells treated with free fatty acid.

CHAPTER 1: ROLE OF OXIDATIVE STRESS IN DEMENTIA

INTRODUCTION

1.1 DEMENTIA

1.1.1 Definition

Dementia (from Latin *de-ment*, "without-mind") is defined by the World Health Organization (WHO) as "an acquire global impairment of higher cognitive function, including memory, ability to deal with daily life, the performance of sensorimotor and social functions, language communication, and control of emotional reactions, without marked of consciousness" (WHO, 1993). Dementia is not a single disease, but a non-specific syndrome. It may be: static, if is the result of a unique global brain injury or progressive if it is the result in long-term decline due to damage or disease in the body. It is not just a problem of memory but is reduces the ability to learn, reason, retain and recall past experience, also can be loss of patterns of feeling and activities. In later stages of dementia condition, the patient can be disoriented in time, in place and in person. Additional mental and behavioral problems often affect people who have this disease and may influence the quality of life. Almost 30% of people with dementia are affected of depression and about 20% of anxiety; psychosis and agitation/aggression may also be present in this pathological condition.

1.1.2 History and epidemiology

Until the end of the 19th century, dementia was a much broader clinical concept; at that time it was referred to anyone who had lost the ability to reason, and was allied equally to psychosis of mental illness. Dementia was called *senile dementia* or *senility*, and viewed as a normal and somewhat inevitable aspect of growing old. In 1907, a specific organic dementing process of early onset, called Alzheimer's disease had been described. This was associated to particular microscopic changes in the brain, but it was seen as a rare disease of middle age. In 1976 Katzmann (Katzmann, 1976) suggested a link between senile dementia and Alzheimer's disease. He showed that the senile dementia occurring after age of 65, was pathologically identical with Alzheimer's disease occurring before age 65 and therefore should not be treated differently. Katzamann stated that "senile dementia", was not a disease, but rather part of aging. At the end, he suggested that Alzheimer's diseases, if taken to occur over age 65, was a common and not rare disease, and was one of the causes of death. All these suggestions opened the view that dementia was a result of a particular disease process, and it was not just a part of the normal healthy aging process, but there were different mechanisms involved in the development of these diseases. In the 21st century, a number of other types of dementia have been differentiated on basis of pathological examination of

brain tissues, symptomatology and by different patterns of brain metabolic activity in nuclear and medical imaging tests.

Worldwide six percent, or even more, of people aged 65 and older suffer from dementia. The number of cases of dementia worldwide in 2013 was estimated at 44.4 million people (http://www.alz.co.uk/research/statistics). Rates increase significantly with age, with dementia affecting 5% of population older than 65 and 20-40% of those older than 85. WHO projection suggest that by 2050, if growth in the older population continues, and there are no changes in mortality or burden reduction by preventive reduction, 71% of 81.1 million dementia cases will be in the developing world (WHO, 1993). In the past years many causes of dementia were known, such as:

- Alzheimer's disease (AD);
- Vascular Dementia (VaD);
- Mixed dementia (AD and VaD);
- Dementia with Lewy bodies;
- Front-temporal dementia;
- Parkinson's dementia.

AD and VaD are the first and the second most prevalent causes of dementia in the world, 62% and 17% respectively.

1.2 ALZHEIMER'S DISEASE (AD)

Alzheimer's disease (AD) is the most common cause of age-related dementia and it is characterized by a loss of neurons in the hippocampus and associative cortex.

Two pathological features of AD, first reported in 1907 by the psychiatrist Alois Alzheimer, are the presence of neurofibrillary tangles (NFT) and senile plaques in the brain (Figure 1.1). NFT are fibrous masses present inside the affected neurons, formed by hyperphosphorylation of a microtubule-associated protein known as tau (τ) protein. Senile plaques are extracellularly localized areas of degenerating and swollen axons or inflamed brain parenchima surrounding a core of aggregated β-amyloid peptides (Aβ). Thus, amyloid plaques and NFT result from an aberrant deposition of the Aβ and the hyperphosphorylated τ protein, respectively and these depositions lead to neuronal loss and neurotoxicity in the AD affected brain (Cotman and Su, 1996; Newman *et al*, 2007).

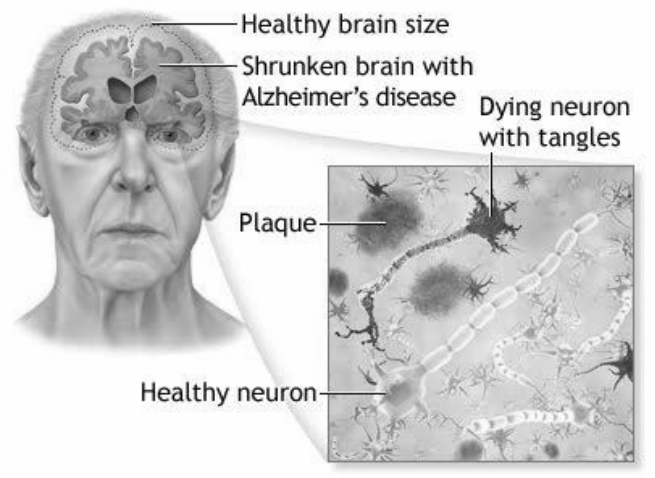


Figure 1.1. Healthy *vs* Azheimer's brain. Patients affected by Alzheimer's disease show a cerebral mass reduced compared to healthy brain. Further typical hallmarks of the disease are neurofibrillary tangles and Aβ plaques. (Taken from A.D.A.M., Inc.)1

In AD, diffuse deposits of Aβ are called "preamyloid" plaques, and can precede neuritic degeneration and senile plaques formation. NFT and senile plaques were also found in young adults affected by Down's syndrome, and in a less extent, in the brain of "normal" elderly individuals. However in the AD brain, soluble and precipitated Aβ are five and hundred times higher than in controls, respectively. Accumulation of Aβ peptides may be the key event in the pathogenesis of AD (Newman *et al*, 2007).

1.2.1 Pathogenesis: Amyloid hypothesis

β-amyloid is a family of peptides produced by amyloid precursor proteins, APPs; these precursor proteins have a short intracellular C-terminus and a longer extracellular N-terminus, and in human are encoded by a gene on chromosome 21, indeed Down's syndrome cases have been shown to develop early onset dementia. APPs are secreted by many cell and tissue types and have several functions; in neurons they may facilitate growth and survival during brain development.

The proteolytic processing of APP that releases the Aβ fragment is a result of cleavage events by secretase proteins. This proteolysis occurs in two distinct pathways: non-amyloidogenic (normal condition) and amylogenic (disease condition) pathways (Figure 1.2).

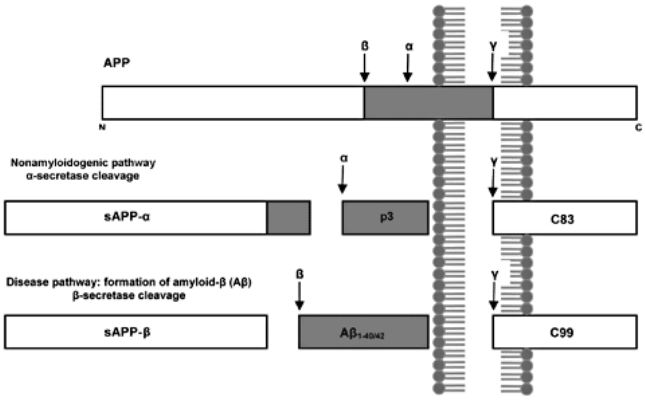


Figure 1.2. Proteolytic process of amiloyd precursor proteins (APPs). The proteolytic processing of APP that releases the Aβ fragment is a result of cleavage events by secretase proteins (α-, β- and γ-secretase). The process occurs in two different patways: non-amyloidogenic (normal condition) and amylogenic (Alzheimer's disease). (Taken from http://journals.prous.com/journals/dnp/20061907/html/dn190411/images/fig1.gif).

The physiological turnover of APP involves proteolytic processing, by α- and β-secretase, to release the N-terminal sequence and leave a fragment in the membrane. α-Secretase is able to cleave in the middle the Aβ sequence and to release soluble αAPP and β-APP into extracellular environment. α-Secretase seems to have a neuroprotective role in the brain; it has been demonstrated that its low level in AD patients may facilitate neuronal damage. In confirmation of that, α-secretase action is the main route of APP cleavage in healthy brain. By contrast, in the amyloidogenic pathway the release of Aβ occurs by the action of γ-secretase enzymes, which contain four proteins, including presenilin. β-Secretase cleaves APP at N-terminus of the Aβ sequence, whereas the cell-associated C-terminus may be either cleaved by γ-secretase to give Aβ or degraded completely. The cleavage by γ-secretase is a heterogeneous event that releases Aβ peptides of different size, with Aβ-40 and Aβ-42 being the most common forms. These forms are both toxic peptides, but Aβ-42 isoform is more prone to aggregate into plaques amyloid because of its insolubility. This process is considered the primary event in AD pathogenesis (Newman *et al*, 2007).

Upon the accumulation of Aβ peptides it is important to highlight that there is a resulting neuronal cytotoxicity that induces neuropathological events causing neurodegeneration in the brain. Aβ-42 can be aggregated into two different conformation states: non-β sheet (non-fibrillar state) and β-sheet (fibrillar state). The fibrillar state is responsible of the neurotoxicity of the Aβ. Aβ-42 exists as both monomer and oligomers, that in turn can become protofibrils and fibrils. The Aβ oligomeric intermediates and mature fibrils are both neurotoxic. However, *in vitro* experiments have showed that the oligomers and protofibrils are more neurotoxic than the mature fibrillar or

amyloid plaques (Dahlgren *et al*, 2002; Newman *et al*, 2007). The final aggregated Aβ plaques may no longer be directly neurotoxic, but, only indirectly, by inducing inflammatory response of microglia.

1.2.2 Forms of Alzheimer's disease: Late and Early Onset Alzheimer's

There are basically two different forms of AD: late and early onset. This classification is based on the symptomatic, biological, genetic, neurophysiological and neurological characteristics, and, mainly on the age of the disease onset (Kensinger, 1996).

Early onset Alzheimer's (EOA) is a rare form of AD (10% of all AD patients), and, even rarer (1% of AD cases) it is the hereditary form of the disease, defined as familial early onset AD (FAD). EOA is called "early onset" because it is diagnosed before 65 years. The late onset Alzheimer's disease (LOAD) is defined as the form that develops in the subjects older than 65 years. LOAD counting about 90% of the total cases of AD, and it is also called sporadic Alzheimer's disease.

The differences between EOA and LOAD patients concern in rate of progression and extent of language dysfunction or visuoconstructional impairment. Compared to LOAD, EOA is associated with more language and cognitive deficits. Additional evidence for the subtypes comes from neurological and neurophysiological studies which confirm that EOA and LOAD have different neurochemical characteristics. At this regard, it is important to mention that cholinergic deficits is more common in early onset than LOAD, where it appears to be confined into the temporal lobe and hippocampus.

1.2.2.1 Mild cognitive impairment (MCI)

Mild cognitive impairment (MCI) is regarded as an intermediate state between normal aging and dementia (Marksbery *et al*, 2006). This pre-clinical condition is characterized by short-term or long-term memory impairment which, at variance of dementia, is not associated with significant daily functional disability (Petersen *et al*, 2001). Importantly, almost one half of these individuals evolve to LOAD, accounting for about 60% of the total cases of dementia in U.S. and western countries (Anchisi *et al*, 2005).

1.2.3 Risk factors of Alzheimer's disease

The 99% of cases of AD have the following risk factors: age, sex, diet, environment enrichment, repeat minor brain trauma (dementia pugilistica), low educational achievement, life-style, smoke, hypercholesterolemia, hypertension, homocysteine, type 2 diabetes, apolipoprotein E (ApoE) and gene mutations. Only FAD subtype is associated with familial risk factor. Other risk factors are also important, and may synergize with genetic predispositions. The three genes that are implicated

in AD pathogenesis, such as APP, presenilin 1 or 2 may be apt to mutation; the mutation in these genes have been identified in cases of FAD, but somatic changes in them can potentially have also a role in sporadic cases of AD. Along with causative mutation in presenilin and APP, the ApoE gene has been identified as a major genetic risk factor in the 99% cases of AD.

1.2.3.1 Apolipoprotein E (ApoE)

Apoliprotein E is expressed in several organs, with the highest expression in the liver, followed by brain. Astrocytes and to some extent microglia, which are non neuronal cells, are the major cell types that express ApoE in the brain (Grehan *et al*, 2001; Bu, 2009); however also neuron can produce this apolipoprotein under certain condition. In plasma ApoE proteins are present on lipoprotein in association with other apolipoproteins; one of its functions is to transport cholesterol to neurons via ApoE-receptors, which are members of the low density lipoprotein receptor family (Bu, 2009). The human ApoE gene exists as three polymorphic alleles: ε2, ε3 and ε4, which have a worldwide frequency of 8.4%, 77.9% and 13.7%, respectively. However, the frequency of ε4 is markedly increased, to about 40%, in patients with AD (Farrer *et al*, 1997; Liu *et al*, 2013). Controversy ε2 allele seems to have a protective effect against AD. Difference between the three isoforms are limited of two amino acid residues, these two affect the structure of ApoE isoforms and influence their ability to bind lipids, receptors and Aβ. Many studies suggest that the different isoforms of ApoE affect Aβ aggregation and clarence. Genome-wide association studies have confirmed that the ε4 allele of ApoE is the strongest genetic risk factor of AD, the presence of this allele is associated with increased risk of both early onset and LOAD; Aβ accumulation in the form of senile plaques is more abundant in ApoE ε4 carriers than in non-carriers (40.7% vs 8.2%, respectively) in the individuals aged 50-59 years, and seems that ε4 increases the risk to develop AD by initiating and accelerating Aβ accumulation, aggregation and deposition in the brain (Figure 1.3).

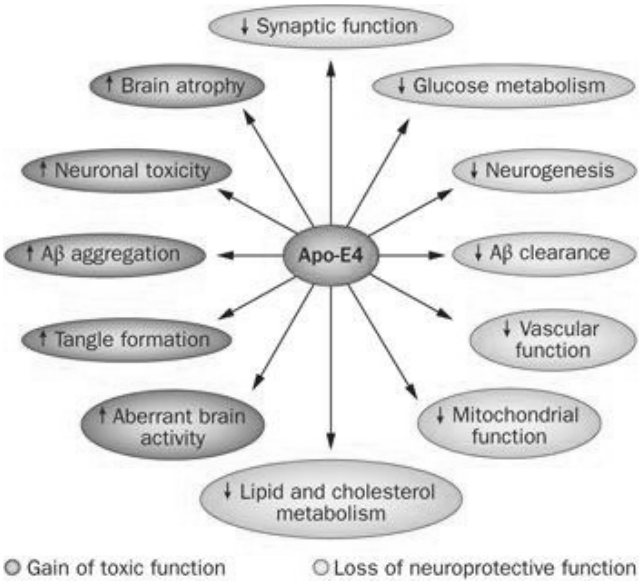


Figure 1.3. Effects of Apo-E4 in the brain. (Taken from Liu et al, 2013)

Furthermore ε4 allele has considerable deleterious effects on memory performing and might be used to predict disease progression in combination AD biomarkers and neuroimaging approaches. Some studies show how ApoE ε4 is associated with impairment memory performance and increase the risk of memory decline in people affected to MCI. Patients who are carriers of this allele had more rapid decline in several cognitive and functional domains. ApoE ε4 genotype in patients with MCI can serve as a predictive factor for determination of clinical outcome and the risk of conversion to AD. The ApoE ε4 genotype is also combines synergistically with atherosclerosis, peripheral vascular disease, and type 2 diabetes mellitus (T2DM) in contributing to an increase risk of AD (Liu *et al*, 2013).

1.3 VASCULAR DEMENTIA (VaD)

VaD, the second cause of dementia in the world (it affects 17% of individuals with diagnosis of dementia), is defined as permanent cognitive impairment produced by vascular damage to the brain. The symptoms of VaD are cannot be distinguished from Alzheimer's symptoms on the basis on history or mental status examination; patients affected by vascular dementia may have a "stair-step" clinical course in which they demostrate significant drops of function following vascular injury to the brain. A significant number of VaD patients have neurological deficits that are not typically of AD, like mild weakness on one side of the body or abnormal reflexes; often they have evidence of cardiovascular diseases, such as: hypertension, past history of heart attack, etc. VaD is indeed a consequence of cumulative vascular damage to the brain, but it is not possible to predict the intellectual decline in base on type, location or size of the damage, prevalently represented by stroke. There are five major vascular brain's damages that can cause VaD are:

1. Strokes produced by atherosclerosis, that is the death of a discrete segment of brain tissue on consequence of cessation of blood flow to that region;
2. Hypertensive change, that can cause disintegration of the blood vessel wall and bleeding into tissue;
3. Anoxic brain damage, this is a consequence of low oxygen or low blood flow. Vulnerable brain regions like the hippocampus are very sensitive to low level of oxygen, and damage to this region produces amnesia;
4. Ischemic white matter damage, the brain white matter contains fibers that connect neurons in each hemisphere and is perfused by small and fragile vessels that are susceptible to damage from high blood pressure and may produce the "white matter abnormalities";
5. Hypotensive brain damage, the connection between low blood pressure and dementia is not still clear, but it seems that hypotension's conditions lead to impairment of cerebral autoregulation.

Vascular cognitive impairment is not a regular pathogenesis entity. Multiple small thromboembolic strokes or strokes in precise position like thalamus, front or temporal lobes may cause cognitive impairment and frequently occur without classical stroke-like symptoms. One of the mechanism involved in the ischemic VaD is under the control of large vessel disease (atherosclerosis) but it is still unclear how the large vessels contribute to the white matter pathology and lacunes associated with the subcortical type of VaD. Some studies suggest that the small vessels may have a important role in this pathology. The alterations of the small vessels can cause damage to the cerebral tissue and are potentially responsible of the development of cognitive impairment. They also may lead to damage affecting the blood brain barrier and chronic leakage of fluid and macromolecules in the white matter.

1.3.1 Risk factors of vascular dementia

The risk factors of VaD are, in part, the same of AD (age, sex, diet, lifestyle, level of homocysteine, genetic factor, inflammation etc.), but they also include atherosclerosis, endothelial dysfunction and other risk factors for stroke.

Genetic risk factors play an important role in VaD and seems to have a pivotal role in the large and small vessels-stroke. The genes involved in this pathology are: (i) genes that predispose individuals to cerebrovascular disease; and (ii) genes that determinate tissue responses to cerebrovascular disease.

The genes that influence the responses to cerebrovascular diseases are the AD's genes. Mutations in APP gene can lead either to AD or to hemorrhagic stroke and dementia depending on the side of mutation and consequent the site of amyloid accumulation. Variants of ApoE gene appear to affect not only susceptibility to cerebrovascular diseases but also recuperative response to it. A recent

meta-analysis has shown evidence of increased risk of VaD in individuals with ApoE ε4 compared to ApoE ε3. Several studies suggest that the contribution of ε4 allele to risk of vascular cognitive impairment is independent of other vascular risk factors including hypertension and dyslipidemia; another evidence shown that age-related cognitive decline among ApoE ε4 carriers is induced by brain damage owing to increased blood pressure (Liu *et al*, 2013).

1.4 DIAGNOSIS OF DEMENTIA: ALZHEIMER'S DISEASE AND VASCULAR DEMENTIA

Dementia can be present in a wide variety of neurological disease entities. Excluding depression is an important part of diagnosis of dementia. Depression is more common in old age than dementia. Also chronic pain and medication side effects can affect the validity of a diagniosis of dementia. For these reasons a "regular" diagnosis of dementia, in particular of AD, is not possible, but it is applied a diagnosis of exclusion.

In 1984, a task force established by the National Institute of Neurological and Communicative Disorders and Stroke the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) put forth a set of clinical criteria for diagnosis of probable AD (McKhann *et al*, 1984). In 1994 American Academy of Neurology (AAN), stated that these criteria are sufficiently reliable and valid, such that a predictive accuracy of up to 90% can be reached for the diagnosis of the major forms of dementia (McKhann *et al*, 1984; Dubois, *et al*, 2007; Yaari and Corey-Bloom, 2007).

1.4.1 Diagnosis of Alzheimer's Disease

NINCDS-ADRDA criteria classify AD into probable, possible and definite levels of diagnostic certainty (McKhann *et al*, 1984; Yaari and Corey-Bloom, 2007). A clinical diagnosis of "probable" AD can be made with confidence without pathological confirmation, because it requires the insidious onset and progressive decline in memory with involvement of at least one of the other cognitive domains that are established by clinical examination and confirmed by neuropsycological tests. Other elements in support of "probable" AD include progressive deterioration of language, praxis, and visual recognition with impaired activities of daily living, family history, and normal results on cerebrospinal fluid (CSF) examination, electroencephalogram and neuroimaging. The diagnosis of "possible" AD can be made in presence of other significant diseases or when the course of cognitive decline is atypical. The category of "definite" AD required histopathological confirmation, it is reserved for patients with probable AD with corroborating data from biopsy or autopsy.

1.4.2 Diagnosis of Vascular Dementia

The diagnosis of VaD is also classified by NINCDS-ADRDA criteria into probable, possible and definitive (Hogervorst *et al*, 2003). The criteria for the diagnosis of "probable" VaD include all the elements describes previously, such as decline in memory, impairment in daily activities etc, if they are concomitant with cerebrovascular diseases. A clinical diagnosis of "possible" VaD may be made in presence of dementia with focal neurologic signs in patients whom brain imaging studies to confirm definite cardiovascular disease are missing, or in the absence of clear temporal relationship between dementia and stroke. The "definite" VaD diagnosis has the same criteria of "probable" VaD, but in addition of them it needs: (i) histopathologic evidence of cardiovascular disease obtained post-mortem; (ii) absence of neuritic plaques and NFT exceeding expected for age; and (iii) absence of other clinical disorders capable to develop dementia.

1.4.3 Diagnosis approaches

The clear cut-off diagnosis differentiating AD and VaD is complicated by the lack of well-recognized discriminatory parameters, in particular as regards the cognitive profile. Moreover, it is acknowledged that some markers of vascular injury typical of VaD might coexist with the diagnosis of AD.

The most accepted criteria that can be used to draw a differentiate diagnosis for these two diseases are based on:

- ***Medical history***: is essential to establish a history of progressive deterioration and for identifying abnormalities in the regular life, such as impaired memory, alteration mood, often delusions and illusions, inability to find the way to a usual destination, inability to use money, difficulties in dressing, reading, and writing etc. A diary maintained by an observer may be helpful in documenting changes;
- ***Clinical examination***: it provides data to exclude or not criteria for the diagnosis of AD and VaD and to document symptoms such as delusion and depression. Mental status testing are an important component of the clinical examination, they include test like, Mini-Mental State Examination (MMSE), for cognitive screening; Blessed Dementia Scale (BDS) for clinical symptoms and social functions; Hamilton Depression State (HDS) for severity depression; Present State Examination (PSE) for anxiety, hallucinations etc; and Hachinsky Scale (HS) for estimating the likelihood of multi-infarct dementia.
- ***Neuropsycological testing***: this test may provide just an additional information for the diagnosis of dementia, because there are not population "standards" for many of these tests, any abnormally can be determinate only by comparison with a normal group matched for sex, age and education. It is considered "abnormal" a score in the lowest fifth percentile of an individual's normal control group. Progressive worsening can be established by

comparison with the patient's previous performance on these tests. Similar series of tests are used to asses less severely affected patients by increasing the complexity of the neuropsycological tests. Even if these tests are used primarily to confirm evidence for the diagnosis of dementia, they are valuable for determinate patters of impairment over time and after treatment or rehabilitation; neuropsycological tests are also used for establishing correlation of abnormal performance and neuropathologic examination.

- ***Laboratory assessments***: clinical and neuropsycological tests provide information to meet criteria for clinically probable AD and probable VaD. But at present there are no specific diagnostic laboratory tests for AD and VaD, but are present laboratory and imaging technologies that can be helpful in ruling out non-AD diagnoses of dementia. There are different tecniques used:
  - *Computerized tomography (CT)* useful in the diagnosis of AD because it permits the exclusion of other disorders such dementia associated with VaD, brain tumor, etc..
    This tecnique can quantitate tissue densities, ventricular size, CSF volume and brain mass;
  - *Regional cerebral blood flow (rCBF)* may be help to differentiate AD and VaD, because in dementia associated to vascular dementia the autoregolation of the system is decrease and rCBF and oxygen consumption are decreased. In AD rCBF is decrease but the autoregulation is preserved;
  - *Positron emission tomography (PET)* is a research technique that allows quantitative measurament of rate of glucose utilization, oxygen consumption and rCBF. It can use to check the pattern of bilateral temporoparietal hypomotabolism that has been shown to have high specificity for AD and may be useful in confirm its diagnosis. However PET imagin can not be widely available for clinical use and it is expensive;
  - *Magnetic resonance imaging (MRI)* reveals the demarcation of gray and white matter of the brain. It may discriminate AD from VaD and other causes of dementia but commonly is used in demyelinating disorder like multiple sclerosis.

  Other tests, not routinely recommended, are used because can be helpful in certain circumstances: chest X-ray, electroencephalography.

  The recommended routine workup for the diagnosis of AD and VaD are the head imaging with CT and MRI. Tough, the utility of these structural brain imaging is limited to the exclusion of other etiologies, for example as the common features of AD are cortical atrophy and ventricular enlargement, which are neither sensitive and specific markers of AD and often accompany normal aging.

- ***Examination of body fluid*** **:** at present there are not specific peripheral body fluid markers for the diagnosis of dementia, in particular AD and VaD. However, there are some markers in CSF and blood that can be helpful for excluding chronic infections, inflammatory disease, vasculositis, or demyelination (McKhann *et al*, 1984). The biomarkers known in CSF are reflected of central pathogenic process of amyloid β aggregation and hyperphoshorylation of tau protein. In AD the concentrations in serum of Aβ42 and t-tau are low and high, respectively, compared with those in healthy controls. Several recent studies suggest that CSF biomarkers may be used to identify prodromal AD. Combinations of abnormal markers reached a hazard ration of 17 to 20 for predicting AD in a follow-up of 4-6 years. Serum and CSF levels of Aβ42 may be predictive for AD in patients with MCI but these measurements have not been formally included in clinical practice guidelines yet (Hansson et al, 2006; Dubois, *et al*, 2007, Jayakar and Huang, 2010; Dickerson and Wolk, 2013). Other laboratory tests in blood are also useful in ruling out other factor that can contribute to dementia, for example: hypothyrodism and B_{12} deficiency, which affected homocysteine and methylmalonyl-CoA metabolism, is associated with irreversible neurological damage (Yaari and Corey-Bloom, 2007; Jayakar and Huang, 2010).

1.5 OXIDATIVE STRESS: CORRELATION WITH AD AND VaD

One of the most metabolically active organs of the body is the brain, including the spinal cord comprising central nervous system (CNS), which, even at rest, utilizes about 20% of the total oxygen uptake (Shukla *et al*, 2011). Any blockage or deprivation of this oxygen supply even for a few seconds can have severe and irreversible effects in brain. Consumption of oxygen leads to the production of free radicals, with consequent development of oxidative stress (OxS). It is been reported that OxS may play a role in both pathogenesis of AD and cerebral ischemia, which causes VaD, because free radicals, including reactive oxygen species (ROS), can react with substrates essentials for the survival of neurons such as proteins, lipids, and nucleic acid, leading to neuropathological lesions and to brain damage (Grimms *et al*, 2001; Bergamini *et al*, 2004). The most abundant source of ROS in the CNS is the respiratory burst system of activate microglia. The brain is particularly sensitive to oxidative damage because: (i) it is rich in oxidizable polyunsaturated fatty acids; (ii) it has a large consumption of oxygen; (iii) it has high levels of iron that predispose it to Fenton reaction with formation of OH and (iv) it has high neuronal calcium input. Furthermore, on the other hand, compared to liver and kidney, the brain has lower levels of antioxidant enzymes: SOD, GPx and catalase; the perfect balance among these enzymes is essential to maintain the cells alive (Torres *et al*, 2011). Moreover, neurons present low levels of GSH, the

antioxidant that is major responsible for the elimination of cytosolic peroxides. Growing *in vitro* and animal evidence (Rinaldi *et al*, 2003; Lovell and Markesbery, 2007; Rodrigues *et al*, 2008) suggest that OxS might caused Aβ accumulation, by increasing the production of the precursor of these peptidase, APP (Zhao and Zhao, 2013). Indeed, it has been shown that these peptides form oligomers that could exert neurotoxicity effects by enhancing ROS level in the brain. More specifically, from these experiments it emerged that Aβ oligomers can directly generate H_2O_2, activate NADPH oxidase in astrocytes as well as induce ROS production in mitochondria, by modulating the activity of enzymes like Aβ-binding alcohol dehydrogenase and α-ketoglutarate dehydrogenase (Valko *et al*, 2007; Mao and Reddy, 2011).

On the other hand, the origin of OxS in VaD can be more associated to the typical vascular abnormalities observed in patients affected by this type of dementia. Indeed, vascular endothelium is able to synthesize, store, and release free radicals in response to stimuli such as injury and hypoxia/hypoperfusion. Vascular OxS is also implicated in the onset of several well-recognized risk factors for VaD (and AD) including diabetes, stroke, atherosclerosis, and hypertension (Zhu *et al*, 2007).

Signs of oxidative damage, especially in proteins and lipids, have been also observed in brains from subjects affected by MCI, suggesting that OxS may be an early event in the pathogenesis of AD (Keller et al, 2005; Lovell and Markesbery, 2007). The mutual interaction between systemic inflammation and OxS can be the explanation for the involvement of the former in the onset and progression of cognitive decline. Some evidences showed that patients affected by AD are characterized by a low-grade systemic inflammation, with increased plasma levels of IL-1β and TNF-α (Zuliani *et al*, 2007).

The relationship between OxS and dementia development have been nicely characterized in animals, *in vitro*, and in post-mortem models, but it still awaits confirmations by studies on living human patients (Matsouka *et al*, 2001; Yao *et al*, 2003; Abramov *et al*, 2001; Marksbery *et al*, 2005). Available clinical data are still conflicting, since both increased markers of oxidative damage and/or decrease antioxidants (Polidori *et al*, 2004; Serra *et al*, 2009; Gustaw-Rothenberg *et al*, 2010), and normal OxS plasma level (Guidi *et al*, 2006) have been reported in demented individuals. The reasons for these discrepancies might lie in several limitations of previous studies including small sample size, lack of control over relevant confounding factors (e.g. age, diabetes, systemic inflammation, CVD and hypertension), and use methods for OxS markers detection with low specificity and sensitivity.

1.6 OBJECTIVES OF THE STUDY

On the bases of the contradiction, the main purposes of our study were:

1. To evaluate in serum a panel of distinct indicators of systemic OxS in a large sample of older patients affected by LOAD, MCI and VaD, and compared them to a cognitively healthy controls;

2. Longitudinally study, to investigate whether baseline serum level of oxidant and/or serum antioxidant capacity might be predictive of the clinical progression from MCI to LOAD.

MATERIALS AND METHODS

1.7 DESIGN OF THE STUDY

The study was conducted according to the Declaration of Helsinki (World Medical Association, http://www.wma.net), the guidelines for Good Clinical Practice (European Medicines Agency, http://www.ema.europa.eu), and the guidelines Strengthening the Reporting of Observational Studies in Epidemiology guidelines (http://www.strobe-statement.org), and it was approved by the local Ethic Committee for human experimentation. A written informed consent for research was obtained from each patient or from relatives or a legal guardian.

Personal data and medical history were collected by a structured interview from patients and caregivers. All patients underwent a general and neurological examination. For neuropsychological assessment, all patients were given a battery of tests as previously described (Zuliani *et al*, 2007). Routine analyses were performed to exclude causes of secondary cognitive impairment, including serum B_{12} vitamin, serum folate, liver function tests including ammonia, kidney function tests, thyroid function tests, blood cell count, and arterial oxygen saturation. Subjects affected by severe congestive heart failure, severe liver or kidney disease, severe chronic obstructive pulmonary disease, and cancer were excluded. There were no evidences of acute illnesses at the time of clinical observation and blood sampling; no subject was taking NSAIDS, antibiotics or steroids at the time of recruitment.

Criteria used for the diagnosis of diabetes, arterial hypertension, and CVD were reported elsewhere (Zuliani *et al*, 2007). Smokers were defined as patients with present or previous significant history of smoking (> 180 packs/years).

The diagnosis of LOAD and VaD during was made according to the NINCDS-ADRDA criteria (McKhann *et al*, 1984). The diagnosis of dementia or MCI was made by trained geriatricians.

1.7.1 Diagnosis of LOAD

Trained geriatricians made diagnosis of LOAD according to the NINCDS-ADRDA criteria in 105 patients (mean age: 78.6 years) that were enrolled in the study. Only patients with “probable” Alzheimer’s disease were selected for the inclusion in the study in order to increase specificity. The Global Deterioration Scale (GDS) ranged from stage 4 to stage 6.

1.7.2 Diagnosis of VaD

Fifty four elderly patients with diagnosis of VaD by the NINDS-AIREN criteria (Hogervorst *et al*, 2003) (mean age: 79.3 years) referring to the same Day Service. Only patients with “probable” VaD were enrolled. The GDS ranged from stage 4 to stage 6.

1.7.3 Diagnosis of MCI

One hundred ninety-nine elderly patients with diagnosis of MCI referring to the same Day Service (mean age: 78.8 years).

MCI was defined as the presence of short/long-term memory impairment, with/without impairment in other single or multiple cognitive domains, in an individual who didn't meet the standardized criteria for dementia (Petersen *et al*, 2001). We also required that the patient with MCI would be still independent in the activities of daily living (ADLs). Subjects with MCI due to known causes (*e.g.* severe depression, extensive white matter pathology, severe vitamin B_{12} deficiency) had been excluded.

The diagnosis of LOAD during the follow-up was made always in according to the NINCDS-ADRDA criteria (McKhann *et al*, 1984). A subgroup (111 subjects) of these MCI patients were followed up for a mean period of 2 years (2.0 ± 0.6 years) and divided into 2 sub-groups on the basis of clinical evolution at follow-up: i) 82 patients whose cognitive performance remained stable or slightly improved (MCI/MCI); and ii) 29 patients converted to LOAD (MCI/LOAD).

1.7.4 Cognitive healthy controls

One hundred eighteen normal older individuals (Controls) without any evidence of dementia and without any functional disability attributable to cognitive impairment were included in the study.

1.8 BIOCHEMICAL ASSAYS

1.8.1 Samples collection

Fresh blood (7 mL) was drawn into vacutainer tubes without anticoagulant by venipuncture after an overnight fast. After 30 minutes of incubation at room temperature, blood samples were centrifuged (3000 g for 10 minutes), and the obtained serum was then divided into aliquots and were stored at – 80°C until analysis.

All the following assays were performed on serum samples using Tecan Sunrise-96 well microplate spectrophotometer (Tecan group Ltd., UK).

1.8.2 dROMs Test

The levels of hydroperoxides (HY) were evaluated by colorimetric assay based on the reaction between these lipid peroxidation by-products and the chromogenic compound, *i.e.* N,N-diethyl-para-phenylendiamine (Sigma-Aldrich, St. Louis, MO, USA) (Alberti *et al*, 2000). Briefly, for each subject, 5 µl of serum or standard (H_2O_2) were added to a solution containing 190 µl of acetate buffer (pH 4.8) and 5 µl of chromogen (0.0028 M). The solution was incubated at 37°C and then

read for optical density after 1 and 4 minutes. The concentration of HY was obtained by the average ΔA_{505}/min and expressed as Carratelli Units (CU), where 1 CU corresponds to 0.023 mM of H_2O_2 (Alberti *et al*, 2000). The intra-assay and inter-assay coefficient of variation was 2.5 and 3.5%, respectively and the limit of detection was 40 CU.

1.8.3 Advanced Oxidation Protein Products (AOPP)

The concentration of advanced oxidation protein products (AOPP) was quantified in according to Capeillère-Blandin (Capeillère-Blandin *et al*, 2004), with minor modifications. The AOPP assay includes a sample preparation procedure to precipitate triglycerides (3000 x g for 10 minutes in presence of 25 mM/L $MgCl_2$ and 0.5 mM/L phosphotungstic acid) which strongly interfere with the determination of the marker. Subsequently, 30 µL of supernatant serum (or the standard chloramine-T) was diluted 1:5 in phosphate-buffered saline. This solution was added into each well and mixed with 10 µL of 1.16 M potassium iodide and 20 µl of glacial acetic acid to each well. AOPP were measured at 340 nm and expressed as µmol/L of chloramine-T (Sigma-Aldrich) equivalents (Capeillère-Blandin *et al*, 2004). Concentrations of AOPP, determined in reference to the calibration were expressed in µmol/L. The intra-assay CV was 5.1%, whereas the inter-assay CV was 9.5%. The limit of detection was 1.5 µmol/L.

1.8.4 Ferric Reduction Antioxidant Power (FRAP) assay

The total concentration of non-enzymatic antioxidants (such as uric acid, ascorbic acid, α-tocopherol etc.) was determined by Ferric Reduction Antioxidant Power (FRAP) assay accordingly to original description by Benzie and Strain with slight modifications (Benzie and Strain, 1999). FRAP method measures the ability of water- and fat-soluble antioxidants to reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}) which absorbs at 593 nm. Briefly, acetate buffer (pH 3.6), TPTZ (10 mM) and $FeCl_3$ (20 mM) were mixed in the ratio 10:1:1 to give the working solution. Serum (10 µL), or standard ($FeSO_4$), was added to 190 µL of this solution. The reaction mixture was then incubated at room temperature for 6 minutes and the absorbance value was recorded at 595 nm. The results of this assay were expressed as FRAP units, where 1 FRAP unit corresponds to 100 µmoles/L of Fe^{3+} reduced to Fe^{2+} in 6 minutes. The intra-assay and inter-assay coefficient of variation was 3.9 and 9.9%, respectively.

1.8.5 Uric Acid (UA) assay

The concentration of serum uric acid (µmoles/L) was determined by the direct enzymatic method (Fossati *et al*, 1980) in which uric acid was oxidized by uricase coupled with peroxidase, and the results were measured colorimetrically. The intra-assay and inter-assay coefficient of variation was 1.2 and 2.2%, respectively. Uric acid, contributes around 60% of total reducing power of serum

measured by FRAP test, at least in healthy subjects (Duplancic *et al*, 2011). The constant stechiometric factor of this test (1 μM= 2 Ascorbic Equivalent Antioxidant Capacity-AEAC) allows the determination of a residual antioxidant power (RAP), by subtracting the contribution of uric acid from the total antioxidant activity (TAP) values. Therefore RAP is a parameter affording a further sensitive index of antioxidant status in uric acid-rich fluids such as serum.

1.8.6 Thiols (TH)

Total concentration of thiols was determined by the colorimetric 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB)-based assay described by Hu (Hu, 1994). Serum (20 μL), or standard (cysteine), was mixed with 160 μL of 0.2 M Na_2HPO_4, 2mM EDTA at pH 8.0 into each well. The absorbance was determined at 405 nm, and 20 μL of 10 mM DTNB (Sigma-Aldrich, St. Louis, MO, USA) in methanol were added to the sample. The absorbance obtained before the addition of DTNB was subtracted from that obtained after incubation with the chormogen. The concentration of thiol groups was expressed as μmoles/L. The intra-assay CV was 6.5%, whereas the inter-assay CV was 8.5%.

1.8.7 Homocysteine

Homocysteine level was determined by the Clinical laboratory of S. Anna Hospital, Ferrara by the Liquid Stable (LS) 2-Part Homocysteine Reagent (Axis-Shield Diagnostics Ltd., UK) using ROCHE COBAS INTEGRA 800 chemistry analyzer following the manufacturer's instructions. Concentrations of homocysteine, determined in reference to the calibration curve, were expressed in μmol/L. The intra-assay CV was 1.5%, whereas the inter-assay CV was 2.6%.

1.8.8 High sensitivity C-Reactive Protein (hs-CRP)

The C-reactive Protein is a marker of chronic inflammation disorders. Concentration of hs-CRP in serum (ng/ml) were measured by using a commercially available non competitive ELISA kit (dbc, Diagnostic Biochem Canada, Canada) according to the guidelines. All the samples were diluted 1:20. The limit of the assay was 10mg/ml, and the CV intra- and inter-assay were 9.5 and 9.0, respectevely.

1.9 LABORATORY ASSESSMENT

1.9.1 Brain computer tomography scan

All patients (LOAD and MCI) underwent a brain Computer Tomography (CT). The instrument used was a third generation SIEMENS SOMATON HQ. The slice thickness was 10 mm. Radiograms were evaluated by trained radiologists who were not informed about the clinical characteristics of the patient. The CT scan information was used to support the clinical diagnosis, and to diagnose possible brain pathologies associated with secondary cognitive impairment.

1.10 STATISTICAL ANALYSIS

Normal distributions of the viariables examined were checked by the D'Agostino Omnibus Test. Since the distribution of hs-CRP and OxS parameters (HY, AOPP, AU, TH, total antioxidant power-TAP and RAP) were skewed, the values were base-10 logarithm tranformed in order to approximate a normal distribution before entering in the statistic analysis. The means of the groups under consideration were compared by analysis of variance (ANOVA) (Fisher's least significant difference as *post-hoc* test), while prevalences were compared by χ^2 test. Analysis of covariance (ANCOVA) was used to check if the difference between groups revealed by univariate analysis were indipendent of potential confunders. The covariates in the multivariate analysis were: age (years), gender (male/female), CVD (yes/no), diabetes (yes/no), hypertension (yes/no), smoke (current/never). Due to the results of our preliminar correlation analysis, hs-CRP was added as covariate in the model for HY, UA, RAP and TH.

Adjusted Odds Ratio (O.R.; 95% confidence interval – 95% CI) for the diagnosis of MCI and LOAD in subjects with high level of HY (above the median value: 310.97 UC) or low levels of RAP (below the median value: 153.7 FRAP units) were calculated by multivariate logisitc regression analysis. High/low HY and RAP values were also combined into four groups characterized by progressively increasing levels of OxS, in order to evaluate the risk for MCI and LOAD resulting from the combination of these two parameters. The four groups were as follows:

- GROUP I: low HY and high RAP values (reference group)
- GROUP II: high HY and high RAP;
- GROUP III: low HY and low RAP;
- GROUP IV: high HY and low RAP.

The risk of receiving a diagnosis of LOAD or VaD in subjects with hyperuricemia or homocysteinemia was calculated by multivariate logistic regression, by using the same covariates of previous analysis. High/low uric acid (UA) and homocysteine serum levels were also combined into three groups in order to evaluate the risk for LOAD and VaD associated with the combination

of these two parameters:

- GROUP I: both parameters within normal limits;
- GROUP II: either one parameter increased;
- GROUP III: both parameters increased.

A two-tailed probability value <0.05 was considered statistically significant. SPSS 17.00 for Windows (Chicago, Illinois, USA) was used for statistical analysis.

RESULTS

The main characteristics of the four groups of subjects are reported in Table 1. As it shown, Controls were younger than LOAD, MCI and VaD. The subjects enrolled for the study were mostly women, and MCI, LOAD and VaD patients presented lower education level compared to Controls group. As expected by selection criteria, the mean of Mini Mental State Examination (MMSE) score was reduced in LOAD and VaD patients, whereas it was within normal limits in MCI and Controls. As regards cormobilities, diabetes, hypertension and CVD were more frequent in MCI, LOAD and VaD compared to Controls. CVD was also more frequent in VaD in comparison with MCI.

Table 1. Principal characteristics of whole sample: non-demented healthy controls, MCI, LOAD and VaD patients

	CONTROLS (n= 118)	**MCI (n= 199)**	**LOAD (n= 105)**	**VaD (n= 54)**
Age (years)	69.5 ± 9.1	77.8 ± 4.1	78.1 ± 5.5 [a]	79.3 ± 5.5 [a]
Female gender (%)	72. 0	64.1	70.1	56.1 [b]
Formal Education (years)	9.1 ± 4.3	5.9 ± 4.0 [c]	5.3 ± 3.5 [c]	5.7 ± 3.8 [a]
MMSE score (/30)	26.7 ± 2.7	25.8 ± 3.2 [a]	20.4 ± 4.4 [a]	21.1 ± 4.2 [a]
GDS (/15)	6.2 ± 3.5	5.6 ± 3.8	5.4 ± 3.3	5.7 ± 4.1
Hypertension (%)	42.1	66.2	64.1[a]	69.1
Diabetes (%)	10.1	14.8	13.7	26.9
CVD (%)	9.5	15.9	16.7	28.1 [a,c]
Smoking (%)	8.5	8.6	8.1	9.6

Continuous variables are expressed as mean ± standard error of the mean (SEM).
Abbreviations: CVD: cardiovascular disease; MMSE: Mini Mental State Examination; GDS: Global Deterioration Scale. [a] $p<0.05$ *vs* Controls ; [b] $p<0.05$ *vs* LOAD; [c] $p<0.05$ *vs* MCI

In Table 2 are reported the mean levels of serum OxS markers, HY, AOPP, TH, UA, RAP (obtained by subtracting UA from TAP) homocysteine in the four groups. Since hs-CRP was correlated to HY ($r=0.180$, $p<0.01$), UA ($r=-0.184$, $p<0.01$), RAP ($r=0.190$, $p<0.05$), and TH ($r=-0.134$, $p<0.05$), it was included as covariate, along with other widely documented confounders (i.e. age, gender, CVD, diabetes, hypertension and smoking) in ANCOVA analysis. Among the OxS markers considered, only HY, RAP, homocysteine showed at least one significant difference between two out of four groups after adjustment for the confounding factors. In particular, compared to Controls, HY were significantly higher, while RAP was significantly lower in both MCI and LOAD (ANCOVA $p<0.05$ and $p<0.01$, respectively). Similarly VaD was characterized by lower RAP (ANCOVA $p<0.01$) and HY (but not significantly), compared to Controls group. Finally, homocysteine levels were slightly higher in VaD compare to Controls ($p<0.058$) and MCI ($p<0.01$).

Table 2. Mean levels (mean ± SEM) of serum OxS markers in non-demented healthy Controls, MCI, LOAD and VaD patients.

	CONTROLS (n= 118)	**MCI (n= 199)**	**LOAD (n= 105)**	**VaD (n= 54)**
HY(CU)	288.1 ± 11.9	294.9 ± 10.0[a]	320.9 ± 12.9[a]	268.5 ± 14.8
AOPP (µmoles/L)	76.8 ± 3.7	71.7 ± 1.6	68.1 ± 1.7	72.7 ± 3.3
Homocysteine (µmoles/L)	14.6 ± 3.4	14.3 ± 1.1	16.8 ± 1.7	18.1 ± 2.2[b]
TH (µmoles/L)	247.7 ± 18.4	170.2 ± 19.0	155.0 ± 15.6	154.0 ± 14.2
UA (µmoles/L)	328.1 ± 17.6	337.9 ± 10.8	344.0 ± 10.5	363.2 ± 12.9
TAP (FRAP units)	663.8 ± 21.0	645.7 ± 16.6	665.2 ± 20.2	683.2 ± 31.2
RAP (FRAP units)	234.5 ± 16.0	178.0 ± 10.0[a]	152.2 ± 15.5[a]	175.2 ± 19.5[a]

ANCOVA: [a] $p<0.05$ or $p<0.01$ *vs* controls; [b] $p<0.05$ *vs* MCI, after adjustment for confounding factors: age, CVD, hypertension, diabetes, smoking, gender and hs-CRP.
Abbreviations: HY, hydroperoxides, CU, Carratelli Units; AOPP, advanced oxidation protein products; TH, thiols; UA, Uric Acid; TAP, total antioxidant power; FRAP, Ferric reduction antioxidant capacity; RAP, residual antioxidant power.

Figure 1 shows the redox status (mean levels of HY and RAP) in Control, MCI, LOAD and VaD. This figure highlights that the presence of a redox imbalance in LOAD and MCI patients compared

to Controls. Instead regarding patients affected by VaD is present an imbalance, but not significative.

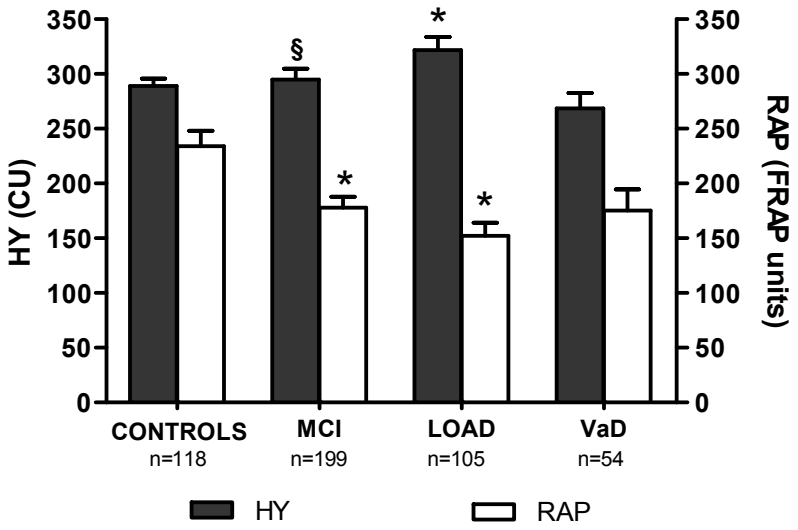


Figure 1: Mean levels of HY and RAP in no-demented healthy patient controls, MCI, LOAD and VaD. Abbreviations: HY, hydroperoxides; CU, Carratelli Units; FRAP, Ferric reduction antioxidant capacity, RAP, residual antioxidant power. In the ANCOVA model the covatiariates considered are: age, gender, hypertension, CVD, diabetes, smoking and hs-CRP. §$p<0.05$ and *$p<0.01$ *vs* Controls

To confirm the evident redox imbalance in MCI and LOAD patients, and to verify the involvement of OxS in the pathology, we calculated the Odds Ratio in according to the median of OxS markers from the whole sample (i.e. 310.97 CU for HY, and 153.7 FRAP units for RAP). In Table 3 are described the probability (O.R.-95% C.I.) for subjects with high HY or low RAP serum levels, of receiving a diagnosis of MCI or LOAD.

Table 3. Odds Ratios (95% C.I.) for diagnosis of MCI or LOAD in subjects with high levels (above the median value) of HY (panel A), or low levels (under the median value) of RAP (panel B).

A) High HY levels	**CONTROLS**	**MCI**
MCI	2.59 (1.08-6.21)	-
LOAD	4.09 (1.36-11.81)	1.71 (0.89-3.25)
B) Low RAP levels	**CONTROLS**	**MCI**
MCI	3.97 (1.62-9.72)	-
LOAD	2.31 (0.83-6.63**)**	0.70 (0.35-1.38)

Covariates included in the models: age, gender, hypertension, CVD, diabetes, smoking and hs-CRP. Abbreviations: HY, hydroperoxides, RAP, residual antioxidant power.

From this analysis it emerged that high levels of HY were associated with a significant increase in likelihood of having MCI and LOAD compared to Controls. Low levels of RAP were also associated with increased probabilities of having MCI and LOAD, but only for MCI the associations was statistically significant.

To check if even MCI or LOAD condition was associated to a derangement of oxidative balance, and not only to a change of one single "scale pan", the whole sample was divided into four groups, on the base of the combinations of HY and RAP levels:

- GROUP I: low HY and high RAP values (reference group);
- GROUP II: high HY and high RAP;
- GROUP III: low HY and low RAP;
- GROUP IV: high HY and low RAP.

In Figure 2 is reported the risk (95% C.I.) for diagnosis of MCI or LOAD. Compared to group I (lower OxS stress), a progressive increase in the risk of MCI diagnosis was observed from group II (O.R.: 3.63; 95% C.I.: 0.87-15.05), to group III (O.R.: 3.77; 95% C.I., 1.05-13.54), to group IV (O.R.: 6.99; 95% C.I., 1.87-25.21) (Figure 2 A). An increase in the probability of LOAD diagnosis was also observed in group II (O.R.: 4.27; 95% C.I.: 0.79-24.02), and group IV (O.R.: 4.87; 95% C.I.: 1.02-23.55), but only the latter resulted statistically significant (Figure 2 B).

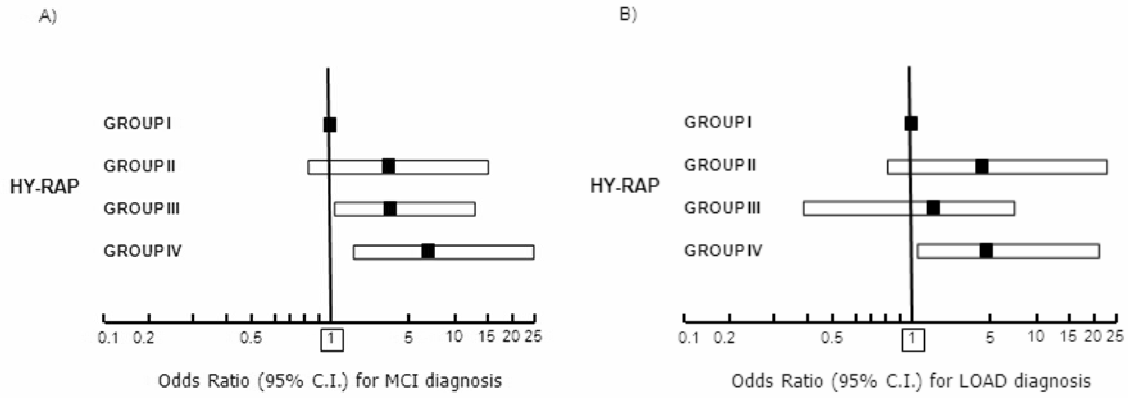


Figure 2: Odds Ratios (95% C.I.) for: A) MCI or B) LOAD diagnosis in subjects with: low HY and high RAP values (group I); high HY and high RAP (group II); low HY and low RAP (group III); high HY and low RAP (group IV). Abbreviations: HY, hydroperoxides; RAP, residual antioxidant power.

A randomly selected subsample (n= 111) of MCI were longitudinally followed up for mean period of 2 years (2.0 ± 0.6 years), to investigate whether baseline levels of HY and RAP might be predictive in the clinical progression from MCI to LOAD. At the end of follow-up, 82 patients showed no cognitive deterioration (so they were designed as MCI/MCI), while 29 converted to LOAD (classified as MCI/LOAD).

As shown in Table 4 we did not found any difference in either OxS markers between these two MCI subgroups.

Table 4. Mean levels (mean ± standard error of the mean, SEM) of serum HY and RAP in MCI/MCI, MCI/LOAD patients.

	MCI/MCI	**MCI/LOAD**
	(n= 82)	**(n=29)**
HY (CU)	295.8 ± 10.0	281.2± 17.5
RAP (FRAP units)	187.5 ± 16.2	205.1± 22.1

MCI/MCI: stable MCI patients; MCI/LOAD: MCI patients converted to LOAD In ANCOVA model: age, CVD, hypertension, gender, smoking, hs-CRP. Abbreviations: CU, Carratelli Units; RAP, residual antioxidant power

As showed in Table 2 the levels of UA and homocysteine, which are markers of both and CVD, were higher in VaD in respect to the other three groups. The results prompted us to evaluate whether hyperuricemia or hyperhomocysteinemia might be independently associated with a

different probability to having either forms of dementia. As shown in Fig. 3 C, higher homocysteine levels (≥ 17.0 μmoles/L) were associated with higher likelihood of VaD diagnosis compared with both Controls (O.R.: 4.17, 95% C.I.: 1.49-11.61) and MCI (O.R.: 3.12; 95% C.I.: 1.34-7.26), after adjusting for age, CVD, hypertension, gender, smoking, hs-CRP. A trend toward a higher risk of VaD compared with Controls, but not with MCI, was found in subjects with high uric acid levels (men: >400 μmoles/L; women: >360 μmoles/L.), but it was no more significant after multivariate adjustment (O.R.: 2.50, 95% C.I.: 0.99-5.80) (Fig. 3 D). The risk of LOAD diagnosis was not associated with higher levels of either levels of homocysteine or UA (Fig. 3 A, 3 B).

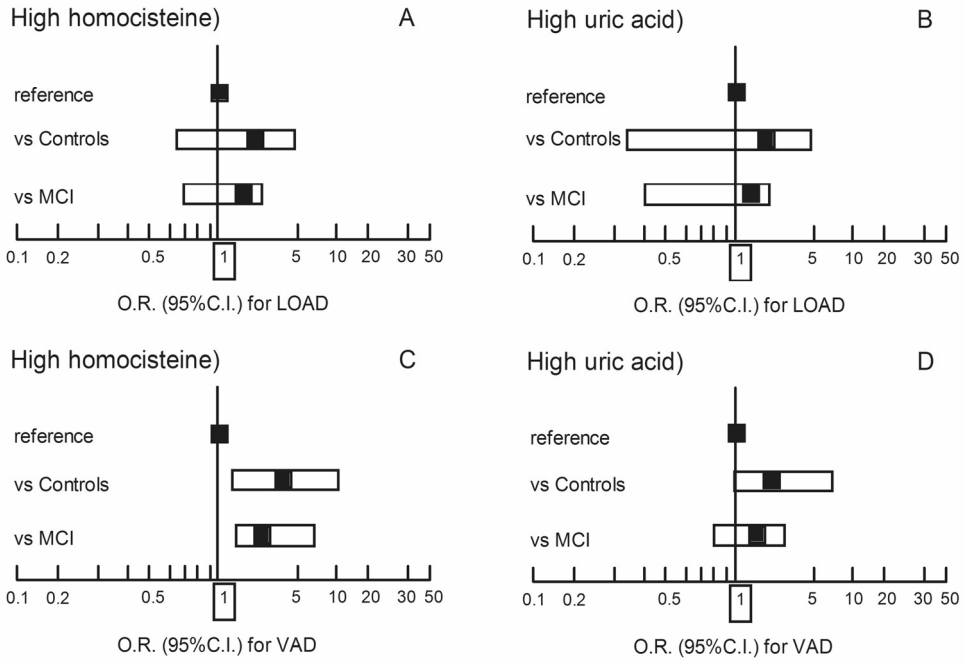


Figure 3: Odds Ratios (95% C.I.) for diagnosis of LOAD or VaD (compared to Controls or MCI subjects) in individuals with high levels of homocysteine (≥ 17.0 μmoles/L) or UA levels (men: >400 μmoles/L; women >360 μmoles/L). O.R. adjusted for covariates: CVD, hypertension, diabetes, smoking and gender.

To further check the relationship between the two markers and the two neurodegenerative diseases, we assessed the adjusted risk (O.R.-95%C.I.) for the diagnosis of LOAD or VaD. To accomplis this aim, we first divided the sample into three groups according to the levels of homocysteine and UA :

- Group I: both parameters within normal levels;
- Group II: increase in homocysteine or uric acid levels;
- Group III: increase in both parameters.

Compared to Group I, a progressive increase in the risk of VAD diagnosis compared with both

Controls (2 C) and MCI (2 D) individuals was observed in Group II (O.R.: 6.32, 95% C.I.: 1.32-29.10 and O.R.: 2.10; 95% C.I.: 0.62-6.52, respectively) and Group III (O.R.: 10.50, 95% C.I.: 2.33-47.22 and O.R.: 4.50; 95% C.I.: 1.31-15.61, respectively); of note, the risk for VaD observed in Group III was remarkably higher compared with Group II. On the contrary, the risk of LOAD diagnosis increased much less and was not significant in either groups (2 A and 2 B).

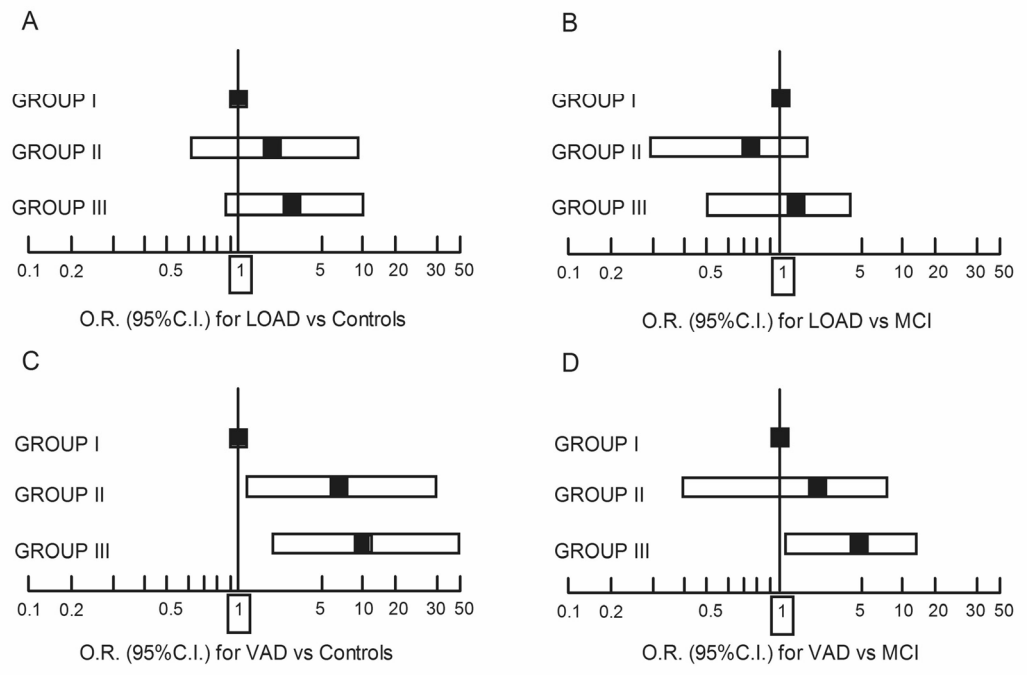


Figure 4: Odds Ratios (95% C.I.) for diagnosis of LOAD or VaD (compared to Controls or MCI subjects) in subjects with: low homocysteine and UA (reference; group I); high UA or homocysteine (group II); high homocysteine and UA (group III). O.R. adjusted for covariates: CVD, hypertension, diabetes, smoking and gender.

DISCUSSION

The condition of OxS has been widely associated to the pathogenesis and progression of several aging-related diseases (Migliore *et al*, 2005; Floyd *et al*, 2011). Indeed, ROS attack is responsible for most of the irreversible damages of major molecular components in cells, organelles and membranes (Bergamini *et al*, 2004). A key-role of OxS in early and late events of AD has been suggested by the accumulation of products of oxidative damage to lipid, DNA, proteins and carbohydrates in *post-mortem* brain tissue from patients affected by MCI, LOAD as well as VaD (Barone *et al*, 2011). In line with these findings there are several studies based on *in vitro* or animal experiments. In contrast, at present, the published data from *in vivo* human studies appear still conflicting.

One of the most important reasons of the unconsistency of *in vivo* studies on humans might be the lack of a gold standard marker for OxS determination. As consequence, currently, the only way to quantify OxS *in vivo* (i.e. in body fluids) is to measure a battery of different indirect markers, preferably reflecting lipid and/or protein oxidative damage as well as antioxidant content. Besides, for a population-based study it would be mandatory to deal with a large sample and, when the condition investigated is related to ageing, to separate the effect of ageing from that of the condition itself. To address these important issues, we measured the serum levels of seven different OxS markers in a large sample of elderly individuals including 118 non-demented healthy controls, 199 MCI, 105 LOAD, and 54 VaD patients.

The main finding of the present study was that MCI, LOAD and VaD are associated with an impairment of systemic oxidative balance. This is more evident in MCI (consider a LOAD "pre-clinical" status) and LOAD patients, since both categories of patients showed a significant and independent, and comparable, decrease antioxidant defence (RAP) and increase of HY. Moreover, subjects with low RAP or high HY had a high probability to be affected by MCI, while an increase in the risk of LOAD was merely found in association with high level of the peroxidation marker (Table 3). Consistently, the risk for both conditions greatly increased when antioxidants and oxidative products changed together from a favorable balance (high RAP-low HY) to a "full blown" OxS state (low RAP -high HY) (Figure 2 A and B). These data suggest that OxS might represent an early event in LOAD pathogenesis, and that the process of redox balance derangement may take place in its prodromal phase (Figure 1).

Globally taken, the longitudinal data on MCI were consistent with a the above mentioned concept of OxS as a condition already present in the prodromal state of AD. Indeed, we found that neither baseline level of the two peripheral markers was able to predict the progression from MCI to LOAD. In other words, the evaluation of OxS by using these markers might not be a helpful tool in indentifying MCI patients who are going to evolve to LOAD. Only a few prospective studies have

been done on this topic, and the results were controversial (Guidi *et al*, 2006; Montine *et al*, 2002). Baldeiras *et al*. (Baldeiras *et al*, 2008), in their prospective study conducted in a sample of 70 MCI subjects, did not find significant differences in any of the baseline indexes of oxidative damage and antioxidant defence measured between stable and progressing to dementia individuals. In contrast, in another study conducted on a similar number of subjects, F_2-isoprostanes measured in cerebrospinal fluid (CSF) were significantly higher in MCI patients who later converted to LOAD compared to stable patients (Brys *et al*, 2009). The discrepancies between these data and ours might be mainly due to the differences regarding the markers (and biological fluids) that were employed for OxS detection as well as regarding the general characteristics (e.g. age and lifestyle habits) of the population-samples. Furthermore, the evidence of similar oxidative, and inflammatory pattern in MCI and LOAD strengthen the widely supported concept of a biochemical equivalence between pre- and clinical conditions which, in turn, might explain the lack of differences between MCI/MCI and MCI/LOAD showed in our study (Table 4).

After demonstrated that OxS can be involved in the pathogenesis of LOAD and MCI, we focused our attention on VaD, which represents the second more frequent cause of dementia. Our aim was to investigate if also VaD could be associated with redox imbalance. As reported earlier, in the patients with VaD levels of HY and RAP appeared less "imbalanced" compared to the other two conditions examined (Table 2). Most of the studies present in literature have demonstrated that LOAD and VaD were associated with a comparable systemic oxidative imbalance (Polidori *et al*, 2004; Guidi *et al*, 2006; Serra *et al*, 2009; Gustaw-Rothenberg *et al*, 2010; Shi *et al*, 2012), but they appear different from ours in relation to methodology and composition/size of the sample. Noteworthy, the only one study dealing with a sample size comparable with ours, found no changes in either antioxidant or lipoperoxidation markers in VaD compared to controls. Nevertheless, it has to be underlined that in this study: i) a remarkable difference in age was observed between the groups, and ii) hypertension, CVD, and diabetes were not included neither among enrollment criteria nor as possible confounders. The hypothesis assuming LOAD as a "vascular disorder" caused by an impairment of cerebral perfusion (De la Torre, 2004; Altman and Rutledge, 2010) might help to explain as OxS emerged as possible common soil for LOAD and VaD in our study. Indeed, it has been shown that some medical conditions such as stroke, CVD, and atherosclerosis might have an important role in the pathogenesis of either forms of dementia. The impairment of oxidative balance observed in VaD and LOAD might derive from vascular OxS; in this light, higher level of HY found in LOAD might derive from a further supply of free radicals
provided by Aβ deposits that both directly (by eliciting superoxide dismutase activity) (Barnham *et al*, 2004) and indirectly (by increasing neuroinflammation and by inducing expression of NADPH oxidase in endothelial (Park *et al*, 2005) and glial cells) enhances neuronal ROS production in this disease.

Interestingly, we also found that individuals with simultaneously high level of homocysteine and UA, which are both well documented risk factors/markers for cardiovascular disorders, had a higher probability to be affected by VaD but not LOAD and MCI (Figure 3 and Figure 4). In one hand, hypeuricemia has been found to be strongly associated (for some Authors by a cause and effect relationship (Nakagawa *et al*, 2006) with metabolic syndrome (Li *et al*, 2011) which, in turn, is an independent predictor of atherosclerosis and cardiovascular mortality (Mottillo *et al*, 2010). In the other hand, high levels of homocysteine might exert even more deleterious effects on cardiovascular health, as demonstrated by the significant reduction in the risk of coronary heart disease, deep vein thrombosis, and stroke observed after lowering homocysteine concentrations by 3 micromol/L from current levels (Wald *et al*, 2002).

Moreover, a number of studies dealing with either of the two markers have shown a significant association with dementia (Schretlen *et al*, 2007; Ruggiero *et al*, 2009), mainly when it was caused by cerebrovascular disorder (Ruggiero *et al*, 2009; Ray *et al*, 2013). The proposed pathogenic pathways linking high levels of uric acid or homocysteine to these diseases imply, besides OxS, endothelial dysfunction and inflammation, which are conditions deeply involved in VaD development. In this context it might be hypothesized that, when simultaneously "high" these two substances might have synergistic detrimental effect on endothelial cell functionality and/or on cytokines production, thus leading to an increase in the likelihood of developing VaD. In apparent contradiction, UA and homocysteine are classically described to elicit an opposite function in systemic redox regulation (Perna *et al*, 2003; Glantzounis *et al*, 2005); homocysteine works as a strong pro-oxidant while uric acid is regarded as the most abundant non-enzymatic circulatory antioxidant. However, urate can also act as pro-oxidant especially in the presence of high concentration of singlet oxygen and peroxynitrite (Sautin and Johnson, 2008). For this reason, we and other investigators (Perna *et al*, 2003) subtracted the contribute of uric acid from the measure of serum TAP since the abundance of uric acid tends to mask the contribution of other antioxidants, (i.e. ascorbic acid and α-tocopherol) which are more effective in contrasting lipid peroxidation processes in neuronal membranes.

In conclusion, taken together our data suggest that MCI, LOAD and VaD are all associated with an impairment of systemic oxidative balance, but regardless of a possible common OxS-related route in their pathogenesis; VaD and LOAD maintain different features. Furthermore, our data are consistent with the concept of OxS as an early event in LOAD. If confirmed by other studies, our results may indirectly support the use of dietary antioxidants in order to rebalance the altered redox homeostasis in patients affected by MCI, LOAD and VaD.

CHAPTER 2: INVOLVEMENT OF OXIDATIVE STRESS IN POST-MENOPAUSAL OSTEOPOROSIS

INTRODUCTION

2.1 OSTEOPOROSIS

2.1.1 Definition, epidemiology and incidence

Osteoporosis (from Greek οστούν/*ostun* "porous bones") is defined as "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue in fracture risk" (Lee and Vasikaran, 2012). Historically, in 1842 Copper (Cooper, 1842) suggested that certain types of fractures may occur due to aged-related reduction in bone mass or quality. He described the original classical epidemiology hallmarks of these fractures: the positive association with age and the higher frequency in women compared to men. Afterwards the term "osteoporosis" entered in the medical terminology in France and in Germany as a descriptive term emphasizing the porosity of the histological appearance of aged human bone (Holroyd *et al*, 2008) (Figure 2.1).

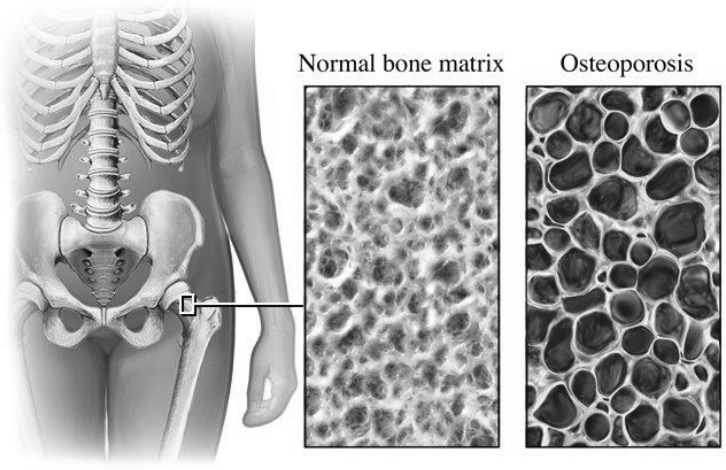


Figure 2.1. Normal bone *vs* Osteoporotic bone.

In the current years osteoporosis is becoming a major public health problem because of its correlation with aging. Elderly people are the fastest growing population in the world and, as people age, bone mass declines and risk of fracture increases. In addition osteoporosis-related fractures are one of the leading causes of significant morbidity and disability in elderly patients and increases the economic burden on the health care system. Worldwide, it is estimated that over 200 million people have osteoporosis (Lane, 2006; Reginster and Burlet, 2006). The reduction in bone strength associated with this disease markedly increases the risk of skeletal and nonskeletal fractures, and the consequent pain and loss of function impinge adversely on quality of life. All fractures (hip, vertebrae, ribs and wrist) are associated with considerable morbidity, a decline in

quality of life, and increased mortality. In Europe, the number of osteoporotic fractures was estimated at 3.79 million. As described above, osteoporotic fragility fractures impose a considerable financial burden on health service due to a reduced mobility, hospitalization, and nursing home requirements. In 1998 in Europe osteoporosis patients occupied 500,000 hospital bed-nights per year, and this was expected to double by 2050 (Lee and Vasikaran, 2012; Reginster and Burlet, 2006).

Osteoporosis may be either a primary or a secondary form. The primary form is the most common and is due to the typical age-related bone loss and it included postmenopausal and senile osteoporosis. Secondary osteoporosis is defined as bone loss consequent of an unrelated underlying cause, such as drug treatment (i.e. chronic corticosteroid use), hypogonadism, malnutrition or eating disorders (i.e. anorexia nervosa), and neoplastic disorders.

2.1.2 Physiological bone regeneration

Skeleton is a highly specialized and dynamic organ that undergoes continuous regeneration. It consists in highly specialized cells, mineralized and unmineralized connective tissue matrix, and spaces that include the bone marrow cavity, vascular canals, canaliculi and lacunae. During development and growth, the skeleton is sculpted to achieve its shape and size by removal of bone from one side and deposition at a different one; this process is called modeling. A healthy skeleton is maintained throughout life by the constant process of bone remodeling that is responsible for the complete regeneration of adult skeleton every 10 years. Bone remodeling is the process by which old bone replaced by new bone; it is regulated by the balanced activities of bone-resorbing osteoclasts and bone-forming osteoblasts, which are essential to maintain the normal physiological structure and mineral content (Figure 2.2).

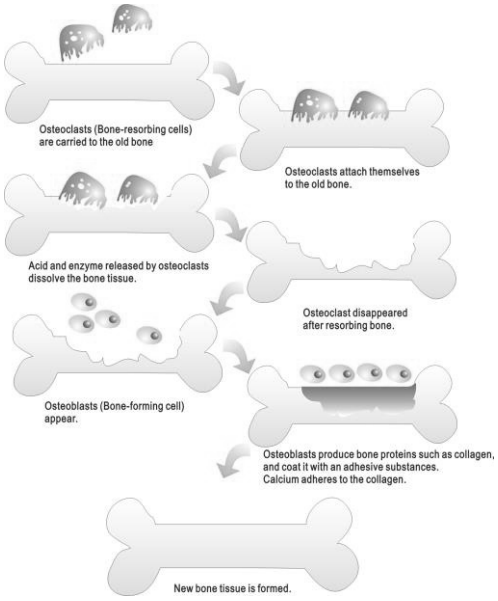


Figure 2.2. Role of bone cells: osteoblasts and osteoclasts. (Taken from Marieb and Brady, 2011).

The physiological bone remodeling cycle involves a series of highly regulated steps that depend on the interactions of osteoclasts and osteoblasts. This process consists in four phases (Figure 2.3):

1. **Resorption**, in which osteoclasts generate an acidic microenvironment between the cell and the surface of the bone, dissolving or resorbing the mineral continent of the bone. Usually this phase takes 3-4 weeks;
2. **Reversal**, in which osteoclasts undergo apoptosis and osteoblasts are recruited to the bone surface;
3. **Formation**, in which osteoblasts deposit collagen, which then, is mineralized to form new bone. Usually it takes 3-4 months;
4. **Resting**, which is the period follow until a new remodeling cycle begins.

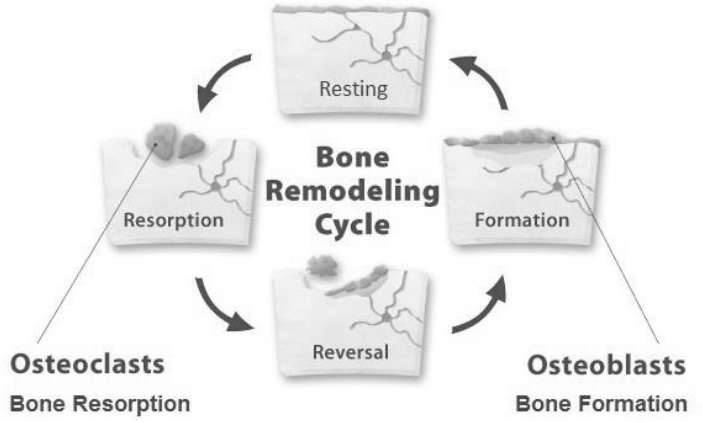


Figure 2.3. Bone remodeling cycle. The process consists in four phases: resorption, reversal, formation and resting. During the resortion osteoclasts dissolve or resorb the mineral continent of the bone (this phase takes 3-4 weeks). The osteoclasts undergo apoptosis and osteoblasts are recruited to the bone surface to form the new bone (reversal and formation phase, respectively). Formation takes about 3-4 moths to be complete. Resting is the phase follow until a new remodeling cycle begins.

Initially, "resorption" stage (or activation stage) involves the interaction of osteoclast and osteoblast precursor cells. This leads to the differentiation, migration and fusion of the large multinucleated osteoclastes. These cells attach to the mineralized bone surface and initiate the resorption by secretion of hydrogen ions and lysosomal enzymes. The attachment of osteoclasts to bone can require specific changes on the bone surface, which can release proteolytic enzymes to uncover a mineralized surface. Once the osteoclasts have completed their work of bone removal, the "reversal" phase starts. In this stage mononuclear cells are present on the bone surface, but their functions is not well understood yet; they may involve further degradation of collagen, deposition of proteoglycans to form the "cement line", and release growth factors to start the "formation" phase. During this final stage of the remodeling bone cycle, the cavity created by resorption can be completely filled in by successive layers of osteoblasts, which differentiate from their mesenchymal precursors and deposit a mineralizable matrix (Raisz, 1999).

Studies on the structural adaptation of the skeleton suggested that there are local factors which regulate bone remodeling. A large number of cytokines and growth factors have been identified as "osteoclasts-activating factors"; furthermore, emerging evidence suggest the involvement of some proteins in the interactions between osteoclasts and osteoblasts. These proteins are in the family of necrosis factor receptors. The activation of osteoclasts is regulated by various molecular signals; one of these is the receptor activator for nuclear factor κB ligand (RANKL). This molecule is produced by osteoblasts and other cells (i.e lymphocytes), and stimulates RANK (receptor activator of nuclear factor κB). The activation pathway of osteoclasts also involves osteoprotegerin (OPG). OPG binds to RANKL on osteoblast cells, blocks the RANKL-RANK ligand interaction between

osteoblast and osteoclast precursor. The last effect of this action is the inhibition of osteoclast precursor into mature osteoclast (Horwood *et al*, 1998; Yasuda *et al*, 1998).

The bone remodeling cycle is also regulated systemically. The metabolic functions of the skeleton are served in large part by two major calcium-regulating hormones: parathyroid hormone (PTH), 1,25-dihydroxy vitamin D. PHT is able to regulate serum calcium level and it can stimulate the bone resorption and has biphasic effects on bone formation. High concentration of this hormone leads to inhibition of collagen synthesis, but on the contrary prolongs intermittent administration of PTH with consequent increase of bone formation. Plasma PTH tends to increase with age, and this may produces an increase of bone turnover and a loss of bone mass. 1,25-dihydroxy vitamin D has a good effect on intestinal calcium and phosphate absorption, and it seems to have a direct effect on bone. It is possible that it can stimulate bone resorption and formation under certain conditions.

Besides PTH and vitamin D, estrogens, in particular 17-βestradiolo (E_2), play an important role, in the maintenance of normal bone turnover. The mechanism by which E_2 regulate bone turnover is still unknown, although studies on animals suggest that this hormone acts by altering the production and activity of local factors that regulate osteoblast and osteoclast precursors (Pacifici, 1998; Lorenzo, 1992).

2.1.3 Pathogenesis of osteoporosis

The underlying mechanism of both primary and secondary osteoporosis involves an imbalance between bone resorption and bone formation. As described before, the processes of resorption and reversal phases of bone remodeling are short (few weeks), while the time required for osteoblast replacement of the bone is longer (few months); accordingly any increase in the rate of bone remodeling will result in a loss of bone mass. The main mechanisms that play a causative role in the development of osteoporosis are:

- Inadequate peak bone mass, because of the insufficient mass and strength of the skeleton during growth;
- Increased bone resorption, that increases fracture risk by twofold;
- Inadequate formation of new bone during remodeling.

The consequence of these processes is the increase in fragility of bone due to a reduction in bone mineral density (BMD). This process is markedly favored in women after menopause because of low levels of circulating estrogens, and, in a less extent, to a condition of low-grade inflammation that seems to be related with postmenopausal transition.

2.1.4 Postmenopausal osteoporosis (PO)

Postmenopausal osteoporosis (PO) is the most common form of osteoporosis among women. In Europe about of 30% of postmenopausal women suffer from osteoporosis, and it has been predicted than more than 40% of them will suffer one or more bone fractures during their remaining lifetime. The first link between menopause and osteoporosis was identified in 1960's (Tella and Gallagher, 2013).

Peak bone mass is reached in the mid-twenties for spine and hip, at the time of menopause there is rapid acceleration in bone loss that starts the year before the menopause and continues for another 3 years, even if the rate of bone loss in the 4-8 years after menopause is still high. The decrease of BMD during the menopause transition is very consistent, about 10% of it can be lost, increasing the risk to develop postmenopausal osteoporosis. During the first phase of menopause bone loss women are in marked negative calcium balance; however this decrease of BMD in postmenopausal women is caused mainly by the cessation of ovarian function. Many evidence have shown that estrogens deficiency-related menopause impairs the normal bone turnover, leading to an acceleration of bone remodeling (Raisz, 2005; Parfit *et al*, 1995; Ebeling *et al*, 1996). Consistently, it has been demonstrated that estrogens have a dominant multifactor role in maintaining cortical bone formation by supporting osteoblasts and preventing bone resorption by suppressing osteoclasts formation and stimulating osteoclast apoptosis (Hughes et al, 1996; Khosla et al, 2011). The mechanisms underlying protective role of estrogens against osteoporosis is still not well understood. However, there are some evidence that suggest that estrogen may act as antinflammatory molecules against specific interleukins and cytokines involved in the development of the disease. Moreover estrogens seem to have a significative action in bone remodeling cycle by increasing OPG secretion and decreasing RANK (Tella and Gallagher, 2013).

2.1.5 Diagnosis of osteoporosis

Osteoporosis is diagnosed clinically when there is a presence of fragility fracture or LOW BMD measured by bone densitometry. The World Health Organization (WHO) has defined criteria for assessing bone status and determining the risk of fracture. These criteria are defined by the T-score, which is the number of standard deviations by which a patient's test result exceeds (positive T-score) or falls below (negative T-score) the mean of the young adult group. BMD is expressed as a relationship to two norms: T-score and Z-score (the expected BMD for the individual's age and sex). WHO has established the following diagnostic guidelines (WHO, 1994; WHO, 2003):

- T-score $\geq$ -1.0 is normal;
- -1.0 $\leq$ T-score < -2.0 is osteopenia;
- T-score $\leq$ -2.5 is osteoporosis.

Osteopenia is defined as condition where bone mineral density is lower than normal. It is considered to be a precursor to osteoporosis. However, not every person diagnosed with osteopenia will develop osteoporosis.

Multiple epidemiological studies have demonstrated that the risk of fracture increases as BMD decline. BMD and bone strength are measured by dual-energy X-ray absorptiometry (DXA). DXA is considered the gold standard for the diagnosis of osteoporosis, because of its low radiation, availability, capacity to evaluate multiple sites, and ease of use. DXA can measure soft-tissue composition (lean and fat mass) and bone mass or bone density at the lumbar spine, hip, and forearm, as well as total-body. Currently, the National Osteoporosis Foundation and the International Society for Clinical Densitometry consider central DXA of the hip and/or spine as the preferred measurement for the diagnosis. The measurement of BMD by DXA is made with greater precision and faster scanning times than the dual-photon absortiometry. The technique relies on transmission measurements made at two photon energies to allow calcium, and thereby bone mineral is assessed (Figure 2.4). The BMD is an area density, expressed in g/cm^2, which is conventionally used to describe the bone mass per unit of projected bone area, or the average mass per pixel (Mazess *et al*, 1990).

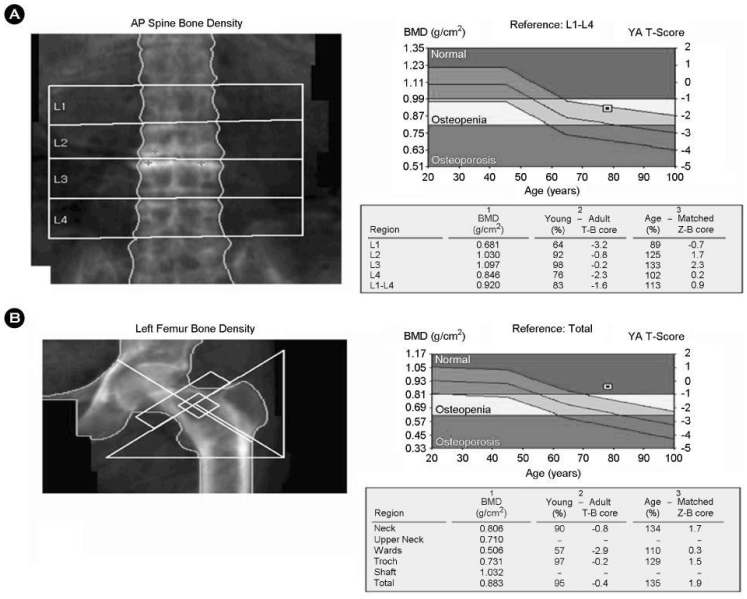


Figure 2.4. Example of medical report by DXA.

BMD cannot relaibly predict the risk of fractures, however investigators from the Study of Osteoporotic Fracture Research Group developed the FRACTURE index (FRAX) (Lane, 2006). FRAX is a fractures risk calculator that is measured by the use of algorithms which include a number of recognized independent risk factors for fracture in addition to BMD, such as age, sex,

body mass index (BMI), family history, past history of fractures, secondary causes of osteoporosis (i.e rheumatoid arthritis, use of medications, smoking and excessive alcohol intake) (Lee and Vasikaran, 2012; Kanis, 2008). FRAX is no calculated routinely in the process of osteoporosis's diagnosis but only in presence of T-score ≤ -2.5 or more.

In the diagnosis of osteoporosis, in addition of BMD, it is also important the bone strength, which is determined by structural and material properties that impact overall bone quality. The structural properties of bone include geometry, as size and shape, and microarchitecture for example trabecular thickness and connectivity and cortical thickness/porosity. The material properties of bone include mineralization, collagen composition (type and cross-links) and damage accumulation, which are all affected by bone turnover.

2.1.6 Biochemical bone turnover markers

Diagnosis of osteoporosis is not based on evaluation of bone markers. They are used for patients with osteoporosis only to evaluate the bone turnover or to detect the common causes of osteoporosis in order to treat them and to monitor the treatment. Biochemical monitoring of bone metabolism depends upon measurement of enzymes and proteins released during bone formation and of the degradation products produced during bone resorption. In fact, biochemical indices of bone cycle remodeling can be divided into two groups:

1. Markers of resorption;
2. Markers of formation.

These markers circulate in blood and/or are excreted in urine, and measuring them provides a quantitative estimate of the current rate of bone remodeling.

2.1.6.1 Bone resorption markers

The bone resorption markers are type I collagen degradation products (Garnero *et al*, 2000; Sunethra, 2012). They reflect the rate of bone matrix breakdown and, indirectly, the number of active osteoclasts. The most used resorption markers are:

- **Pyridinoline crosslinks**, which include pyridioline and deoxypyridinoline. These are small and cyclic amino acid structures linking peptide chains of collagen molecules. During resorption these structures are released into the circulation, and can be detected in urine. The urinary concentration of pyridioline and deoxypyridinoline reflect the rate of collagen degradation.
- **Telopeptides**, which consist in the N- and C-terminal ends of mature collagen that are released during bone resorption and can be detected in the circulation. Between these markers C-terminal telopeptide (CTX-1) is by far the most used and reliable indicator of

bone resorption. High concentration of CTX-1 has been found to be associated with and increased risk of fractures independent of BMD. The most common clinical application of this marker is in the monitoring the response to antiresorptive drugs (i.e bisphosphonates).

2.1.6.2 Bone formation markers

Bone formation markers are products of osteoblasts and their anabolic activity (Garnero *et al*, 2000; Sunethra, 2012). This group of markers includes:

- **Propeptides of type I procollagen**, which are released from osteoblasts. The concentration of them reflected the rate of bone formation;
- **Alkaline phosphatase**, which consists of several isoforms originated from live, bone, intestine, kidney and placenta. Bone-specific alkaline phosphatase (BAP) is synthesized in osteoblasts and reflects osteoblasts activity during bone formation. The concentration of BAP is significantly associated to fracture risk regardless of BMD in postmenopausal women;
- **Osteocalcin**, which is a protein synthesized by osteoblasts and binds to hydroxyapatite in the bone matrix. Osteocalcin is unstable once collected, for this reason resting is not widely offered.

2.1.7 Risk factors of osteoporosis

Many risk factors of osteoporosis have been identified. Several interaction factors contribute to the risk to develop this disease, including clinical, medical, nutrition and genetic variables. Risk factors of osteoporosis can be divided in two big groups:

- **Modifiable risk factors**;
- **Nonmodifiable risk factors**.

Modifiable factors include all those situations that can be modifyed through changes in the life style. The potentially modifiable risk factors are:

- **Excess alcohol**;
- **Vitamin D deficiency**;
- **Smoking**;
- **Malnutrition**;
- **High dietary protein**;
- **Low body mass index**;
- **Endurance training**.

Nonmodifiable risk factors include those entire factors that cannot be changed even with healthy life style. This group included:

- **Race**: it has been demonstrated that European or Asian ancestry are predisposed to develop osteoporosis;
- **Genetic**: people with a family history of fractures or osteoporosis are at an increased risk.;
- **History of fragility fracture**: this is an important risk factor for further fracture, evidence have demonstrated that risk of fracture of the hip of increased by more than two-fold after previous fracture of the hip and spine (Ross *et al*, 1993 of 9);
- **Sex**: It is well known that postmenopausal, but not premenopausal women are more susceptible to develop osteoporosis than man of the same age.;
- **Age**: with sex, age is the most important independent risk factor in the development of osteoporosis.

For all these risk factors WHO guidelines for osteoporosis suggest routine screening and treatment over 65 years of age and for postmenopausal women with history of fragility or who have one or more risk factors (Reginster and Burlet, 2006).

2.2 OXIDATIVE STRESS AND OSTEOPOROSIS

Oxidative stress (OxS) is considered to be responsible of aging process and a number of aging-related diseases such as osteoporosis, and in particular PO. A strict relationship between menopause and both development and progression of OxS-related diseases has been documented (Pansini *et al*, 2005). Consistently, a number of *in vitro* evidence suggest that ROS could be involved in the pathogenesis of PO. One of the most intriguing hypothesis at this regard considers the ability of estrogens to protect bone against OxS by acting as antioxidant. *In vitro* and animal experiments, indeed, showed that estrogens withdrawal alters the generation of reactive oxygen species (ROS) and the antioxidant defence capacity of the cell (Lean *et*, 2003), leading to an accumulation of these oxidant species, which, in turn, are able to stimulate osteclast formation and resorption activity (Lean et al, 2004; Hodge *et al*, 2011). Furthermore, more recently, it has been shown that the increase of ROS, mostly due to xanthine/xanthine oxidase activity (Bai *et al*, 2005), stimulates the resorption process by triggering osteoclastogenic Nuclear Factor-kappa B (NF-κB) ligand (RANKL)-RANK signalling between osteoblasts and osteoclast precursors (Bai *et al*, 2005; Hodge, *et al* 2011). RANKL binding to RANK initiates osteoclast differentiation and activation and is critical for maintaining their survival and for promoting bone resorption (Bai *et al*, 2005). However the definitive consensus on the involvement of OxS in the derangement of bone homeostasis is still lacking, due to the controversial results of the few *in vivo* human studies so far conducted.

2.3 OBJECTIVES OF THE STUDY

The scarcity of *in vivo* data on the relationship between OxS and PO prompted us to conduct the present cross-sectional study on women in pre-, peri- and post-menopausal status. The main purposes of this research were to evaluate:

- If OxS is correlated with BMD in women with high and low physiological levels of estrogens;
- If OxS is associated with PO development;
- If OxS might be an influencing factor for the bone turnover impairment underlying postmenopausal osteoporosis development.

MATERIAL AND METHODS

2.4 DESIGN OF THE STUDY

The subjects (n=261) examined in the present study were recruited among women undergoing bone densitometry evaluation at the Menopause and Osteoporosis Centre (MOC) of University of Ferrara (Ferrara, Italy). This study was carried out in accordance to the Declaration of Helsinki (World Medical Association, http://www.wma.net), the guidelines for Good Clinical Practice (European Medicines Agency, http://www.ema.europa.eu) and it was approved by the Human research ethics committee of the University. Eligible participants were Caucasian, apparently healthy women aged between 21 and 65 years. Initial evaluation was made by a detailed questionnaire that included demographic information, medical and reproductive history, main lifestyle habits and diet. All participants consumed a Mediterranean diet. Exclusion criteria were pregnancy, use of antioxidant supplements (vitamins, selenium etc.), excessive alcohol intake (> 20 g/day), chronic diseases (diabetes, malabsorption, cancer etc.) and medications (antiobesity, thyroid hormone, diuretics, insulin, antihypertensives, oral contraceptives and hormone replacement therapy etc.) that might affect accumulation and distribution of fat in the body and oxidative stress level. Menopausal status of the participants was defined according to the recent ReSTAGE's modification of the Stages of Reproductive Aging Workshop (STRAW) staging criteria (Soules MR *et al*, 2001). Women reporting a regular menstrual cycle were classified as premenopausal; perimenopausal women were those presenting 2-11 months of amenorrhea; subjects reporting longer periods of amenorrhea were classified as postmenopausal. Body weight, standing height, waist and hip circumferences were assessed by trained personnel and used to calculate values of BMI, waist to hip ratio (W/H) and waist to height ratio (W/height) according to standard protocols.

2.5 BONE DENSITOMETRY ASSESSMENT

Areal bone density was assessed at lumbar spine, hip and total body by Discovery dual energy X-ray absorptiometry scanner (Hologic Inc, Bedford, MA). Postmenopausal osteoporosis was diagnosed when BMD T score (the number of standard deviations below the average for a young adult at peak bone density) was lower than 2.5 standard deviations from BMD peak at either femoral neck or lumbar spine, according to WHO guidelines (WHO, 2003). In accordance with these criteria, women with T score at either skeleton area between -2.5 and -1.0 were classified as osteopenic and those with a value higher than -1.0 as normal.

2.6 BIOCHEMICAL ASSAYS

2.6.1 Samples collection

Fresh blood (7 mL) was drawn into vacutainer tubes without anticoagulant by venipuncture after an overnight fast. After 30 minutes of incubation at room temperature, blood samples were centrifuged (3000 g for 10 minutes), and the obtained serum was then divided into aliquots.

Urine (10 mL) was collected after an overnight fasting. Urine samples were centrifuged (3000 x g for 10 minutes) and the supernatant was divided into aliquots. In some of them was added 0.05% of 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT). Serum and urine samples were stored at – 80°C until analysis.

All the following assays were performed on serum samples using Tecan Sunrise-96 well microplate spectrophotometer (Tecan group Ltd., UK).

2.6.2 8-hydroxy-2-deoxy Guanosine (8-OH-dG) EIA assay

The DNA damage was measured in urine by using a commercial available competitive "sandwich" EIA kit (StressMarq Bioscience Inc., Victoria, British Columbia, Canada) according to the manufacturer's guidelines. The urine samples were diluted 1:200. The detection limit of the assay was 33 pg/ml. The intra- and inter-assay coefficient variation, were 7.9% and 6.4%, respectively. Levels of 8-OH-dg were normalized for levels of urine creatinine (ml/dL).

2.6.3 F_2-Isprostane (F_2-iso) EIA assay

8-Isoprostane was measured by a commercial competitive "sandwich" EIA kit (Cayman Chemical's, USA) according to the manufacturer's guidelines. The detection limit of it was approximately 2.7 pg/ml. The intra- and inter-assay coefficient variation were 12.4% and 15.6%, respectively. Levels of 8-isoprostane were normalized for levels of urine creatinine (ml/dL).

2.6.4 Other markers of oxidative stress

The markers of oxidative stress, such as: dROMs (hydroperoxides-HY), total antioxidant power-TAP (FRAP), advanced oxidation protein products (AOPP), uric acid (UA) and thiols (TH) were assayed in serum as described previously (Material and Methods Chapter 1-1.8 Biochemical Assays).

2.6.5 Cerulosplasmin ELISA assay

Levels of ceruloplasmin in serum (expressed as μg/mL) were measured by using a commercially available non competitive "sandwich" ELISA (AssayPro, St Charles, USA) according to the

manufacturer's guideline. All serums samples were diluted 1:400. The limit of sensitivity of the assay was about 0.6µg/ml. The intra- and inter-assay coefficient variation were 4.6% and 7.1%, respectively.

2.6.6 Estradiol (E_2) and Follicle-stimulating hormone (FSH)

Serum concentrations of E_2 and FSH were determined by conventional chemiluminescent microparticle immunoassay using the commercial kits Architect Estradiol and Architect FSH from Abbot Laboratories (Abbott Park, IL, USA), respectively. The assays were carried according to manufacturer's instructions. Minimal detectable amounts were 10 pmol/L for E_2 and 0.05 IU/L for FSH. Detection limit values were taken into account for statistical analysis when immeasurable hormone levels were obtained. E_2 intra-assay and inter-assay coefficient of variation for was 6.0 and 6.7%, respectively, and FSH coefficient of variation were 3.1 and 3.4%, respectively.

2.6.7 Bone turnover markers: Bone Alkaline Phosphatase (BAP) and C-terminal telopeptide (CTX-1) ELISA assay

The measurements of BAP and CTX-1 concentrations were performed using commercially available non competitive "sandwich" ELISA kits: OCTEIA Ostase® BAP immunoenzymometric assay and ß Cross-Laps Siero (CTX-I), respectively, (both kits were from Immunodiagnostic Systems Ltd., Boldon, Tyne and Wear, UK) according to the manufacturer's guidelines. The serum samples were not diluted. The limits of sensitivity of the assays were: 0.7µg BAP/L for BAP and 0.020 ng/mL for CTX-1. The intra- and inter-assay coefficient variation were:

- CTX-1: 2.2% and 7.7%, respectively;
- BAP: 4.1% and 5.5% respectively.

2.7 STATISTICAL ANALYSIS

Data were analyzed using SPSS 18.0 for Windows (IBM, Chicago, IL, USA). Continuous variables were first analyzed for the normal distribution by the Kolmogorov-Smirnov and the Shapiro-Wilkinson test. Since the distribution of OxS parameters (HY, F_2-iso, 8-OH-dg, AOPP, TH, TAP and UA), lumbar and spine BMD were skewed, the values were log10-transformed. To minimize the negative effect on statistical power of the analysis due to unequal size of groups considered, one-way analysis of variance (ANOVA) for unequal variance was performed. Analysis of covariance (ANCOVA) was performed to evaluate whether the differences in OxS markers evidenced by previous analysis retained significance after adjustment of age, body mass index (BMI) and waist to hip ratio (W/H). ANCOVA was implemented with Tuckey's *post-hoc test*, which was used to compare two groups at once. The correlations of OxS with age, BMI, W/H,

estrogen (E_2) and follicle-stimulated hormone (FSH) and bone health status markers (BMD and CTX-1) were checked by Pearson's and Spearman's analysis if they were normal and non-normal distributed, respectively.

Preliminary multiple regression analyses were performed to evaluate the possibility of collinearity problem among variables to include as covariates in multivariate analysis. Values of variance inflation factor (VIF) above 2.5 were regarded as indicative of multicollinearity. After this analysis, BMI was not included in the covariates set, because of its collinearity with waist circumference and of its weaker correlation with the variables of interest. Finally, univariate (by Pearson's correlation test) and multivariate (by partial correlation or multiple regression) analyses were performed to check the associations between continuous variables.

A two-tailed probability value 0.05 was considered statistically significant.

RESULTS

Table 1 shows the general characteristics of the sample subjects according to menopausal status. The final sample was composed of 290 women, subdivided in: 81 healthy reproductive age (HRA) women; 39 perimenopausal women (PERI) and 170 postmenopausal women (POST). As disclosed by ANOVA analysis, all anthropometric parameters considered (age, BMI and W/H) were different among groups ($p<0.001$) with a trend towards increase passing from HRA to PERI and POST. With regard to OxS markers, HY, TAP and TH showed significant differences among the three groups considered in the analysis ($p<0.001$ for both). However, upon adjustment for age, BMI and W/H, TAP was the only marker retaining significance ($p<0.01$) with mean values still significantly ($p<0.05$) higher in PERI and POST with respect to HRA as showed by *post-hoc* test. On the contrary, age, but not menopausal status, was the main determinant for the differences in HY and TH in the sample detected by ANOVA.

Table 1. Principal characteristics of whole sample: healthy reproductive age, peri- and post-menopausal women

	HRA (n= 81)	**PERI (n= 39)**	**POST (n= 170)**
Age (years)	34.9 ± 10.7	50.6 ± 3.0[a]	56.8 ± 4.76[b,c]
BMI (kg/cm^2)	22.6 ± 3.4	26.1 ± 4.3[a]	24.8 ± 3.5[b]
W/H	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
E_2 (pg/mL)	12.3 (10-15)	6.1 (3-9)[a]	1.6 (1-2)[b,c]
FSH (mIU/mL)	8.0 (6-11)	57.0 (41-72)[a]	77.0 (70-86)[b,c]
HY (UC)	248.2 ± 9.4	253.3 ± 9.1[a]	255.1 ± 9.7[b]
F_2-iso (pg/mg creatinine)	2.7 ± 0.02	2.9 ± 0.3	2.9 ± 0.3
8-OH-dg (ng/mg creatinine)	1.8 ± 0.1	2.1 ± 0.2	2.1 ± 0.3
AOPP (μmol/L)	188.2 ± 16.6	188.9 ± 22.5	192.5 ±16.29
TAP (FRAP units)	649.4 ± 147.7	714.4 ± 140.2[a]	706.0 ± 148.5[b]
UA (μmol/L)	231.7 ± 16.7	234.8 ± 16.4	236.9 ± 13.7
RAP (FRAP units)	313.8 ± 133.7	341.6 ± 132.7	319.7 ± 122.4
TH (μmol/L)	219.8 ± 17.3	224.9 ± 19.9[a]	233.1 ± 16.9[b#]
Ceruloplasmin (μg/mL)	56.9 ± 34.2	50.3 ± 8.8	45.4 ± 33.2

Data presented are mean ± standard deviations (SD) for continuous variables. Values for E_2 and FSH were expressed as median (25th -75th percentile) because non-normally distributed. Abbreviations: BMI, body mass index; W/H, weight to hip ratio; E_2, estradiol; FSH, follicle stimulating hormones; HY, hydroperoxides; F_2-iso, F_2-isoprostane; 8-OH-dg, 8-hydroxy-2-deoxy guanosine; AOPP, advanced oxidation protein products; TAP, total antioxidant power; UA, uric acid; RAP residual antioxidant power; TH, thiols; CU, Cartelli Units; FRAP, Ferric reduction antioxidant capacity.

[a] $p<0.05$ (by Bonferroni's post-hoc test) between HRA vs PERI; [b] HRA vs POST; [c] PERI vs POST. #$p<0.05$ (p value for comparison among HRA, PERI and POST by ANCOVA with BMI, W/H and age as covariate).

The lack of association between OxS markers and endocrine change, decrease in E_2, related to menopausal transition, the correlation between these markers and E_2 levels was checked. Pearson's correlation analysis did not show any significant association between E_2 and OxS markers (Table 2). From the analysis of correlations among OxS markers and other continuous variables considered in our study (e.g. FSH, months of amenhorrea) did not arise any meaningful results.

Table 2. Simple Pearson's correlation coefficients (r) between E_2 and OxS markers in total sample

	HY (r)	**F_2-iso (r)**	**8-OH-dg (r)**	**AOPP (r)**	**TAP (r)**	**UA (r)**	**RAP (r)**	**TH (r)**
E_2	-0.15	-0.17	-0.02	-0.07	-0.16	-0.06	-0.19	0.06

Abbreviations: HY, hydroperoxides; F2-iso, F_2-isoprostane; 8-OH-dg, 8-OH-dg, 8-hydroxy-2-deoxy guanosine; AOPP, advanced oxidation protein products; TAP, total antioxidant power; RAP, residual antioxidant power; UA, uric acid; TH, thiols.

Then, we focused our attention on the possible relationship between OxS and PO. To accomplish this aim, the mean levels of OxS markers were compared between three different subgroups of the sample: HRA, health (HPOST) and osteoporotic postmenopausal (OPOST) women (Table 3).

Table 3. Characteristics of sample groups including healthy women in reproductive age (HRA), healthy women in postmenopause (HPOST) and osteoporotic women in postmenopause (OPOST).

	HRA **(n= 81)**	**HPOST** **(n= 63)**	**OPOST** **(n= 30)**
Age (years)	34.9 ± 10.7	53.9 ± 5.0[a]	57.7 ± 4.9[b]
BMI (kg/m2)	22.6 ± 3.4	25.4 ± 3.5[a]	24.4 ± 3. [b]
Lumbar spine BMD (g/cm2)	1.01 ± 0.11	0.94 ± 0.13[a,c]	0.75 ± 0.09[b]
Lumbar spine T-score	-0.56 ± 1.12	-0.86 ± 0.70[a,c]	-2.72 ± 0.12[b]
Femoral neck BMD (g/cm2)	0.79 ± 0.11	0.75 ± 0.07[a,c]	0.61 ± 0.08[b]
Femoral neck T-score	-0.49 ± 0.98	-1.00 ± 0.09[a,c]	-2.17 ± 0.13[b]
E_2 (pg/mL)	12.3 (10-15)	14.0 (11-19)[a]	12.0 (11-17)[b]
FSH (mIU/ml)	8.0 (6-11)	50 (68-87)[a]	67 (52-76)[b]
HY (CU)	248.2 ± 9.4	343.3 ± 83.2	347.8 ± 81.2[b]
TAP (FRAP units)	649.4 ± 147.7	702.4 ± 151.1 [a]	708.6±127.2[b]

Data presented are: n - (% in the group) for categorical and mean ± standard deviations for continuous variables. Values for E_2 and FSH were expressed as median (25^{th} -75^{th} percentile) because non-normally distributed [a] Significant ($p<0.05$) difference (by Bonferroni's *post-hoc* test) between HRA and HPOST; [b] HRA and OPOST; [c] HPOST and OPOST.
Abbreviations: BMI, body mass index; E_2, estradiol; FSH, follicle stimulating hormones; HY, hydroperoxides; TAP, total antioxidant power; CU, Cartelli Units, FRAP, Ferric reduction antioxidant capacity.

As expected, BMD at both skeleton sites considered was significantly decreased in OPOST with respect to that detected in the other two groups ($p<0.0001$ for all) with densitometric values significantly lower in HPOST compared to HRA ($p<0.01$) (Table 3). In accordance with Table 1 that showed the reproductive and menopausal stage of women, E_2 and FSH was significantly lower and higher respectively, in the HPOST and OPOST respect to HRA ($p<0.0001$ for both). In regard to the serum markers of oxidative balance, HY and TAP showed significant difference between HPOST and HRA ($p<0.05$ and $p<0.01$, respectively). Interestingly none of these outcomes concerning OxS markers retained significance after adjustment for well-known potential confounding factors as age, BMI and smoking. Of note, the results obtained with the other markers of OxS (reported in Table 1) are not shown in Table 3 (and in the following tables and figures) because their level did not significantly change across the groups.

In addition, we checked for possible links between OxS markers and BMD parameters through Pearson's correlation analysis. In order to unveil if reproductive or postmenopausal phase of women's life were independently determinant in this possible OxS-linked bone loss, we checked the above association separately in HRA and a second group including HPOST and OPOST women. These analyses showed that spinal BMD was still negatively correlated with HY in postmenopausal subsample (r=-0.251, p=0.012), while no statistically significant link between these two variables there was in HRA (r=-0.022, p=0.833) (Figure 1a) and 1b). Noteworthy, the correlation in the former group retained significance after adjusting for age, BMI, smoking habit and diagnosis of osteoporosis (r=-0.330, p=0.001).

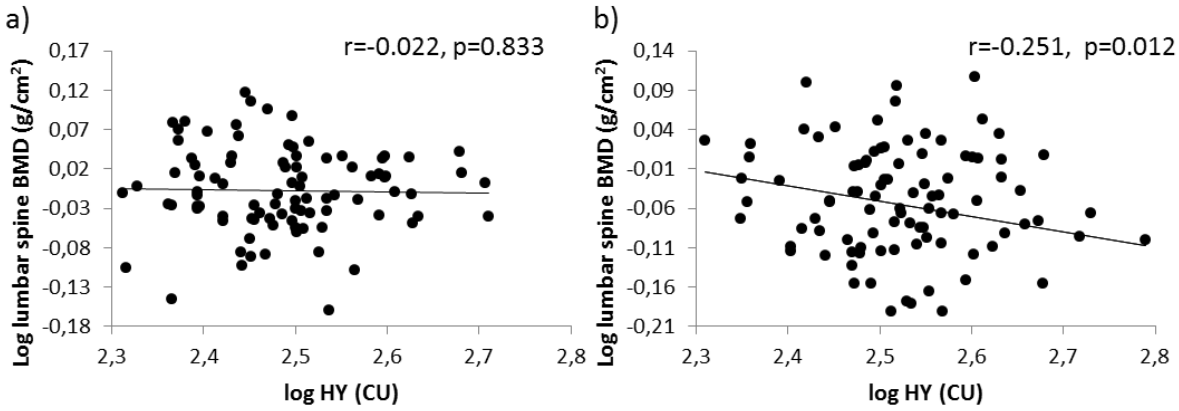


Figure 1. Scatter plots of the relationship between lumbar spine BMD and HY in the sample groups including: a) women in reproductive age (HRA, n=81) and b) in postmenopause (HPOST+OPOST, n=93).

Subsequently, the level of OxS and bone markers were evaluated in relation to PO or osteopenia diagnosis, in a larger postmenopausal group. Table 4 shows the main characteristics as well as the mean values for BMD and bone markers of this population. Osteopenic and osteoporotic women presented lower mean values of years since menopause, BMI, waist circumference compared to the healthy ($p<0.05$ for all). On the contrary, frequency of smokers and serum levels of E_2 and FSH did not significantly vary across the groups. By definition, total, neck and lumbar spine BMD, and the correspondent T-score values, were significantly ($p<0.01$) higher in healthy with respect to osteopenic and osteoporotic women. Finally, the levels of CTX-1 and BAP were not different among the groups.

Table 4. Principal characteristics of healthy, osteopenic and osteoporotic postmenopausal women

	Healthy (n= 38)	**Osteopenia (n= 73)**	**Osteoporosis (n= 56)**
Age (years)	53.7 ± 4.6	55.6 ± 4.5	58.4 ± 4.3[a,b]
Years since menopause (years)	7.4 ± 0.8	7.0 ± 0.8[a]	6.9 ± 0.7[a]
BMI (kg/m^2)	26.4 ± 4.1	24.4 ± 2.9[a]	24.2 ± 3.2[a]
E_2 (pg/mL)	21.1 ± 7.1	12.9 ± 1.8	13.8 ± 2.6
FSH (mIU/mL)	72.1 ± 7.2	85.2± 4.2	79.2 ± 5.2
DXA parameters			
Lumbar spine BMD (g/cm^2)	1.04 ± 0.09	0.88 ± 0.09[a]	0.75 ± 0.08[a,b]
Lumbar spine T-score	-0.09 ± 0.77	-1.46 ± 0.73[a]	-2.77 ± 0.70[a,b]
Femoral neck BMD (g/cm^2)	0.81 ± 0.06	0.69 ± 0.06[a]	0.62 ± 0.08[a,b]
Femoral neck T-score	-0.28 ± 0.59	-1.39 ± 0.6 [a]	-2.06 ± 0.74[a,b]
Total hip BMD (g/cm^2)	0.90 ± 0.06	0.81 ± 0.07[a]	0.62 ± 0.07[a,b]
Total hip T-score	-0.21 ± 0.51	-1.04 ± 0.66[a]	-1.72 ± 0.62[a,b]
Total body BMD (g/cm^2)	1.09 ± 0.16	1.05 ± 0.07[a]	0.95 ± 0.07[a,b]
Total T-score	0.35 ± 0.99	-0.67 ± 0.68[a]	-1.60 ± 0.74[a,b]
Bone markers			
CTX-1(ng/mL)	0.60 ± 0.21	0.66 ± 0.39	0.67 ± 0.40
BAP (μg/L)	27.7 ± 2.7	25.7 ± 1.2	25.1 ± 1.7

Data presented are expressed as: % within the group for categorical and mean ± standard deviations for continuous variables. [a] $p<0.05$ vs. healthy; [b] $p<0.05$ vs. osteopenia.
Abbreviations: BMI, body mass index; E_2, estradiol; FSH, follicle stimulating hormones; BMD, bone mass density; CTX-1, C-terminal telopeptide of type I collagen; BAP, bone-specific alkaline phosphatase

As shown in Table 5, there were no significant differences in serum levels of OxS markers among the subgroups. However, osteopenia and osteoporosis appeared to be associated with a worse oxidative balance. Indeed, compared to healthy women, those affected by these two conditions, presented higher levels of the lipid oxidative damage marker, hydroperoxides, and lower levels of total antioxidant power and ceruloplasmin.

Table 5. OxS markers mean levels in healthy, osteopenic and osteoporotic postmenopausal women

	Healthy (n= 38)	**Osteopenia (n= 73)**	**Osteoporosis (n= 56)**
HY (CU)	349.3 ± 12.3	352.7 ± 11.7	370.6 ± 10.8
AOPP (μmoles/L)	82.1 ± 8.8	76.6 ± 2.9	87.4 ± 7.6
TH (μmoles/L)	225.9 ± 13.9	215.2 ± 12.7	225.0 ± 17.3
TAP (FRAP units)	734.1 ± 28.2	675.7 ± 15.7	697.2 ± 21.6
Ceruloplasmin (mg/dL)	52.6 ± 6.7	49.2 ± 4.0	45.9 ± 5.1

Data presented are expressed as: mean ± standard errors for continuous variables.
Abbreviations: HY, hydroperoxides; CU, carratelli units; AOPP, advanced oxidation protein products; TH, thiols; TAP, total antioxidant power; FRAP, Ferric reduction antioxidant capacity.

From the correlation analyses on this sample emerged that, besides the correlation between HY and lumbar spine BMD already observed in the smaller sample (Figure 1), the marker of lipid peroxidation was correlated negatively with lumbar (r= -0.225, p=0.009) and total (r=-0.180, p=0.040) BMD and positively with CTX-1 (r=0.197, p=0.018) (Figure 2).

Actually, as shown in Table 5, the strengths of the multivariate correlations between the OxS marker and the biochemical and densitometric bone parameters appeared to be stronger that the respective univariate. This effect was more evident for the correlation between HY and total hip BMD that resulted to be significant ($p<0.05$) only in the multivariate analysis.

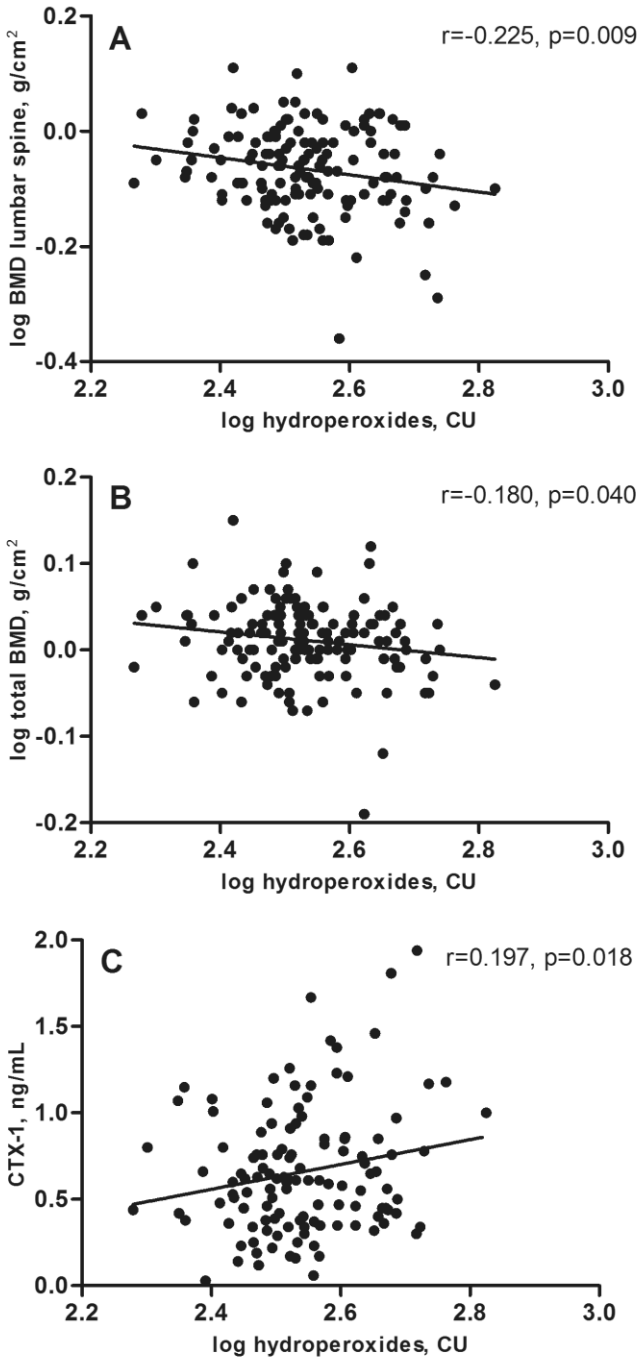


Figure 2. Scatter plots of the relationship between HY and lumbar spine BMD (A) total body BMD (B) and CTX-1 (C), in the subgroup (healthy, osteopenic, and osteoporotic women) (n=167). Abbreviations: BMD, bone mass density; CTX-1, C-terminal telopeptide of type I collagen.

Table 6. Simple and partial correlation coefficients for the association of HY with total, lumbar spine and total hip BMD, and CTX-1 in the total sample (n=167)

	Simple correlation (r)	Partial correlation (r)
Lumbar spine BMD (g/cm^2)	-0.225[b]	-0.282[b]
Total hip BMD (g/cm^2)	-0.120	-0.208[a]
Total body BMD (g/cm^2)	-0.180[a]	-0.192[a]
CTX-1 (ng/mL)	0.197[a]	0.233[a]

[a] $p<0.05$, [b] $p<0.01$. Adjusting variables for partial correlation: age, years since menopause, smoking and waist circumference. Abbreviations: BMD, bone mass density; CTX-1, C-terminal telopeptide of type I collage.

Since higher levels of CTX-1 and HY, were found to be predictors of lower lumbar spine BMD, multiple regression analyses were run to unveil whether these associations were independent to each other (Table 6). To this aim, three separate multiple regression models were performed (Table 7), where each one included age, years since menopause, smoking and waist circumference plus: HY (Model 1), CTX-1 (Model 2); HY and CTX-1 (Model 3). As displayed in Table 5, the correlation between hydroperoxides and lumbar spine BMD was significant ($p<0.01$) regardless of the presence of CTX-1 among the covariates. On the contrary, the association between CTX-1 and lumbar spine BMD did not persist when the OxS marker was included in the multivariate model.

Table 7. Multiple regression analysis of the association of HY and CTX-1 with lumbar spine BMD

Model 1			**Model 2**			**Model 3**		
Expl variables	B* (SE)	β#	Expl variables	B* (SE)	β#	Expl. variables	B* (SE)	β#
HY	0.262[b] (0.061)	-0.321	CTX-1	-0.044 [a] (0.023)	-0.172	HY	-0.246 [b] (0.066)	-0.301
						CTX-1	-0.026 (0.023)	-0.100
	adjusted R^2 = 0.25			adjusted R^2 = 0.16			adjusted R^2 =0.25	

Model 1: age, years since menopause, smoking, waist circumference + hydroperoxides
Model 2: age, years since menopause, smoking, waist circumference + CTX-1
Model 3: age, years since menopause, smoking, waist circumference + hydroperoxides and CTX-1
[a] $p<0.05$; [b] $p<0.01$
* unstandardized regression coefficient; # standardized regression coefficient
Abbreviations: HY, hydroperoxides; SE, standard error; CTX-1, C-terminal telopeptide of type I collagen.

DISCUSSION

Following previous reports (Pansini *et al*, 2008; Cervellati *et al*, 2009), we investigated the potential link between postmenopausal osteoporosis (PO) and OxS, which has an important role in the development of several menopause-related diseases in elderly women (Miquel et al, 2006).

The menopausal transition leading to the cessation of E_2 activity, might contributes to postmenopausal osteoporosis onset by unlocking the potential adverse effects of reactive oxygen species (ROS) against bone (Almeida *et al*, 2007). It is widely accepted, indeed, that these sexual hormones, through an antioxidant action can protect women against bone loss during the reproductive age (Weitzmann and Pacifici, 2006). The data in support of the interplay between OxS and PO onset have been mostly obtained from *in vitro* and animal experiments, which, overall, suggest a potential role of reactive species in uncoupling bone turnover. However, so far, the definitive consensus on the involvement of OxS in the derangement of bone homeostasis is still lacking, because of the controversial results of the few in vivo human studies conducted on small size samples. Out cross-selectional study compares for the first time the relationship between BMD and OxS markers in a large cohort on pre- and postmenopausal women.

The first finding of our study was that the physiological decline of E_2 level occurring during menopausal transition does not influence OxS. Our data indicate that change in E_2 level is not associated with modification in oxidative balance, refusing the commonly held hypothesis that endogenous E_2 acts as antioxidant at systemic level. Thus, the worsening of systemic OxS in post-menopausal women observed in this and other studies might be linked with aging, rather than menopause itself. Subsequently, we found that, although systemic OxS is not increased in women with PO with respect to the healthy counterparts (still in postmenopause), it does result negatively and independently associated with BMD in areas of the skeleton which are highly susceptible to PO-related fractures. Noteworthy, these correlations were not found to be significant among premenopausal women.

The contextualization of our data showing the marked difference in correlation coefficient for spinal BMD against HY (Figure 1) measured and in HRA postmenopausal subsample, is challenging due to the lack of studies involving the former population. The few studies present in literature involved just a small size of sample and not comparable with our. However, our findings of a correlation between markers of oxidative balance (HY) and BMD could be consistent with results in other reports, albeit focusing on population samples not completely superimposable with ours. In particular, two lipid peroxidation markers, urinary F_2-isoprostanes and serum malondialdehyde (MDA), and a serum marker of DNA oxidative injury, 8-hydroxy-2'deoxyguanosine, were found to negatively correlate with BMD at spine and femoral neck (Basu et al, 2001; Mangiafico et al, 2007; Ostman *et al*, 2009). Some studies have shown that ROS are

able to directly and indirectly (e.g. by activation of the osteoclastogenic transcription factor NF-κB) stimulate bone resorption. Among the findings of our study, the significant positive association between serum levels of HY and CTX-1, *i.e*. marker of bone resorption, was the one that mostly adds to the current literature. Indeed, to the best of our knowledge, only another population-based study found this association among postmenopausal women, by using, however, different markers for OxS, *i.e*. 8-hydroxy-2'-deoxyguanosine and bone resorption, *i.e.* cross-linked carboxyterminal telopeptide of type I collagen (ICTP). These methodological differences between the two works are not entirely negligible, mostly in relation to the latter marker. Indeed, as nicely described by Garnero et al. (Garnero *et al*, 2003) and others (Delmas *et al*, 2000; Sanchez-Rodriguez *et al*, 2007), ICTP and CTX-1 reflect different collagenolytic pathways, which, in turn, take place in distinct bone pathologies. When compared to the other marker, CTX-1 serum level showed to be a more reliable indicator of the osteoclastic resorptive activity. Consistently, the assessment of CTX-1, but not of ICTP, is recommended for monitoring the effectiveness of anti-resorptive therapy in patients affected by postmenopausal osteoporosis (Garnero *et al*, 2003). Further insight into the understanding of the "weight" of OxS in bone loss of postmenopausal women was provided by multivariate analysis. As shown in Table 6, the relationship between decrease in lumbar spine BMD and increase in CTX-1 serum levels was obvious after taking into account a set of strong potential predictors of bone loss (*i.e.* smoking, age, years since menopause and waist circumference), but it disappeared after including also HY in this set. This statistical outcome led us to speculate that the degradation of the main collagen component of bone organic matrix might be, at some extent, dependent on OxS.

In conclusion, our observations, although preliminary, may represent an important basis to evaluate the potential beneficial effects of nutritional antioxidants on bone health.

CHAPTER 3: ROLE OF (-)-EPICATHECHIN AND ITS METABOLITES IN THE MODULATION OF HEPATIC NADPH OXIDASE IN HEPG2 CELLS TREATED WITH PALMITATE

INTRODUCTION

3.1 METABOLIC SYNDROME (MetS)

Metabolic syndrome (MetS) affects one third of the population in throughout the world. In recent years, MetS has been become a very important public health problem in the world's adult population because it leads to the obesity, diabetes (T2DM) and cardiovascular disease (CVD).

3.1.1 History and definition of MetS

It was over 80 years ago that one of the first description of MetS appeared. In 1923 Kylin described a syndrome involving hypertension, hyperglycemia and hypeuricaemia (Kylin, 1923); twenty years later Vague (Vague *et al*. 1979) wrote, in a landmark paper, that a particular obesity phenotype, upper body, android or male-type obesity, was associated to the metabolic abnormities often seen with diabetes and CVD. Following this, an abstract was presented at the European Association for the Study of Diabetes, which described a syndrome that involved hypertension, hyperglycemia and obesity. The clinical importance of the syndrome was highlighted 40 years later by Reaven, who described "a cluster of risk factor for diabetes and CVD" with insulin resistance as a central phatophysiological feature, and named it "Syndrome X"; Reaven didn't include obesity as a factor linked to the syndrome (Raven, 1988). MetS was renamed many times, "The deadly Quarter" and "The insulin Resistance Syndrome", but now the term "metabolic syndrome" is used globally and is the most usual description of this cluster of metabolic abnormalities. A number of expert groups have attempted to develop a unifying definition for the MetS. The first major organization that proposed a set of clinical criteria for MetS was the World Health Organization (WHO), which in 1999 published a document entitled "Definition, diagnosis and classification of diabetes mellitus and its complications" (Alberti and Zimmet, 1998; Balkau and Charles, 1999). The definition is based on the assumption that insulin resistance is the major underlying contributors to MetS, and features impaired glucose regulation. In addition this requires the presence of two risk factors from: hypertension, obesity, raised triglycerides or low glucose. Critics of the WHO definition identified several limitations, of which the most important related to the use of euglycemic clamp to measure insulin resistance, for this reason the European Group for the Study of Insulin Resistance (EGIR) developed a modified version to be used in non-diabetic subjects only (Balkau and Charles, 1999); it proposed the use of fasting insulin levels to estimate insulin resistance and impaired fasting

glucose (IFG) as a substitute for impaired glucose tolerance, also EGIR added in the definition the waist circumference (94 cm for men and 82 cm for women) as the measure of adiposity. After that, in 2001, the National Cholesterol Educational Program (NCEP) of the USA introduced the ATPIII (Adult Treatment Panel III) definition, which was designed to facilitate diagnosis in clinical practice and therefore does not include a measurement of insulin resistance (Zimmet *et al,* 2005). The ATPIII guidelines establishes that MetS can be diagnosed when a patient has three or more of five clinically following identifiable risk factors: abdominal obesity defined by the measurement of waist circumference (cut-points higher than in EGIR definition >102 cm for men and >88 cm for women); high levels of triglycerides (> 150 mg/dL); low cholesterol HDL level (<40 mg/dL for men and <50 mg/dL for women); high blood pressure (systolic ≥130 mmHg or diastolic ≥85 mmHg); and high fasting plasma glucose concentration (≥110 mg/dL) (Alberti *et al*, 2009) (Figure 3.1).

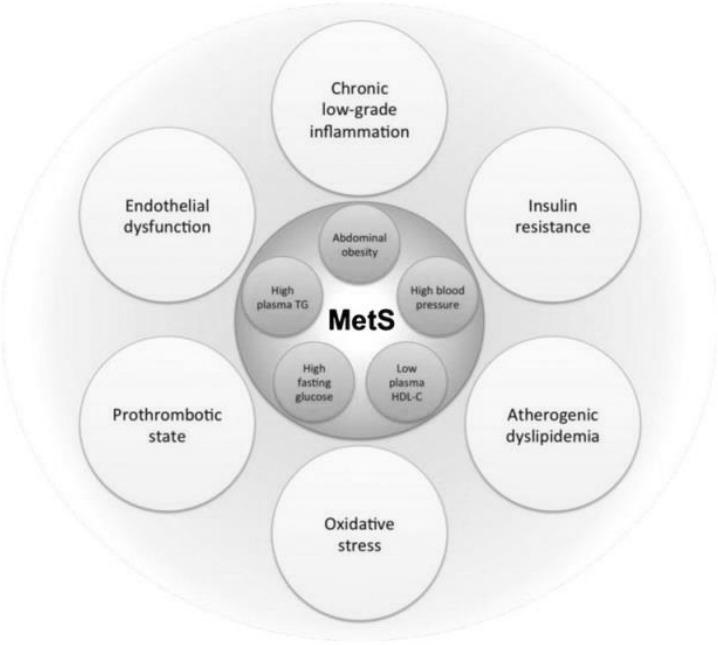


Figure 3.1. Overview of MetS parameters. Regardless of the metabolic syndrome (MetS) definition used, individuals classified with MetS possess a combination of key parameters. In addition, MetS is associated with chronic low-grade inflammation, insulin resistance, atherogenic dyslipidemias and dysfunctional lipoproteins, elevated oxidative stress, a prothrombotic state, and endothelial dysfunction (outer circles). All of these factors contribute to the increased risk of cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM). (Taken from Adersen and Fernandez, 2013)

Prevalence data for the metabolic syndrome in different countries and in different ethnic groups clearly show that the syndrome is a large problem everywhere in the world and that the number of people affected continues to grow. It is estimated 366 million people around the world had diabetes in 2011, and this number is predicted to rise to 522 million by 2030 (Whiting *et al*, 2011). For the

high incidence of this disease in the world, to understand and control the causes of MetS will be a very important step to improve the quality of daily life both people already affected or not.

3.1.2 Etiology of Metabolic Syndrome

MetS represents a multi-component disorder that is characterized by impaired insulin sensitivity, dyslipidemia, abdominal obesity and hypertension. It is associated with high risk of subsequent development of T2DM (Schivo *et al*, 2013), CVD and premature death (Bruce and Byrne, 2009). Various mechanisms have been proposed for the etiology of the MetS. Genetic predisposition, physical activity, smoking, an unhealthy pattern of daily life, aging, proinflammatory states and hormonal changes may have a causal effect in MetS; but the factors that are most commonly blamed in the development of the syndrome are insulin resistance and obesity, which are related to each other (Figure 3.2).

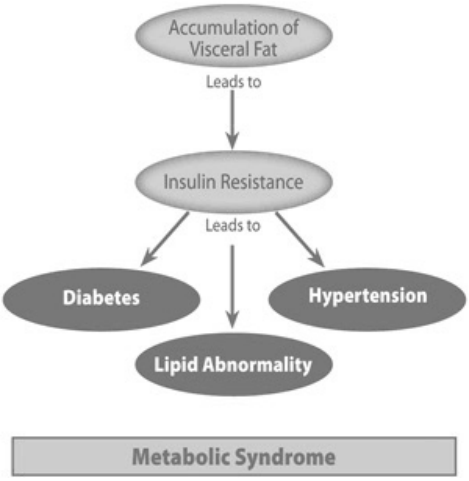


Figure 3.2. Principal factors involved in the etiology of MetS. Obesity and insulin resistance are related to each other in the development of MetS. (Taken from http://www.healthyfellow.com/anti-aging-nutrition)

3.1.2.1 Relationship between obesity and insulin resistance

Obesity is a result of excess calorie intake and genetic predisposition. There are at least two ways whereby adipose tissue may influence glucose homeostasis, then in the genesis of insulin resistance, and act as key etiological factor in the development of MetS. First, excessive adipose tissue energy storage results in increase fatty acid flux to other tissues and increases triglyceride storage in peripheral tissues, which promote insulin resistance. Second, adipose tissue is an important endocrine organ that secretes several inflammatory factors such as adipocytokines and adipokines, which have a direct effect on insulin sensitivity. Indeed obesity, insulin resistance and T2DM are associated with "chronic" inflammation characterized by abnormal cytokine production.

Mounting evidence highlight the contribution of adipose tissues to a systemic inflammatory state, that plays an important role in metabolic regulation. The most important proinflammatory adipocytokines secretes from adipose tissue are tumor necrosis factor (TNFα) and interleukin (IL)-6. TNFα is a major player mediating the activation of signaling cascade in adipocytes; it is involved in the development of insulin resistance and glucose intolerance because it is able to promote and inhibit the phosphorylation of serine and tyrosine, respectively, of insulin receptor (IR) in adipocytes.

Evidence in obese and insulin resistance animal models knock out TNFα and knock out TNFα receptors genes have demonstrated that these animals improve their response to insulin. Regarding (IL)-6, it is a multifaceted cytokine which is secreted by the adipose tissue, skeletal muscle and liver. Because one-third of circulating (IL)-6 in healthy individuals is estimated to originate from adipose tissue it is considered an adipokine. (IL)-6 plays an important role in inflammation. High plasma (IL)-6 levels are correlated with increased body mass, waist circumference and free fatty acids (FFAs) levels. Indeed it can directly affect lipid metabolism and activate pathways to promote increased energy turnover, it stimulates lipolysis in humans, with a consequent increase of plasma FFAs concentration and whole body fat oxidation. A number of *in vitro* and *in vivo* studies demonstrated that (IL)-6 is capable of inducing insulin resistance. In adipocyte cells, (IL)-6 production is strongly increase by TNFα and induces insulin resistance by inhibiting glucose uptake and impairing insulin signaling and action.

Even if obesity and insulin resistance are related to each other and overweight is considered the main component in the development of the MetS, evidence has shown that not all obese individuals suffer from an impaired metabolic profile (Sueleyman *et al*, 2014). However, considering the close correlation between insulin resistance and obesity, disassociation of these two major underlying factors in the pathogenesis of MetS has been hard. Generally, insulin resistance is related to an increase of body mass index, in fact most obese people show a broad range of insulin sensitivities (Reza and Khosrow, 2009).

Thus, correcting imbalance of hormones, nutrients, and inflammation may provide opportunities and challenges for the prevention and treatment of MetS, and consequent prevention of T2DM. In the relationship between obesity and insulin resistance, FFAs levels seem to have an important role, because an excessive release of FFAs, cytokines and other proinflammatory products from adipose tissue induces insulin resistance in muscle and liver. Solid evidence has suggested that FFAs, derived from nutritional intake or conversion from carbohydrates, not only act as an important energy source, but also may have important roles in the modulation of intracellular protein kinases involved in insulin resistance.

3.2 INSULIN RESISTANCE

3.2.1 Role of insulin

Insulin is the primary hormone involved in glucose homeostasis and the stimulation of glucose transport; it is produced by β-cells of the pancreas, and is central to regulating carbohydrate and fat metabolism in the body. Its roles are many; first is to regulate blood glucose concentration by keeping the balance between glucose absorption from the intestine, production by liver and uptake and metabolism by peripheral tissues. Insulin increases glucose uptake in muscle and fat, and inhibits hepatic glucose production by inhibition of glycogenolysis and gluconeogenesis, that are the key mechanisms by which insulin controls glucose homeostasis. Insulin also regulates lipid metabolism by increasing fatty acid synthesis, increasing sterification of FFAs, and decreasing lipolysis; it also exerts anabolic effects on protein metabolism by increasing DNA replication and protein synthesis via control of amino acid uptake and decreased proteolysis. Insulin is involved in other important processes, such as: cell growth, cell proliferation, survival and differentiation. When these processes are not under tight regulation a phatophysiological condition called insulin resistance is present.

3.2.2 Definition of insulin resistance

Insulin resistance is defined as a phatophysiological condition in which normal insulin concentration does not adequately produce a normal insulin response in target tissues as adipose, muscle and liver. Under this condition, pancreatic β-cells secrete more insulin to develop hyperinsulinemia to overcome the hyperglycemia. Over-time the inability of the pancreas to produce sufficient insulin to correct worsening tissue insulin resistance leads to hyperglycemia and over T2DM (Gual *et al*, 2005; Reza and Khosrow, 2009). Solid evidence in human and animal studies showed defects in the insulin signaling pathway in these pathologies. The earliest anomaly observed is a decrease in insulin-induced glucose uptake in skeletal muscle and adipose tissue, and a reduced ability of the hormone to suppress glucose production by the liver (Gual *et al*, 2005). Moreover insulin resistance is induced by several molecules, including glucose, insulin, FFAs, and certain cytokines, like TNFα. Hepatic insulin resistance has been suggested as the main underlying cause of MetS.

3.2.3 Insulin-signaling pathway

Despite the well established role of insulin in fat, carbohydrates and protein metabolism, the molecular mechanism of its action remain hard to define. Once insulin binds to its receptor (IR) and activates its tyrosine kinase activity the insulin pathway diverges. One pathway proceeds through the IRS and depends on activation of the enzyme phosphatidylinositol-3'-kinase (PI3-K)

which mediates the metabolic effects of insulin (glucose, lipid and protein); another pathway proceeds trough Grb2/Sos and Ras, leading to activation of the mitogenic-activated protein kinase (MAPK) family: ERK1/2, JNK and p38 MAPK (Figure 3.3), which control the mitogenesis, growth and cell differentiation.

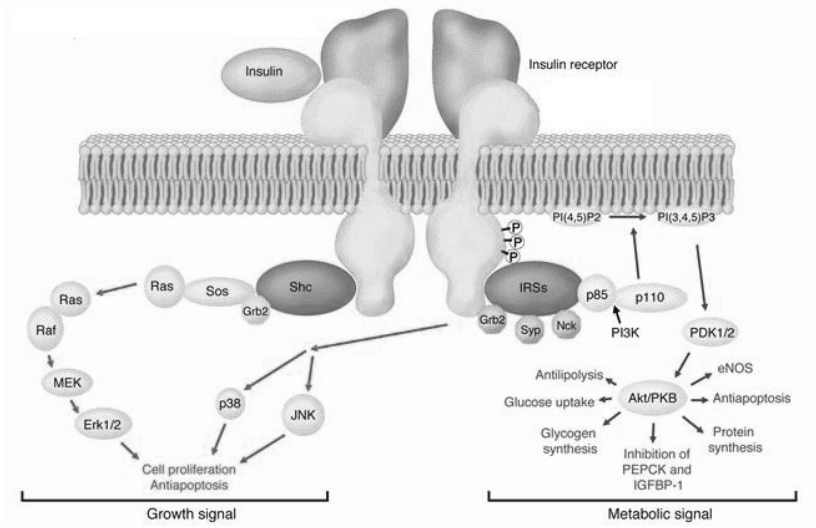


Figure 3.3. Insulin signaling pathway. (Taken from van de Berge G, 2004)

Insulin exerts most of its metabolic actions through the PI3-K pathway; in contrast, not much is known about the MAPK pathway in insulin-stimulated glucose transport or glycogen synthesis, but is well known its role in the mitogenic responses (Cusi *et al*, 2000; Kelley *et al*, 1992; Ogawa *et al*, 1998). Insulin signaling has many important roles in biological system: it has growth and mitogenic effects, by activation of the Ras/MAPK pathway; ii) inhibits the production and release of glucose by the liver by blocking gluconeogenesis and glycogenolysis; and iii) promotes fatty acid synthesis. The insulin receptor protein is a heterotetramer composed of two identical extracellular α-subunits and two transmembrane β-subunits; the α-chain is in the extracellular portion of the cell membrane, while the β-chain spans the cell membrane in a single transmembrane segment, with part of it located in the intracellular face of the membrane. The subunits are held together by disulphide bonds. The IR is a tyrosine kinase that has two different ligand binding regions: high- and low affinity sites. Insulin binding to the IR leads to activation of "second messenger" acting as intracellular mediators, triggering a cascade of phosphorylation and dephosphorylation activities, which are responsible for a series of pathways and metabolic mechanisms, including glucose transport. In physiological conditions, on detection of high levels of glucose released from ingestion and uptake of food, leads to the release of insulin by the β-cell in the pancreas. Binding of

insulin to the IR, causes the activation of the IR tyrosine kinase occurs by autophosphorylation of β-subunit, at the intracellular domain of the receptor. The activated IR tyrosine kinase ensues the phosphorylation of tyrosine residues on insulin receptor substrate (IRS) proteins, some of which are recognized by the Src homology 2 (SH2) domains of the p85 regulatory subunit of PI3-K that is a lipid kinase. The PI3-K has a major role in insulin function; its pathway leads to the phosphorylation of phosphatidylionositol-(4-5)-bisphosphate (PIP_2) into phosphatidylionositol-(3,4,5)-trisphosphate (PIP_3). The generation of PIP_3 activates sets of specific proteins, enzymes, substrates, and molecules. One substrate of PI3-K is the phosphoinositide-depend kinase 1 (PDK1), which initiates the activation of a number of downstream proteins including protein kinase B (Akt). Once active, Akt phosphorylates and inactivates of glycogen synthase kinase 3 (GSK3), which has as major substrate the glycogen synthase, an enzyme that catalyses the final step in glycogen synthesis. Phosphorylation of glycogen synthase by GSK3 inhibits glycogen synthesis; therefore the inactivation of GSK3 by Akt promotes glucose storage as glycogen. Akt also has an important and central role in insulin-stimulated glucose uptake directly associating upstream insulin signaling with glucose transporter 4 (GLUT4) translocation.

3.2.4 Insulin resistance pathway

At the molecular level, the mechanisms responsible of insulin resistance are poorly understood. Several mechanisms including abnormal insulin production, mutation in IR and IRS, and insulin antagonists have been proposed. However it appears that defects in post-receptor signaling are the major cause of insulin resistance in target tissues (adipose, muscle and liver). Many evidences suggest that the development of insulin resistant affects the PI3-K pathway. On the other hand, there is not much evidence that the MAPK pathway is equally affected by insulin resistance as the PI3-K/Akt pathway.

As it has been described above, stimulation of IRS phosphorylation by insulin seems to be the crucial event in the insulin-signaling pathway. Both *in vivo* and *in vitro* evidence has shown that this step is defective in most cases of insulin resistance. Several factors have been proposed linking inflammation with insulin resistance. Studies have suggested that agents such as TNFα, FFAs and cellular stress can be involved in the development of insulin resistance because they can activate serine/threonine kinases that phopshorylate IRS and inhibit its function (Figure 3.4). TNFα interferes with early steps of the insulin-signaling pathway by inhibiting IRS tyrosine phosphorylation through the promotion of its serine phosphorylation (Gual *et al*, 2005; Kanety *et al*, 1995). The serine phosphorylation can induces a conformational change of IRS that can lead to a decrease in its interactions with IR. Also, once phosphorylated on serine residues, IRS may bind unknown signaling molecules that inhibit the IR kinase activity. Thus, TNFα leads to progressive

accumulation of IRS molecules that are phosphorylated in serine, which are less efficient to couple to the IR. In addition, suppressor of cytokine signaling proteins (SOC) proteins seem to inhibit insulin action at the level of IRS, although through different mechanisms. These kinds of proteins can attenuate insulin signaling by binding to the IR and by reducing their ability to phosphorylate IRS proteins (Gual *et al*, 2005).

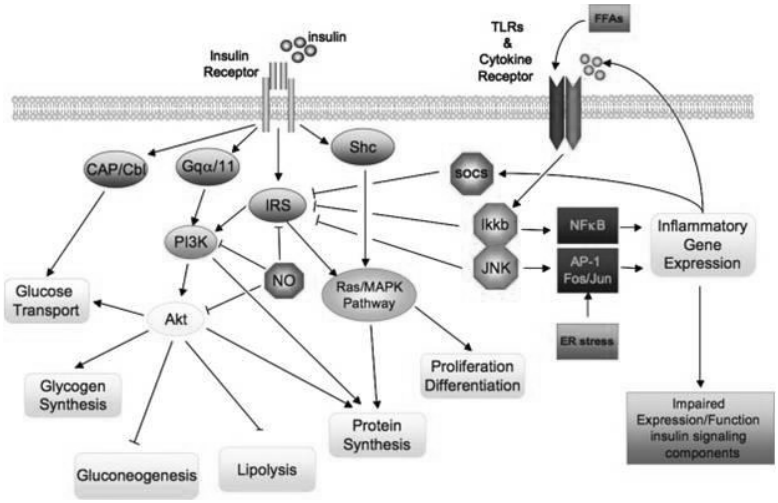


Figure 3.4. Insulin resistance pathway. Under stress condition (TNFα or FFAs) JNK and IKK are activated. This activation leads to inhibition of IRS with consequent insulin resistance.

Among the serine/threonine kinases activated by TNFα, c-Jun NH_2-terminal kinase (JNK) and IκB kinase (IKK), have been showed to be involved in this phosphorylation with consequent inactivation of insulin signaling. JNK and IKK have been demonstrated to promote insulin resistance though inhibition of insulin's action by serine phosphorylation, and through the induction of proinflammatory cytokine in different cell types, especially macrophages. Effectively they have powerful effects on gene expression, including promoting further inflammatory gene expression through activation of the activator protein (AP-1) complex and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). It has been demonstrated that IKK overexpression in hepatocytes causes local and systemic induction of proinflammatory genes and systemic insulin resistance in absence of obesity (Cai *et al*, 2005). Furthermore liver-specific deletion of IKK has provided evidence that its activity in liver influences systemic metabolism (Reza and Khosrow, 2009).

Also FFAs are involved in the inactivation of insulin signaling by modulation of intracellular protein kinases JNK, IKK and also protein kinase C (PCK); and decreasing insulin-stimulated GLUT 4 translocation. Saturated FFAs can interact with a liver-secreted glycoprotein that binds and actives a receptor involved in NF-κB activation and c-SRC recruitment for the activation of JNK and inhibition of insulin action. Moreover, in adipocytes it has been demonstrated that FFAs

induce insulin resistance by activating JNK and by increasing of TNFα expression, which in an autocrine/paracrine fashion acts on cells to impair adipocytes function, amplifying the proinsulin resistance signals (Nguyen *et al*, 2005). Fatty acid metabolism, such as diaglycerol and acyl CoA can activate tissue specific PCKs, PCK-θ in muscle and PCK-δ in liver which increase serine phosphorylation of IRS leading to inhibition of the insulin signaling. Excessive dietary FFAs flux into the liver via the portal vein may cause fatty liver disease and hepatic insulin resistance. Indeed experimentally, lipid infusion or a high fat diet that increases circulating FFAs levels promotes insulin resistance in the liver. However, molecular mechanism linking FFAs to hepatic insulin resistance remain poorly understood. Candidate events linking FFAs to insulin resistance *in vivo* are the up-regulation of sterol regulatory element-binding transcription protein 1 (SREBP-1c), which regulates genes required for glucose metabolism, FFAs and lipid production; inflammation caused by activation of JNK or IKK. However, it is still not clear which is the direct and initial target of FFA in the liver.

For all the reasons described above, the inhibition of IRS function could be representing a unifying mechanism linking between all factors involved in insulin resistance. Moreover is evident that JNK and IKK occupy key roles in linking obesity to insulin resistance, T2DM and MetS, because almost all metabolic stressors that causes insulin resistance activate these protein kinases. *In vivo* and *in vitro* studies demonstrate that activation of JNK and IKK appears to be sufficient to induce systemic insulin resistance.

Among the factors promoting the development of insulin resistance oxidative stress is proposed to play a central role. Overproduction of oxidants has been proposed as a link between FFAs and hepatic insulin resistance (Nakamura *et al*, 2009, Gao *et al*, 2010).

3.3 ROLE OF OXIDATIVE STRESS IN HEPATIC INSULIN RESISTANCE

Evans *et al.* (Evans *et al*, 2003) proposed that "oxidative stress induced by elevation in glucose and FFA plays a key role in causing insulin resistance and β-cell dysfunction". The suggested mechanisms proposed to cause impaired glucose homeostasis (Figure 3.5).

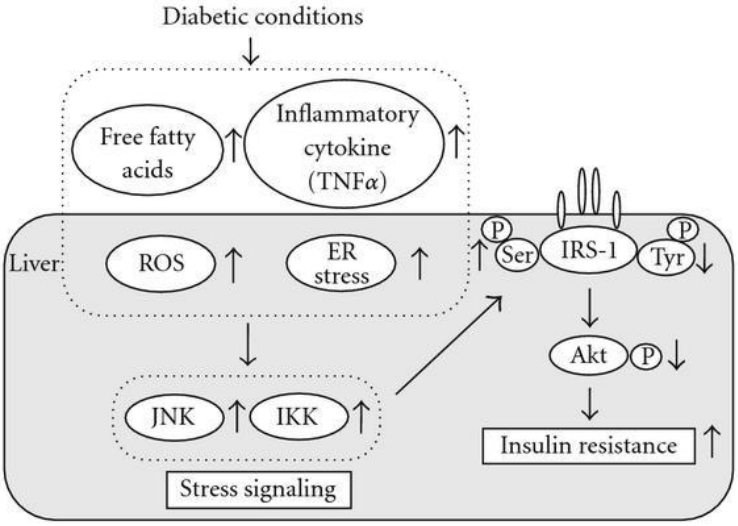


Figure 3.5. Role of ROS in the development of insulin resistance in type 2 diabetes. The JNK and IKK pathway are activated by various factors such as ROS, ER stress, free fatty acids (FFAs), and inflammatory cytokines such as TNFα. (Taken from Kaneto H *et al*, 2010).

Under condition of elevated metabolism, many tissue-specific cells are continuously subject to insults from ROS such as O_2^- and H_2O_2. It is thought that the chronic nutrient overload in obesity and T2DM can result in an exaggerated flux of glucose through glycolysis and Krebs cycle resulting in NADH and FADH production that, when excessive, may overload the mitochondrial respiratory chain that is one of the main sources of ROS. Likewise, increased flux of lipids during the β-oxidation may also contribute to an excessive electron flow, leading to the conversion of O_2 to O_2^-, which can then be converted to H_2O_2. O_2^- and H_2O_2 can react with other molecules in the cells to form different ROS. Another important source of ROS in context of obesity and above all hepatic insulin resistance is the NOX family of NADPH oxidases. Several NOX isoforms are expressed in the liver and pancreatic β-cells; moreover, an increase of ROS production is one of the earliest events in cases of glucose intolerance, and it may cause of pancreatic β-cell dysfunction as well as hepatic pathologies. NOX involves increased JNK-mediated-serine/threonine phosphorylation of IRS and PI3-K activity causing reduced Akt phosphorylation. Enhanced JNK-dependent liver injury is correlated with acute induction of NOX-dependent O_2^- production. This means that JNK may act to control NOX gene expression in the liver, which influences ROS-dependent AP-1 response. Furthermore, oxidative stress may lead to insulin resistance via stimulation of proinflammatory cytokines expression; indeed, ROS has been shown to activate NF-κB inducing production of cytokines including TNFα and IL-6. In turn, as has described previously, TNFα and IL-6 can cause decreased insulin sensitivity by promoting inhibition of IRS. Both inflammatory and oxidative mechanisms are related to each other because of their ability to

inhibit IRS via the common "avenue of stress-signaling" which includes the activation of JNK and IKK. The activation of these stress-protein kinases may be originated also by ER stress, which is able to activate proinflammatory pathways. ER stress is another source of ROS involved in metabolic disorders, the production of ROS derived from induction of the unfolded protein response. Proper folding of many proteins requires the formation of inter- and intra-molecular disulfide bonds involving the oxidation of cysteine residues and the release of electrons; the oxidation of cysteine residues and the release of electrons lead to the ER oxidoreduction and then to the conversion from O_2 to $O_2^{\cdot}$ (Tiganis, 2011).

As have been described Evans *et al*., also FFAs are involved in oxidative stress, and there is solid evidence that suggest FFAs association with the development of insulin resistance by initially disrupting the phosphorylation process in the insulin-signaling pathway and consequently reducing glucose oxidation and glycogen synthesis. Reduced glucose oxidation and glycogen synthesis increases FFAs oxidation, which causes an increase and accumulation of glucose-6-phosphate, inhibiting the glycogen synthesis pathway. This inhibition causes an increase of cell glucose which immediately blocks the glucose uptake with a consequent increase of the glucose levels in the bloodstream. This, may lead to insulin resistance and diabetes as a long term impact. Increased FFAs oxidation causes increased of ROS production, which can lead to increased fat accumulation.

All of the above strongly supports the theory that the pro-oxidant environment associated with metabolic disorders can lead to insulin resistance. Important role in the regulation of oxidant production and proinflammatory signals. Indeed, experimental evidence supports an inverse relationship between consumption of flavonoid-rich foods and pathologies with inflammatory components, like MetS.

3.4 FLAVONOIDS

3.4.1 Chemistry and biological action

Flavonoids represent a large class of at least 6,000 phenolic compounds found in fruits, vegetables, herbs, cocoa, chocolate, tea, soy, red wine and other plant food and beverage products. These compounds are responsible for many functions in the plant, including the regulation of growth and UV protection. In humans, flavonoids may have several beneficial health properties (Babu *et al*, 2013).

Chemically, flavonoids have 15 carbon atoms and they consist of two aromatic rings (A and B) linked by a chain of three carbon that form an oxygenated heterocyclic ring (C ring) (Figure 3.6).

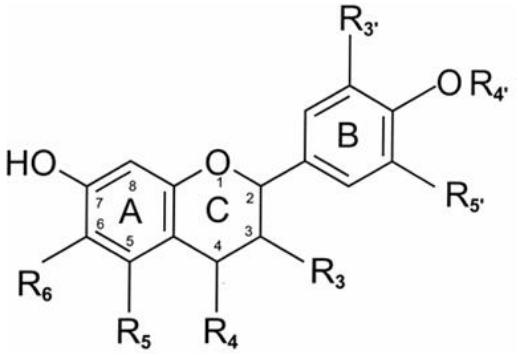


Figure 3.6. Chemical structure of flavonoids. Flavonoids have 15 carbon atoms and they consist of two aromatic rings (A and B) linked by a chain of three carbon that form an oxygenated heterocyclic ring (C ring).

Their biosynthesis is a metabolic pathway that involves acetate to produce ring A; while ring B is derived by shikimic acid synthesis (Bravo, 1998). The chemical characteristics of the C ring define the various subfamilies of flavonoids (Figure 3.7) by providing difference arrangements of hydroxyl, methoxy, and glycosidic group, and the bonding with other monomers. These various subfamilies are: flavanols, flavanones, flavonols (essentially, flavan-3-ols), isoflavones, anthocyanidins and chalcones (Crozier *et al*, 2009).

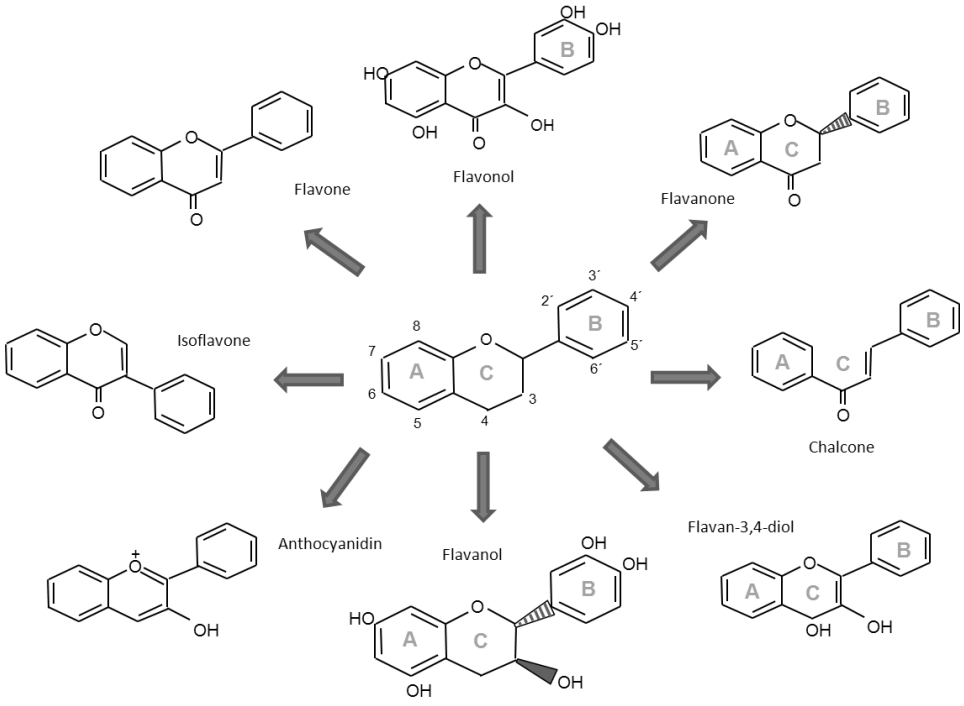


Figure 3.7. Chemical structures of the main flavonoid families present in plants. In the center: the basic generic flavonoid structure showing A, B, and C rings and the numbers for the various positions in the flavan structure. For the flavanol family, the structure of (−)-epicatechin is shown. (Taken from Fraga *et al*, 2011).

Flavonoids are in plants often present in O-glycosylated forms, in fact they are linked to sugar such as monosaccharides, disaccharides or oligosaccharides; for this reason the presence of sugar in the molecules can affect the bioavailability of flavonoids (Bravo, 1998, Manach *et al*, 2004).

To explain several polyphenols biological effects it has been proposed that the interaction of them with protein could be one of the relevant mechanisms. The chemical features shared by most of the flavonoids, for example the phenol group, are key to their biological action; on the other hand, a select chemical structure of a particular polyphenol becomes relevant for a specific mechanism of action. Using molecular modeling techniques, it has been shown that besides the basic chemical structure, the tridimensional structural characteristics of flavonoids are critical for their biological action (Mackenzie *et al*, 2008). The interaction of flavonoids proteins can be highly specific. The biological effects of flavonoids would then depend on the functions of the protein involved, including modification of enzymatic activities, transcription factors binding to their specific sites etc. In fact polyphenols can be efficient inhibitors of the activity of a broad number of enzymes, such as NADPH oxidase.

The research interest of mechanism of action of flavonoids was initially mostly centered on the antioxidant activity of these compounds. Epidemiological studies and meta-analysis suggested an inverse relationship between the consumption of flavonoid-rich diets, and the development of many aging-associated diseases, therefore oxidative stress-associated diseases including: cancers, CVD, osteoporosis, neurodegenerative disorders (Alzheimer's and Parkinson diseases) and diabetes (Temple, 2000). Indeed frequent consumption of natural antioxidant, through fruit and vegetable diet can reduce the risk of these diseases. Numerous *in vivo* and animals studies support this beneficial effect of dietary flavonoids, in particular flavanols, on glucose homeostasis; they can regulate carbohydrates digestion, insulin secretion, insulin signaling and glucose uptake in insulin-sensitive tissues through various intracellular signaling pathway.

3.4.2 Flavonols (flavan-3-ols): bioavailability and antioxidant capacity

Flavanols and procyanidins are polyphenols occurring in plants, in which they are secondary metabolites with various characterized function. Flavanols are a subfamily of flavonoids in which ring C is a saturated heterocycle with a hydroxyl group in position 4 (Figure 7). This subfamily of flavonoids includes: (-)-epicatechin (EC), (+)-catechin, epicatechin gallate (ECG), gallocatechin, epigallocatechin (EGC) and epigallocatechin gallate (EGCG). EC and catechin are the main flavan-3-ols in fruit and cocoa, the others are found mainly in tea, grapes and seeds of certain leguminous plants.

Flavan-3-ols are present in plants as algycones, as oligomers, or esterified gallic acid and most of them are present in nature as stereoisomers in *cis* and *trans* configuration respect to carbon 2 and 3:

EC and (+)-catechin. Even minor change in isomerization, monomer bonding type and degree of polymerization may have a major impact on the biological action of these compounds. The molecular action of flavanols and procyanidins are largely depend on their bioavailability at the target tissue, which depends of their absorption, metabolism at the gastrointestinal tract, tissue and cellular distribution and tissue metabolism after absorption. Solid evidence from human studies have shown that the flavan-3-ols are stable during the gastric transit, given their presence throughout in the gastrointestinal tract after ingestion of flavonoids-rich food. In the colon, the microflora can also modify flavanols and procyanidins, including the breakage of the flavan structure to form simple phenol and ring-fission metabolites. In the small intestine flavanols are extensively glucuronidated and partially methylated, allowing negligible amounts of native catechin or epicatechin in the mesenteric circulation. In blood and in plasma EC and its metabolites can reach concentration in the low micromolar range. Although flavan-3-ols and their related procyanidins can act as oxidant scavengers, and trapping pro-oxidant metals in *in vitro* conditions, their low concentration in tissues after dietary human consumption does not make this direct antioxidant activity relevant. However, they can reach high concentration in the gastrointestinal tract where an antioxidant action would be feasible. Very importantly, flavan-3-ols and their related procyanidins can exert indirect antioxidant action by modulating the activity of select cellular components, for example NADPH oxidase.

3.4.3 (-)-epicatechin (EC): structure, EC-derived metabolites and mechanisms of action

EC is a flavan-3-ol widely distributed in nature and one of the most abundant flavonoids in human diet. It is found in large amount in several beverages like tea and red wine, grapes, cocoa and many other fruits and vegetables (Arts *et al*, 1999). The chemical structure of EC, as has been described previously, has two aromatic rings linked by an oxygenated heterocycle with a hydroxyl group in position 4 (Figure 3.8)

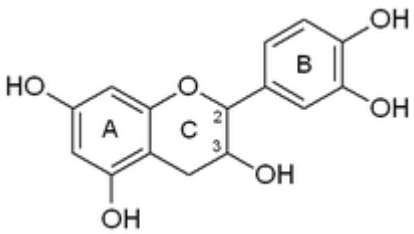


Figure 3.8. Chemical structure of (-)-epicatechin.

Regarding the bioavailability, some evidence has shown that among the procyanidin-containing food ingested, only plasma oligomers of EC, and not (+)-catechin, were detected (Crozier *et al*, 2009). The simple stereochemical orientation of the OH in position 3 of the C ring, that distinguishes EC from catechin makes a remarkable difference in bioavailability, and stresses that

the interactions of these compounds with cellular components has to be highly specific. It has been demonstrated that after 2 hours of ingesting an EC-rich cocoa drink, small amount of EC were found in human plasma, with the majority begin derived metabolites (ECM). After ingestion of an EC-rich food, EC is metabolized into a range of metabolites which maintain an intact flavanol ring and ring-fission metabolites, originated from EC breakdown by microflora in the colon. There are several studies that have reported on the absorption and metabolism of EC and other mammalian species: but thus far, it has been clearly established that ingested EC is extensively metabolized into ECM by O-methylation, O-sulfonation, O-glucuronidation and combination of those. Ottaviani *et al.* (Ottaviani *et al*, 2012) identified and measured in plasma eight different ECM (Figure 3.9) 2h after consumption of a test drink containing EC. This study showed that ECM consist of: (-)-epicatechin-3'-β-D-glucuronide, (-)-epicatechin-3'-sulfate, (-)-epicatechin-5-sulfate, (-)-epicatechin-7-sulfate, 4'-O-methyl(-)-epicatechin-5/7-sulfates and 3'-O-methyl(-)-epicatechin-5/7-sulfates.

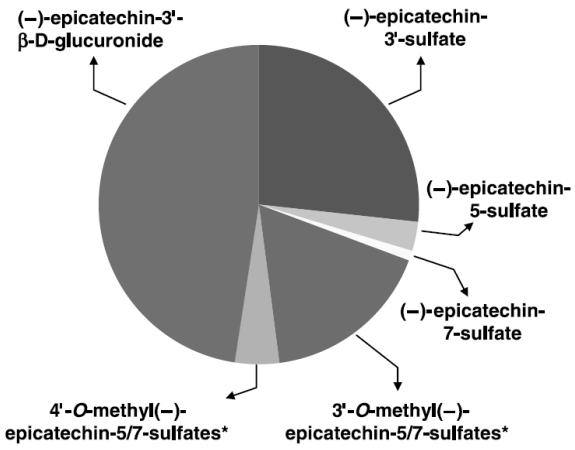


Figure 3. 9. Relative amount of individual (-)-epicatechin metabolites in plasma. (Taken from Ottaviani et al, 2012)

Although Figure 9 shows that (-)-epicatechin-3'-β-D-glucuronide is the most predominant O-glucuronidated metabolic derivative of EC present in human circulation, the consumption of diet-relevant amounts of EC leads to presence in the system circulation of this three main ECM: (-)-epicatechin-3'-β-D-glucuronide, 3'-O-methyl(-)-epicatechin-5/7-sulfates and (-)-epicatechin-3'-sulfate. EC and its metabolites may have an antioxidant biological effect in the prevention of oxidative damage through direct and indirect mechanisms (scavenging oxidants, and/or regulating protein synthesis and activities). The mechanisms of action of EC as antioxidant include: i) modulation of endothelial nitric oxide phosphorylation and nitric oxidase availability in endothelial cells (Fraga *et al,* 2011); ii) regulation of calcium channel that modulate cellular calcium influx (Verstraeten *et al*, 2008); iii) modulation of NADPH oxidase activity which can result in a decrease

in ROS leading to alterations in the modulation of redox signals (Steffen *et al*, 2007; Steffen *et al*, 2008); and iv) direct interaction with signaling molecules, e.g. NF-κB.

3.4.4 Antioxidant indirect effects of EC and ECM

The main kind of indirect antioxidant effects is the modulation of pro-oxidant enzymes by flavonoids, in particular EC and ECM, is through the inhibition of NADPH oxidase. It has been demonstrated that the ECM, especially (-)-epicatechin O-methylated metabolite share structural similarities with apocynin, a typical NADPH oxidase inhibitor (Figure 3.10). (-)-Epicatechin glucuronide is able to inhibit the activity of this enzyme.

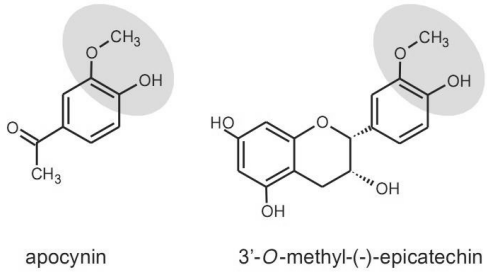


Figure 3.10. Structural features of apocynin and 3'-O-methyl(-)-epicatechin as NADPH oxidase inhibitors. (Taken from Fraga et al, 2010)

The inhibition of NADPH oxidase by EC and ECM can be regulates the redox signaling through decrease of O_2^- production. The mechanism underlying this inhibition can be related to the ability of EC and ECM to act as the specific inhibitors of NADPH oxidase (apocynin, DPI and VAS 2870) on the expression of NOX subunits, such as $p22^{phox}$; and on the translocation of $p47^{phox}$, and $p67^{phox}$ from the cytosol to the membrane (Figure 3 introduction).

Another indirect action of flavanols and procyanidins is the alteration of oxidant production as a result of inhibiting the binding of ligand to its receptor; in fact EC can decrease the transient increase in oxidants associated to TNFα-triggered signaling by the inhibition of the binding between TNFα and its receptor which leads to the activation of NADPH oxidase. Also has been demonstrated *in vitro* that EC can inhibits at different levels the NF-κB pathway involved in inflammation and insulin resistance. Evidence suggested that flavanols may reduce T2DM risk and inflammatory biomarkers by enhancing insulin sensitivity and secretion, protecting pancreatic β-cell functions, and improving glucose homeostasis through up-regulation of GLUT4 gene expression. Vazquez-Prieto *et al.* (Vazquez-Prieto *et al*, 2012) have shown that EC improves parameter of inflammation and insulin sensitivity in adipocytes *in vitro* through the prevention of deregulation of key signaling cascades, as NF-κB, MAPKs and AP-1, triggered by inflammatory stimuli (TNFα). Although is well known that EC have an important role against insulin resistance

in adipocytes it is still unclear the role of them and its metabolites on the activity of hepatic NADPH oxidase and its consequences on the insulin pathways.

3.5 OBJECTIVE OF THE STUDY

For all the reasons described above the aim of this study was to evaluate if EC and ECM could improve insulin sensitivity through the modulation of hepatic NADPH oxidase in human hepatocytes (HepG2 cells) treated with the fatty acid palmitate (Pal).

MATERIALS AND METHODS

3.6 MATERIALS

HepG2 cells were from the American type culture collection (Rockville, MA). Cell culture media and reagents were from Invitrogen Life Tecnologies (Carlbad, CA). Primary antibodies for NOX3 (sc-67005), α-tubulin (sc-23948), actin (sc-1615), $p47^{phox}$ (sc-17845), pJNK (sc-6254), and JNK (sc-572) were from Santa Cruz Biotechnology (Santa Cruz, CA). Primary antibodies for pIKKα/β (2681) and IKKβ (2684) were from Cell Signaling Technology (Danvers, MA). 5-(and-6)-carboxy-2,7dichlorodihydrofluorescein diacetate (DCF) was from Invitrogen/Molecular Probes (Eugene, OR, USA). (-)-Epicatechin, VAS 2890, and lucigenine were from Sigma Aldrich (USA). The Cell Viability kit was from Promega (Wisconsin, USA). (-)-Epicatechin metabolites were a kind gift of Mars Inc (Hackestown, NJ, USA).

3.7 METHODS

3.7.1 Palmitate (Pal) preparation

Palmitic acid is the most saturated fatty acid found in animals, plants and microorganism. Pal was prepared 8 mM in 10.5% of bovine serum albumin (BSA) fatty acid free. BSA was dissolved in DMEM and 1M HEPES at room temperature (do not adjust the pH), was and subquently added with sodium palmitate (8 mM) (Sigma Aldrich). The solution was mixed at 37°C for 3 hours in a beaker of water on a hot plate to monitor the temperature. When the solution was homogeneous, it was filtered through a cellulose acetate filter unit, 0.22μM filter (Millipore Steriflip). The Pal stock was stored at -20°C until further use.

3.7.2 ECM composition

Of the EC metabolite mixture (ECM) used in this study corresponds to a representative mixture of structurally related (-)-epicatechin metabolites (SREM) present in human circulation after EC consumption (Figure 9, Table 1) (Ottaviani *et al*, 2012). ECM was a kind gift of Mars Inc. (Hackestown, NJ, USA). The mixture was prepared by dissolving ammonium salt of the metabolites in ethanol:water (1:1).

SREM	Abundance (%)	Abundance (µmol/L)
(−)-epicatechin-3′-β-D-glucuronide	25%	250
(−)-epicatechin-3′-sulfate	25%	250
3′-*O*-methyl(−)-epicatechin-5-sulfate	25%	250
3′-*O*-methyl(−)-epicatechin-7-sulfate	5%	50
3′-*O*-methyl(−)-epicatechin-4′-sulfate	5%	50
4′-*O*-methyl(−)-epicatechin-5-sulfate	5%	50
4′-*O*-methyl(−)-epicatechin-7-sulfate	5%	50
(−)-epicatechin-5-sulfate	5%	50
Sum of SREM	100%	1000

Table 1. Composition of SREM. In the table are shown the concentrations of each metabolite present in the ECM used in the study. The sum of all SREM present in the solution is equivalent to 1mM (Taken from Mars Inc.)

3.7.3 Cell culture and incubation

The human liver carcinoma cell line HepG2 cell is derived from the liver tissue of fifteen years old Caucasian American male with differentiated hepatocellular carcinoma. These cells are epithelial in morphology and have a model chromosome number 55. HepG2 cell are usually used for a variety of biochemical and cell biology research, because they have a high degree of morphological and functional differentiation *in vitro*.

HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM low glucose) supplemented with 10% FBS, antibiotics (100 IU/ml penicillin and 10 mg/ml streptomycin) and 0.1M of sodium pyruvate. Cells were maintained at 37°C with humidified air and 5% CO_2 until confluence. Confluent cells were switched to Minimum Essential Medium (MEM) supplemented in the same way as DMEM and they were incubated with different concentration of Palmitate (0.25, 0.35, 0.5, 0.75 mM), in presence or absence of EC and ECM (0.25-1 µM) for 24 hours.

3.7.4 Western blot analysis

To prepare total extract, cells were rinse with PBS, scraped and centrifuged (800 x g for 8 min). The pellet was resuspended in 200 µl of lysis buffer (50 mM HEPES, 150 mM NaCl, 1mM Na_3VO_4, 100 µM NaF, 1% (v/v) Iegepal, 0.5 mM EDTA, 1mM PMSF and proteases inhibitors (Roche, Switzerland). Samples were exposed to one cycle of freezing and thawing, incubated at 4°C for 30 min, and centrifuged at 10,000 x g for 30 min. The supernatant was decanted and protein concentration was measured (Bradford 1976). Aliquots of total cell fractions containing 25-50 µg protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. Colored molecular weight standards (Biorad Laboratories, Hercules, CA, USA) were ran simultaneously. Membranes were blotted for 1 h in 5% (w/v) non-fat milk, incubated overnight at 4°C in the presence of the corresponding antibodies (1:1000 dilution

for all antibodies except for NOX3 1:250) in 5% (w/v) bovine serum albumin in TBS buffer (50 mM Tris, 150 mM NaCl, pH 7.6), containing 0.1% (v/v) Tween-20. After incubation for 90 min at room temperature in the presence of the secondary antibody (HRP-conjugated) (1:10,000 dilution) the conjugates were visualized by chemiluminescence detection in a Phosphoimager 840 (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA).

3.7.5 RNA isolation and real-time PCR (RT-PCR)

For quantitative RT-PCR studies, RNA was extracted from cells using TRIzol reagent (Invitrogen). cDNA was generated using high-capacity cDNA Reverse Transcriptase (Applied Biosystems, Grand Island, NY, USA). Expression of NOX3 and $p47^{phox}$ was assessed by quantitative real-time PCR (iCycler, Bio-Rad) with appropriate primers:

Primer p47 Forward: TCACCGAGATCTACGAGTTC
Primers p47 Reverse: TCCCATGAGGCTGTTGAAGT

Primer NOX3 Forward: GTGAACAAGGGAAGGCTCAT
Primer NOX3 Reverse: GACCCACAGAAGAACACGC

3.7.6 Cell viability

The effect of Pal, EC, on HepG2 cell viability was determined by using CellTiter-Glo Luminiscent Cell Viability Assay (Promega, Madison, WI, USA) which measures cellular ATP levels. Briefly, cells (5,000/well) were plated in 96-well plated and grow for 24 h. Cells were incubated in the absence or in the presence (0.25-0.75 mM) of Pal for 24 h and cell viability tested following the manufacturer's protocol.

3.7.7 Cell oxidant levels evaluation

Cell oxidant levels were evaluated using oxidant-sensitive probe 5-(and-6)-carboxy-2,7 dichlorodihydrofluorescein (DCF) (Figure 3.11).

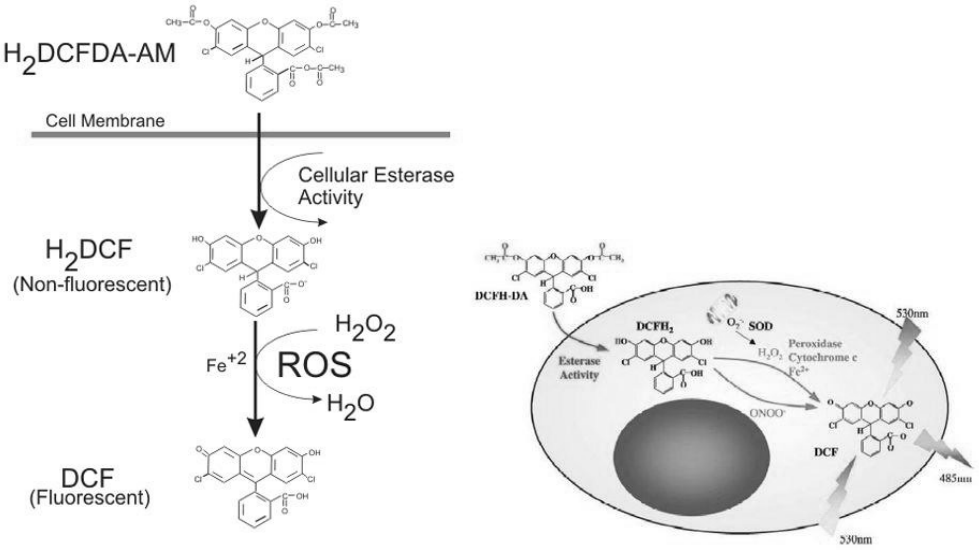


Figure 3.11. Diagram of the intracellular oxidation of the probe 5(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCDCDFH). Cell esterase activity renders the redox-sensitive non fluorescent form 5(and 6)-carboxy-2',7'- dichlorodihydrofluorescein (DCDHF) which in the presence of oxidant species is oxidizes to the fluorescent compound 5(and 6)-carboxy-2',7'- dichlorohydrofluorescein (DCF).

HepG2 cells ($0.3x10^6$ cells/dish) were grown in 35 mm dishes until confluence. After 24 hours of incubation with Pal, EC and ECM, the media was discarded and changed to FBS-free MEM containing 20 µM DCF. After 30 min of incubation at 37°C, the media was removed; cells rinsed with PBS; scraped and centrifuged at 800 x g for 8 min. The cell pellet was resuspended with 150 µl PBS, an aliquot separated for protein determination, and the rest transferred to 96 well plates. Fluorescence (λ excitation: 475 nm; λ emission: 525 nm) was measured in a plate reader (Wallac 1420 VICTOR2™, PerkinElmer Life Science, Waltman, USA). Results are expressed as the ratio DCF fluorescence/mg protein.

3.7.8 NADPH oxidase activity

3.7.8.1 Isolation cytosolic and membrane fraction

To measure NADPH activity after the corresponding incubations cytosolic and membrane fractions were isolated. HepG2 cells (2.2 x 10^6 cells/dish) were grown in 100 mm dishes. After 24 hours of incubation with Pal, EC and ECM, cells were rinse with PBS; scraped and centrifuged at 800 x g for 10 min. Pellets were resuspended in 300 µl of Krebs Buffer (20 mM HEPES, 119 mM NaCl, 4.7 mM KCl, 1mM $MgSO_4$, 0.4 mM NaH_2PO_4, 0.15 mM Na_2HPO_4 and 1.25 mM $CaCl_2$) containing 1mM PMSF and Roche protease inhibitor cocktail (Roche, Switzerland). Cells were homogenized and then centrifuged at 800 x g for 8 min. The supernatant was collected and

centrifuged 53,000 rpm for 1 h at 4°C in an ultracentrifuge (Sorvall RCM120 GX, Thermo Scientific, New York, NY, USA). After the ultracentrifugation, the supernatant (cytosolic fraction) and the pellet (membrane fraction) were collected. The supernatant was used to measure the $p47^{phox}$ levels by Western blot and the pellet, after resuspension in 150 µl of Krebs Buffer, was used to measure NADPH oxidase activity. Proteins of each sample were measured by Bradford assay (Bradford, 1976).

3.7.8.2 NADPH oxidase activity assay

NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence assay. Samples (10 µg of protein) (10 µl) were placed in 96 well plates, in the absence or the presence of 1mM VAS 2870 (2 µl), then 170 µl of Krebs buffer, 5 µM of lucigenin (5 µl) and 40 µM of NADPH (20 µl) were added in sequence. The reaction was done under temperature controlled conditions (37°C). Light emission was measured every 30 seconds for 10 min in a plate reader (Wallac 1420 VICTOR2™, PerkinElmer Life Science, Waltman, USA). NADPH oxidase activity was calculated as the area under the curve and expressed as relative units (counts per second (CPS)/mg protein).

3.7.9 Oil Red O Staining

Oil red O staining was used to measure cell lipid accumulation. HepG2 cells ($0.1x10^6$ cells/well) were grown in 12 well plates and after 24 h incubation with Pal, EC and ECM, cell monolayers were rinsed with PBS and fixed with a solution 10% (w/v) formaldehyde in PBS for 15 min at room temperature. After the fixative solution was removed, cells were stained for 1 h at room temperature with Oil red O solution, prepared fresh immediately before use (6 ml of 0.05 % (w/v) Oil red O 2-isopropanolplus 4 ml of distilled H_2O). Subsequently cells were rinsed 3 times with distilled H_2O to remove the excess dye e. Images were recorded in an inverted microscope (Olympus IX71, Center Valley, PA, USA). Subsequently, cells were added with 2 ml 2-isopropanol for 15 minutes at room temperature to extract the dye from the cells, and the absorbance of 2-isopropanol extract was measured spectrophotometrically at 520 nm (Wallac 1420 VICTOR2™, PerkinElmer Life Science, Waltman, USA).

3.8 STATISTIC ANALYSIS

Data were analyzed by one-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC, USA). Fisher least significance difference test was used to examine differences between group means. A p value < 0.05 was considered statistically significant. Data are shown as mean±SEM.

RESULTS

3.9 PAL AFFECTS HEPG2 CELLS VIABILITY AND CAUSES LIPID ACCUMULATION

To evaluate the effects of Pal HepG2 cell viability, cells incubated with different concentration of Pal (0.25-0.75 mM) in the absence and the presence of EC and ECM. Figure 1 shows that after 24h of incubation, Pal decreased cell viability at 0.35 mM and up to 0.75 mM Pal concentrations. Pal did not affect cell viability at 0.25 mM concentration. Moreover, EC and ECM did not further affect cell viability.

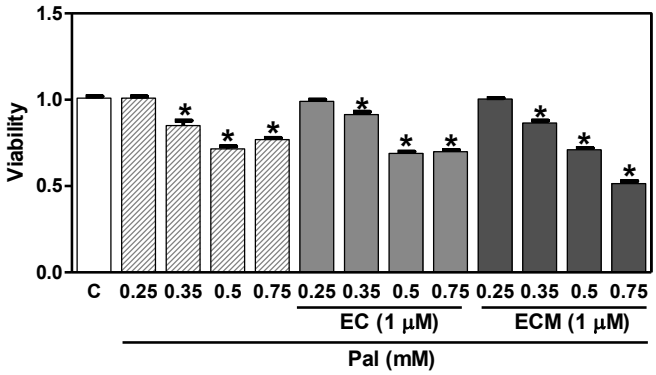


Figure 1. Cell Viability. Cell viability in HepG2 treated with Pal (0.25-0.75 mM) with/without EC (1 μM) or ECM (1 μM). Results are shown as means ± SEM of 3 independent experiments. * Significantly different respect to C, (One Way ANOVA, $p < 0.05$)

Lipid accumulation was assayed by Oil Red O staining, Figure 2A shows images of Oil red staining of HepG2 treated with Pal, EC and ECM. After 24h of incubation with 0.25-0.75 mM of Pal in absence and in presence of EC and ECM cells were fixed on the wells and stained with Oil Red O. Images illustrate that compared to the control (C-without Pal) Pal induced lipids accumulation. Cell morphology confirmed the results of the cell viability (Figure 1), hepatocytes incubated with 0.35, 0.5 and 0.75 mM Pal were morphologically different compared to cells incubated in the absence and in the presence of 0.25 mM Pal. HepG2 cells treated with concentration 0.35 mM and above displayed the cytotoxic effects of Pal at high concentrations. For this reason subsequent experiments were done at 0.25 mM Pal.

On the other hand Pal at concentration of 0.25 mM significantly increased lipid accumulation compared to the control cells (Figure 2B). EC and ECM did not affect on Pal-induced lipid accumulation in HepG2 cells.

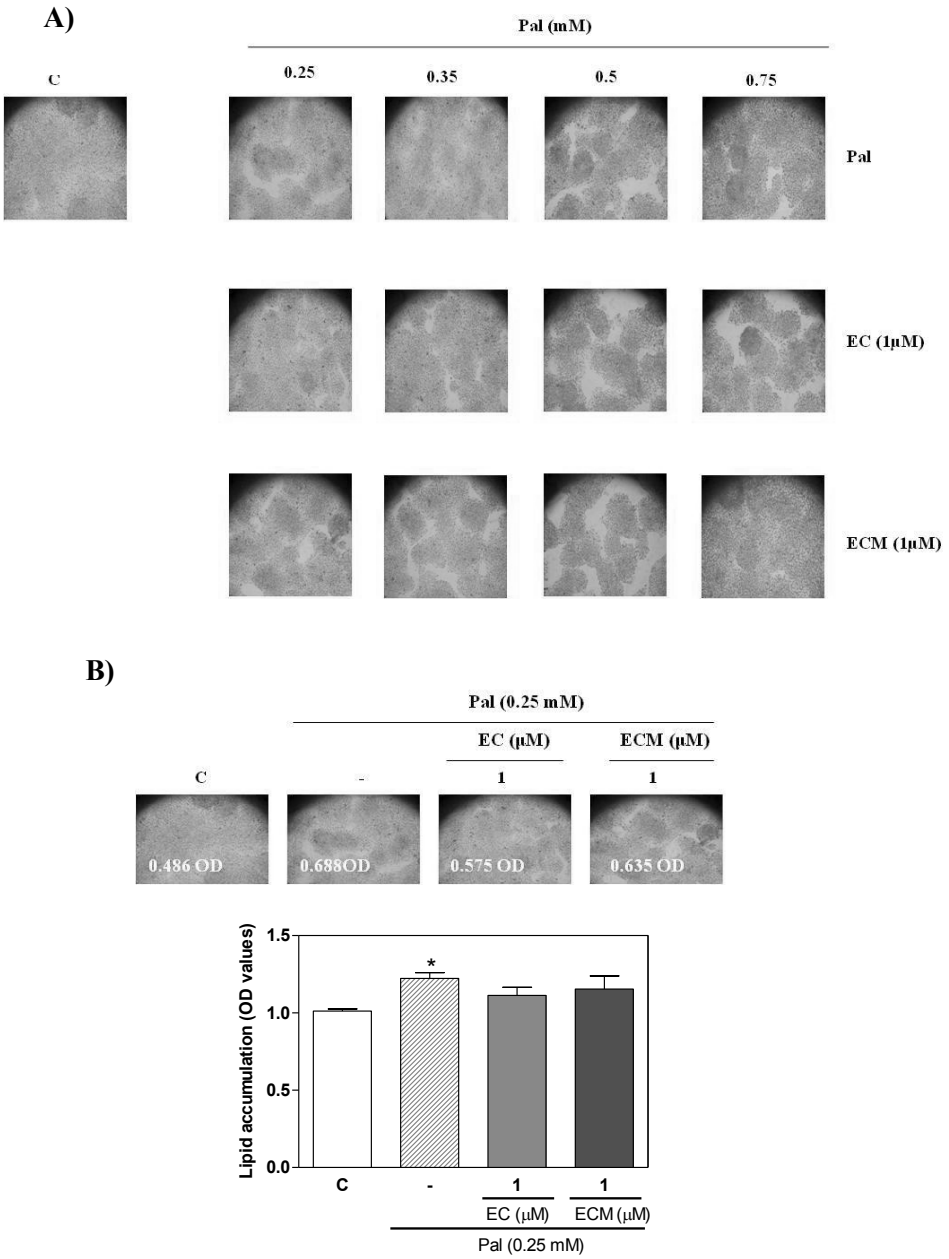


Figure 2. Oil Red O Staining. HepG2 cells were incubated for 24 h in the absence (C) or the presence of 0.25-0.75 mM Pal with or without 0.1 µM EC or ECM. Cells were subsequently stained with Oil red O to evaluate lipid accumulation**. A)** Representative microscope images **B)** After extracting Oil Red O staining from HepG2 cells, the absorbance at 520 nm was measured. Results are shown as means ± SEM of 5 independent experiments. * Significantly different respect to C, (One Way ANOVA, $p < 0.05$).

3.10 EC AND ECM INHIBIT PAL-MEDIATED INCREASED NOX3 EXPRESSION

To investigate if FFAs could have a role in the development of oxidative stress in liver by regulating the expression of NOX, we measured the expression (protein and mRNA) of NOX3

protein subunits in HepG2 cells treated with different concentrations of Pal. Among all the members of the NOX family we measured protein levels of NOX3 because solid evidence has demonstrated the expression of NOX3 and its subunits (but not of NOX1, NOX2, NOX4 and NOX5) in HepG2 cell line and in rat hepatocytes (Gao *et al*, 2010). In HepG2 cells, Pal (0.25-0.75 mM) caused a significant increase (70-78%) of NOX3 protein levels (Figure 3).

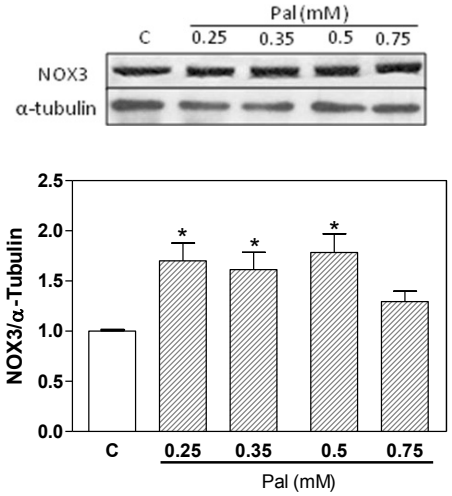


Figure 3. Palmitate (0.25-0.75 mM) increases NOX3 protein levels in HepG2. HepG2 cells were incubated for 24 h in the absence or the presence of 0.25-0.75 mM Pal. NOX 3 protein levels were measured by Western blot and referred to α-tubulin content as loading control. Results are show as means ± SEM of 8 independent. * Significantly different respect to C, (One Way ANOVA, $p < 0.05$)

On the other hand, Pal did not have any effect on the expression of the NOX3 subunits, $p22^{phox}$ and $p47^{phox}$ (Figure 4).

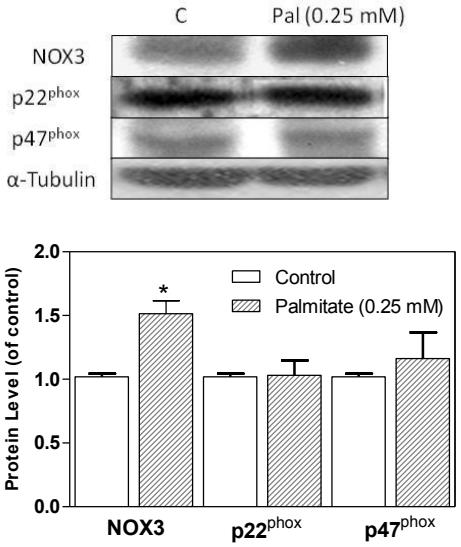


Figure 4. Pal significantly increases the protein levels of NOX3 but not of the $p22^{phox}$ and $p47^{phox}$ subunits in HepG2 cells. HepG2 cells were incubated for 24 h in the absence or the presence of 0.25 mM Pal. NOX 3, $p22^{phox}$ and $p47^{phox}$ subunit protein levels were measured by Western blot and referred to α-tubulin content as loading control. Results are shown as means ± SEM of 8 independent experiments * Significantly different respect to C (One Way ANOVA, $p < 0.05$)

Since the data on cell viability showed that Pal at 0.35, 0.5 and 0.75 mM concentration decreases cellviability, 0.25 mM was subsequently used for further experiments. To investigate if EC and ECM play a role in the modulation of Pal-induced NOX3 expression, HepG2 cells were treated with 0.25 mM of Pal in the absence or the presence of EC (0.25-1 μM) and ECM (1 μM). Different concentrations of EC were used to verify an eventual dose-response between this flavanol and NOX 3 protein levels.

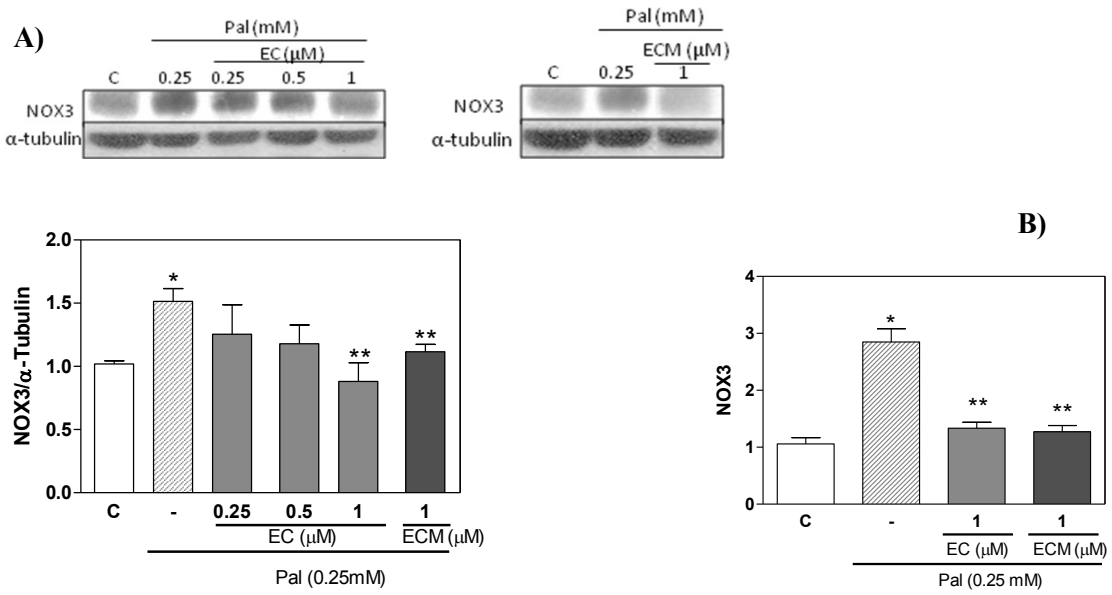


Figure 5. **Pal (0.25 mM) increases NOX3 protein and mRNA expression in HepG2 cells: effects of EC and ECM.** HepG2 cells were incubated for 24 h in the absence or the presence of 0.25 mM Pal with or without the addition of 0.25-1 μM EC or ECM. **A)-** NOX 3 subunit protein levels were measured by Western blot and referred to α-tubulin content as loading control. **B)-** mRNA levels for NOX3 were measured by quantitative real-time PCR, normalized against TATA-Box binding protein (TBP) and referred to control group values (C). Results are shown as means ± SEM of 4-8 independent experiments. * Significantly different respect to C (One Way ANOVA, $p < 0.05$)

EC caused a dose (0.25-1 μM)-response inhibition of Pal (0.25 mM)-induced increase in NOX 3 protein content (Figure 5A). ECM (1 μM) had a similar effect compared to EC indicating that both EC and EC metabolites are effective inhibiting Pal-induced NOX 3 protein increase. Pal also caused a significant increase in NOX3 mRNA levels, which was inhibited by both EC and EC metabolites (Fig. 5B).

3.11 EC AND ECM DECREASE PAL-INDUCED NADPH OXIDASE ACTIVATION AND OXIDANT PRODUCTION IN HEPG2 CELLS

As demonstrated in the previous results Pal increased both protein and mRNA levels of NOX3 in hepatocytes, which was inhibited by EC and its metabolites. We next investigated if EC and ECM were able to act also on the regulation of the activity of hepatic NADPH oxidase, which is a main source of superoxide anion in cells. Hepatic NADPH oxidase activity was directly assayed in the membrane fraction and indirectly in the cytosolic fraction evaluating the membrane translocation of $p47^{phox}$, that is required for NADPH oxidase activation.

Pal (0.25 mM) increased the activity of NADPH oxidase; while EC and its metabolites prevented Pal-induced NADPH oxidase activation, as is shown in Figure 6A. Figure 6B shows that the cytosolic subunit $p47^{phox}$, in presence of Pal, translocated from the cytosol to the membrane, because $p47^{phox}$ cytosolic protein levels are lower in the cells treated with 0.25 mM Pal than in controls. EC and ECM did not have any effect on $p47^{phox}$ membrane translocation. These results indicate that EC and ECM act in part exerting a direct inhibitory effect on NADPH oxidase activity.

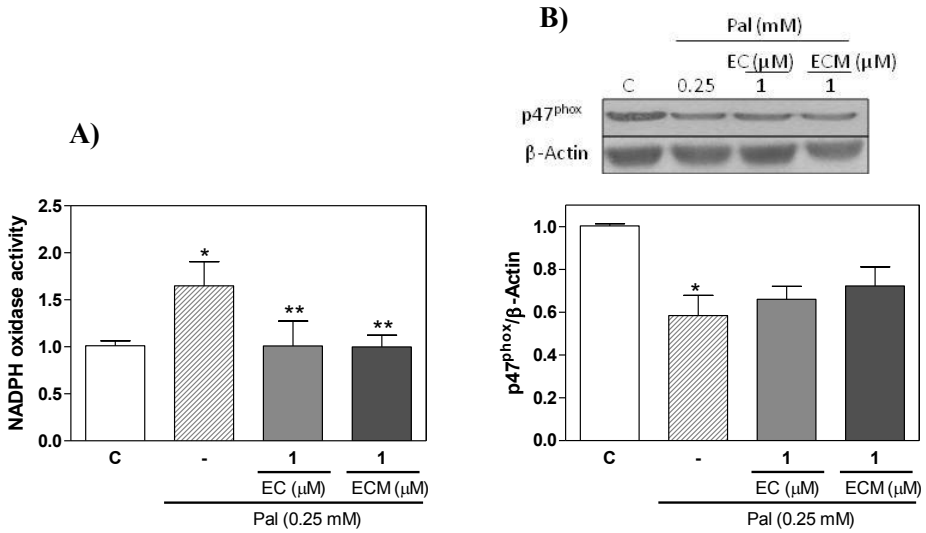


Figure 6. **NADPH oxidase activity and $p47^{phox}$ translocation in HepG2 cells treated with Pal 0.25 mM and EC (1 μM) or ECM (1 μM)**. HepG2 cells were incubated for 24 h in the absence or the presence of 0.25 mM Pal with or without the addition of 0.25-1 μM EC or ECM. Membrane and cytosolic fractions were isolated as described in methods. **A)**- NADPH oxidase activity measured in the membrane fraction. **B)**- Protein $p47^{phox}$; Levels in the membrane fraction were measured by Western blot. Results are shown as means ± SEM of 7 independent experiments. * Significantly different respect to C, ** significantly different respect to Pal (One Way ANOVA, $p < 0.05$)

The increase in activity of NADPH oxidase in response to different stimuli leads to the overproduction of $O_2^{\cdot-}$. Accordingly, HepG2 cells treated with Pal for 24 h showed significantly higher levels of oxidant levels, as evaluated with the probe DCF, compared to controls. EC and ECM, significantly reduced Pal-induced oxidant increase (Figure 7).

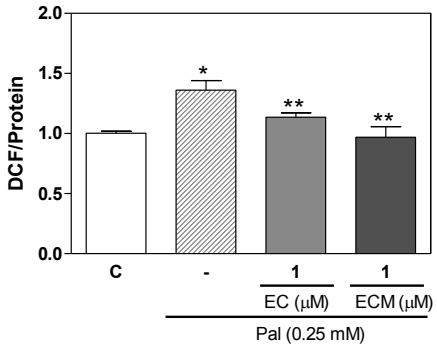


Figure 7. **ROS quantification in HepG2 cells treated with Pal (0.25 mM) and EC (1 μM) or ECM (1 μM).** HepG2 cells were incubated for 24 h in the absence or the presence of 0.25 mM Pal with or without the addition of 0.25-1 μM EC or ECM. Cells oxidants were measured with probe DCF as described in methods. Results are shown as means ± SEM of 6 independent experiments. *Significantly different respect to C, ** significantly different respect to Pal (One Way ANOVA, $p < 0.05$)

3.12 EC AND ECM MAY MITIGATE HEPATIC INSULIN RESISTANCE IN HEPG2 CELLS THROUGH DOWN REGULATION OF JNK AND IKK

As shown previously, Pal increases ROS production in part through the up-regulation of NOX3 expression and indirectly increasing NADPH oxidase activity. Literature evidence suggests that chronic elevation of ROS can lead to impaired insulin signaling. Oxidative stress may be the link between FFAs and insulin resistance. Thus, we next investigated if NOX3-derived ROS may drive Pal-induced hepatic insulin resistance through the regulation of two kinases involved in the development of this condition, JNK and IKK. The capacity of EC and ECM to mitigate hepatic insulin resistance through the regulation of JNK and IKK protein expression was also investigated. Figure 8 shows a trend for increased JNK ($p=0.070$) and IKK ($p=0.062$) phosphorylation compared to controls (C). EC (0.25-μM) (Figure 8A) showed a dose-response decrease trend, but not significant compared to Pal. A similar effect was observed for ECM ($p=0.072$). For IKK (Figure 8B) EC decreased the phosphorylation of IKK only at concentrations of 0.5 and 1 μM ($p=0.068$); while ECM significantly decreased IKK phosphorylation.

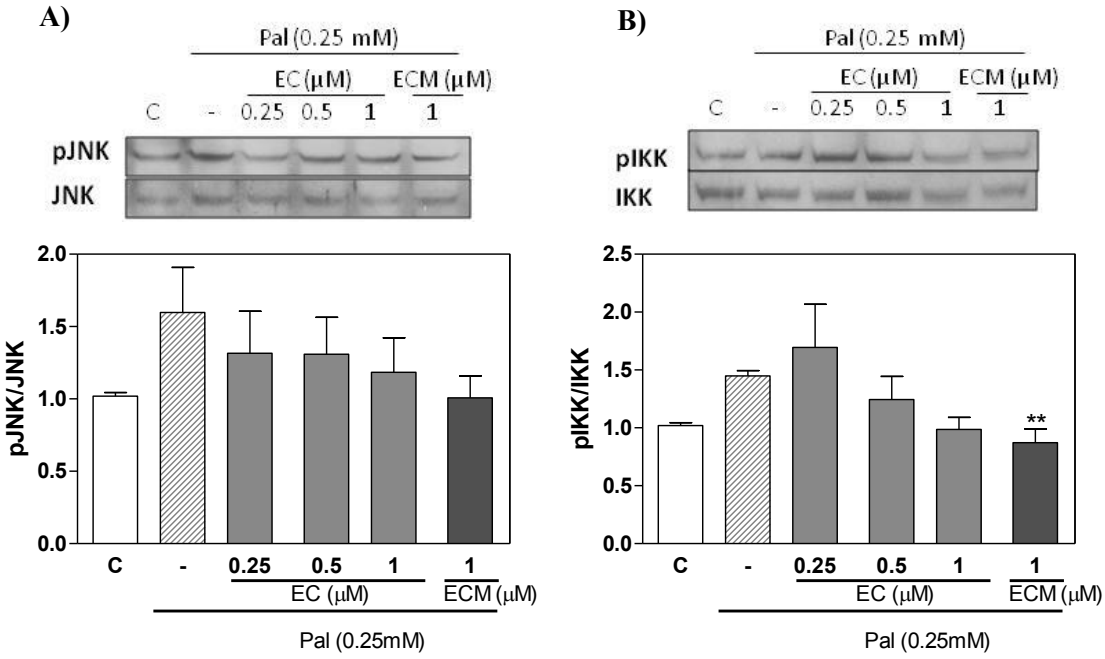


Figure 8. **JNK and IKK phosphorylation in HepG2 cells treated with Pal (0.25 mM) and EC (0.25-1 μM) or ECM (1 μM).** HepG2 cells were incubated for 24 h in the absence or the presence of 0.25 mM Pal with or without the addition of 0.25-1 μM EC or ECM. **A-** JNK (Thr183, Tyr185) and **B-** IKKα/β (Ser178/180) phosphorylation levels were measured by Western blot and the ratio phosphorylated/unphosphorylated protein calculated. Results are shown as means ± SEM of 4-8 independent experiments. * Significantly different respect to C (One Way ANOVA, $p < 0.05$)
*Significantly different respect to C, ** significantly different respect to Pal (One Way ANOVA, $p < 0.05$).

DISCUSSION

Metabolic syndrome (MetS) is becoming one of the major health problems in the adult world's population. MetS is diagnosed when three out of the five following risk factor are present: elevated waist circumference (according to population- and country-specific definitions); blood triglycerides ≥ 150 mg/dL; blood high density lipoproteins (HDL)-cholesterol ≤ 40 or 50 mg/dL in men and women, respectively; blood pressure ≥ 130/85 mmHg, and fasting glucose ≥ 100 mg/dL. In fact MetS is considered a highly complex multifactorial endocrine disorder, which shares not one, but several common underlying mechanisms, including fat accumulation, impaired insulin sensitivity and increased systemic inflammation. It has been demonstrated that obesity and insulin resistance are involved in the etiology of this syndrome and, in turn, they are related to each other. Solid evidences in animals and humans have shown that the link between obesity and insulin resistance can be an excessive release of free fatty acids (FFAs). Increased plasma concentration of FFAs leads to intracellular lipid accumulation in humans, suggesting that FFAs may play a critical role in initiating and developing insulin resistance. It has been reported that saturated FFAs (i.e. palmitate) but not monounsaturated FFAs (i.e oleate) induced insulin resistance in rat hepatocytes H4IIEC3 (Nakamura *et al*, 2009). We recently found (Bettaieb *et al*, FRBM in revision) that high fructose consumption induces hyperlipemia, oxidative stress and insulin resistance in rats which is mitigated by EC dietary supplementation. Thus, this study investigated if EC and ECM could improve insulin sensitivity through the regulation of palmitate-induced NADPH oxidase activation and consequent oxidative stress.

Our results showed that 0.25 mM Pal caused lipids accumulation in hepatocytes, NAFPH oxidase activation, oxidative stress, and activation of kinases (JNK, IKK) involved in insulin resistance.

JNK and IKK are stress-cytokines which once activated are able to promote insulin resistance though inhibition of IRS1 tyrosine phosphorylation through serine phosphorylation, and by induction of proinflammatory cytokines in different cell types, especially within macrophages. *In vivo* and *in vitro* studies demonstrate that activation of JNK and IKK appears to be sufficient to induce systemic insulin resistance. Among the factors promoting the development of insulin resistance, and consequently progression of MetS, oxidative stress seems to play an important role. In this regard, increased ROS production is one of the earliest events in cases of glucose intolerance, as it may cause pancreatic β-cell dysfunction as well as hepatic pathologies. An important source of ROS is the NOX family of NADPH oxidases. Several NOX isoforms are present in liver, but Gao *et al.* demonstrated that only NOX3 and its subunits (Rac, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$) are expressed in HepG2 cell line and in rat hepatic tissue. Overproduction of ROS has been proposed as a link between FFAs and hepatic insulin resistance (Nakamura *et al*., 2009; Gao *et al*, 2010). To confirm that hypothesis our results showed that HepG2 cells treated with Pal has a

significant overexpression of NOX3 both in protein and mRNA levels and also increases NADPH oxidase activity with an associated overproduction of ROS compared to untreated cells. Pal-mediated increase in NADPH oxidase activity was confirmed through the measurement of $p47^{phox}$ the translocation of NADPH subunit which is responsible of activation of NADPH oxidase complex. Moreover we demonstrated that NOX3-derived ROS may drive Pal-induced hepatic insulin resistance through up-regulation of JNK and IKK (Figure 8) respect to control. Many evidences suggest that NADPH oxidase, JNK and IKK pathways can be good targets to act on prevention of insulin resistance, then MetS and T2DM. Epidemiological studies have shown that flavonoids may have an important role in the regulation of oxidant production and proinflammatory signals; indeed have been demonstrated an inverse relationship between consumption of flavonoid-rich foods and pathologies with inflammatory components, like MetS. Among all the flavonoids known, (-)-epicatechin (EC) (family of flavanols) is one of the most abundant in humans diet, being high concentration in grapes, cocoa, tea and many others fruits and vegetables. It is believed that flavanols acts mostly by providing antioxidant protection by trapping radicals and chelating redox-active metals, but increasing body evidence support the participation of this compound in the regulation of cell signaling and enzyme activity. Our data shown that EC and also its metabolites (ECM) are able to decrease the expression and the mRNA level of NOX3 induced by Pal. Downregulation of NOX3 expression by EC is related to the concentration of this favanols, high concentrations of EC lead to a high decrease of Pal-induced NOX3 expression (Figure 5A). Since the important role of NADPH oxidase in the overproduction of ROS and is involves in the development of insulin resistance, it can be consider a good target for therapies or prevention of this condition. EC and ECM have been demonstrated to decrease the activity of NADPH oxidase (Figure 6A) in HepG2 cells in which was mimic a state of insulin resistance through Pal treatment. This activity regulation by EC and ECM is associated to their signaling regulating cytosolic $p47^{phox}$ subunit expression even if was not significant. Moreover EC and ECM decrease significantly the ROS production in these cells. As demonstrate and described previously, activation and increase of JNK and IKK expression induced by FFAs and NADPH oxidase play an important role in the development of insulin resistance; to contrast that our data suggest that EC and ECM can mitigate the expression of both JNK and IKK, through a dose-response action.

In summary out data showed that EC and ECM may play a fundamental role in the prevention of insulin resistance because of: their action on the modulation of hepatic NADPH oxidase, at a level of expression and activity, in conditions of hyperlipidemia *in vitro*; their direct action on the regulation of stress-cytokines JNK and IKK which are directly involved in the insulin resistance signaling pathway. In conclusion we can suggest that consumption of EC-rich foods could mitigate MetS-associated insulin resistance.

GENERAL CONCLUSIONS

Late onset Alzheimer's disease (LOAD), postmenopausal osteoporosis (PO) and metabolic syndrome (MetS) are becoming three of the most spread diseases in the world's adult population. Understanding the underlying pathogenic mechanisms of these disorders is a mandatory issue, because only after this step forward is taken it will be possible to find an effective care for these still incurable diseases. The solid link among AD, PO and MetS is the aging. The involvement of oxidative stress (OxS) in these age- and metabolic-related pathologies has been nicely demonstrated i*n vitro* and in animal models. In contrast, the data from human studies, manly those investigating AD and PO, are scarce and highly controversial. In our opinion, one of the most important reasons of these inconsistencies might be the lack of a gold standard marker for OxS determination *in vivo*. As consequence, currently, the only way to quantify OxS in body fluids such as serum, plasma and urine, is to measure (as we have done) a battery of different indirect markers, preferably reflecting lipid and/or protein oxidative damage as well as antioxidant content. Furthermore, the population-based studies on AD and PO evidence important differences (in composition or size of the sample, in exclusion and inclusion criteria etc.) that make very complicated any critical and systematic comparison among data.

Our findings derived from a large sample of patients affected by AD or PO suggested that systemic OxS is involved in the pathogenesis of these diseases. Indeed, a redox "imbalance" was associated with LOAD onset and with bone loss process affecting postmenopausal women.). Concerning MetS, data obtained *in vitro* on hepatic cells treated with free fatty acid, to mimic insulin resistance (then MetS), showed an oxidative stress condition through increasing of NADPH oxidase (which represents the main source of reactive oxygen species-ROS) activity with consequent overproduction of ROS.

A number of *in vivo* and *in vitro* evidence suggest that Mediterranean diet may has a role against OxS and related diseases. However, there are also several other studies that report altogether different results. Unfortunately, the ideal "mix" of antioxidants able to contrast effectively the action of ROS has not been discovered yet. However, our findings *in vitro* might represent an important step forward in this direction. Indeed we found that (-)-epicatechine is a potent inhibitor of NADPH oxidase activity, and, thus, it might be an important ingredient of a dietetic intervention against the deleterious effects of OxS on human health.

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