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Disturbance of redox and lipid homeostasis in Alzheimer's disease pathogenesis

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List of Abbreviations

4-HNE	4-hydroxynonenal
ACAT	acetyl coenzyme A cholesterol acyltransferase
AOPP	Advanced protein products
CNS	central nervous system
Cp	ceruloplasmin
CVD	Cariovascular diseases
EOA	Early Alzheime's disease
F2-iso	F2-isoprostanes,
FC	free cholesterol
FeOx	Ferroxidase activity
GPx	Glutathione peroxidase,
GSH	Glutathione
HCYS	Homocysteine
HDL	High dentsity lipoproteins
HY	Hydroperoxides
LDL	low density lipoproteins
LOAD	late Alzheimer's diseas
MCI	Mild cognitive Impairment
MDA	Malondialdehyde
MPO	Myeloperoxidase
NFT	neurofiblillary tangles
NOX	NADPH oxidase
OR	Odds Ratio
OxS	Oxidative stress,
RAP	residual antioxidant power
SOD	Superoxidodismutase,
VaD	Vascular dementia

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General introduction

The present thesis is divided in three main chapters.

Chapter I describes the current knowledge on the epidemiology and pathogenesis of the two most frequent forms of dementia, Alzheimer's disease and Vascular dementia.

Chapter II reports and discuss our data in support of a possible early and tight association between systemic oxidative stress and dementia-related diseases.

Chapter III describes our findings of an interplay between brain/systemic lipid metabolism and chronological/endocrine aging and discusses how a derangement of lipid homeostasis can be implicated in dementia pathogenesis.

CHAPTER I

Dementia-related disorders: epidemiology and pathogenesis

1.1. Introduction

Dementia is a term used to describe a chronic and progressive syndrome characterized by a severe impairment of cognitive functions. Demented patients show a decline in memory, orientation, learning, thinking, calculation and speaking skills that mainly affect emotional control, social behavior and motivation, and thus overall daily activities.

The syndrome can be divided in three different stages: *early stage* with feeble symptoms, often overlooked; *middle stage* with more evident signs such as becoming lost at home, having difficulty in communication, needing help for personal care; *late stage* with inactivity and total dependence of the patient to a caregiver.

Different diseases, which are all associated with brain degeneration, can cause dementia including:

- Alzheimer's disease (AD), the most common form after 60 years old and contributing to 60-70% of total cases of dementia. The etiopathogenesis of this neurodegenerative disorder are still unknown;
- Vascular Dementia (VaD), the second most common form of dementia (around 20% of cases), caused by low blood flow to the brain, often occurring in people with a history of strokes or cardiovascular disease (CVD).
- Dementia with Lewy bodies, a form of dementia caused by the formation of abnormal clumps of protein structure, called Lewy bodies, between brain cells. Symptoms are similar to AD and patients often have sleeping disorders.
- Frontotemporal disease (FTD), dementia which causes neuronal cells loss in frontotemporal lobes. It arises in 50-70 years old and causes impairment in speaking and understanding.
- Parkinson's disease (PD), in which dementia can occur in the late phase of the disease.

Worldwide, 47 million people suffer from dementia (5-8% of the population aged 60 and over), with 7.7 million of new cases every year (1). The projections estimate 75.6 million people with dementia in 2030 and 135.5 million in 2050. World Alzheimer's report 2015 (2) estimates that 58% of all people with dementia live in countries currently classified as low or middle-income countries and that the proportion of people with dementia living in these countries is estimated to increase much more than in high income countries, since rapid

rising in life expectancy and not adequate economic and human professional resources, Fig. 1.1.

Fig. 1.1: Estimated number of people living with dementia in each world region in 2015.
Reprint from World Alzheimer's report 2015 (2).

1.2. **Alzheimer's disease**

AD is the most common form of dementia, characterized by neurodegeneration leading to a progressive functional and behavioural decline. The fact that AD is strictly connected with many of physical and systemic conditions suggests that this the pathological traits of this disease are not solely confined in CNS, but also in periphery. It seems that brain damage starts 10-20 years before symptoms. AD is often preceded by a preclinical condition, defined as mild cognitive impairment (MCI), which is characterized by memory impairment but it is not severe enough to constitute dementia. Not all MCI patients develop the pathology, but they have higher risk for AD, since almost one half of these individuals evolve to AD with a rate of approximately 10-15% per year (3).

The pathology was first reported in 1906 by the German psychiatrist Alois Alzhiemer. He followed for five years the case of a 50-years old woman in Munich hospital with "untreatable paranoid symptomatology", "sleep disorders, disturbance of memory, aggressiveness, crying and progressive confusion". After the patient's death he investigated morphologically and histologically the brain and he described the presence of neurofibrillary tangles (NFT) and senile plaques (SPs), two distinct features that are still used today for AD diagnosis (4). In addition, AD patients have neuronal loss and astrocyte proliferation that

appears as cortical atrophy (temporal and frontal lobes), ventricular dilatation and reduction of hippocampus neuronal loss (Fig. 1.2). NFT and SP can be found also in normal aged people, but in fivefold lower quantity compared to AD.

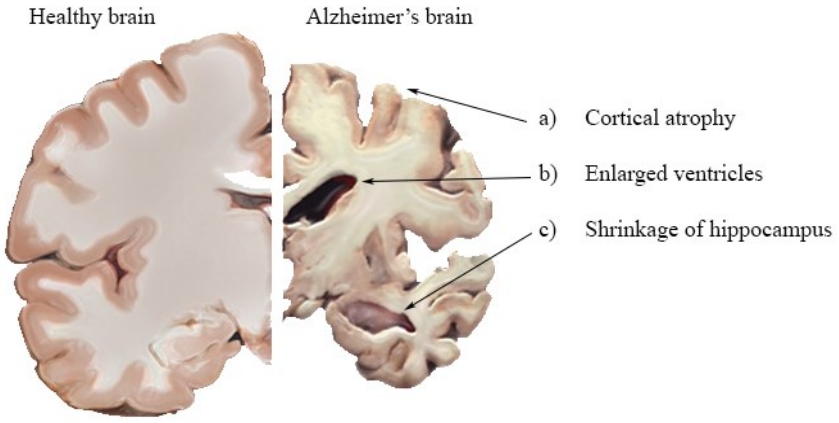

Fig. 1.2: Healthy and Alzheimer's brain.
The AD section (on right) compared to the healthy one shows cortical atrophy (a), enlarged ventricles (b) and shrinkage of hippocampus (c) caused by AD. Reprint from NIH publication (5).

Alzheimer's disease is subdivided in two forms:

- Early onset AD (EOA) with an important genetic component,
- Late-onset AD (LOAD) also known as sporadic that usually arises after 65 years and affects approximately 90% of AD patients.

EOA is associated with more pronounced language and cognitive deficits than LOAD. Moreover, evidences suggest different neurochemical characteristics with higher cholinergic deficits in temporal lobe and hippocampus in EOA as compared to LOAD.

To date, several missense mutations have been found associated to EOA, but not to LOAD. In particular, autosomal dominant mutation of three genes localized in chromosome 21, 14 and 1 coding amyloid precursor protein (APP); preselin 1 (PSEN1) and preselin 2 (PSEN2), respectively have been associated to EOA. APP is an integral membrane protein expressed in several tissue and it is constitutively released from cells following α-secretase cleavage. Aβ peptides derive from the cleavage of APP by β-secretase. APP biological function is still partially unclear, but separate evidence suggest that it could act as neurons grow regulator and/or synaptogenesis promoter(6). PSEN-1 is a subunit of γ-secretase complex and it is involved in cleavage of proteins, including APP. PSEN-2 is a probable catalytic subunit of γ-secretase complex and may have a role in intracellular signaling, activating gene for cells

growth and maturation. Evidences suggest a role of PSEN-2 in the conversion of APP into soluble APP peptide, promoting cells growth.

1.2.1. **Brain abnormalities in Alzheimer's disease**

1.2.1.1. *β-Amyloid*

Amyloid plaques in AD brain are formed by β-amyloid peptides generated from amyloid precursor protein (APP) cleavage (7).

APP underwent to proteolytic processes in several sites near to C-terminal end by α- , β- and γ- secretase. α-secretase enzymes, which belong to ADAM (A Disintegrin and Metalloproteinase) protease family, cleave APP 83 amino acid from C-end. On the contrary, β-secretase enzymes, consisting oftwo complexes named beta-amyloid precursor protein 1 and 2 (BACE1 and BACE2), cleave C-end of APP proteins, mainly generating C99 fragments. The γ-secretase activity is mediated by preselin-1 and -2 , which cuts APP in different sites: 50 amino acid from C-end generating amyloid intracellular domain (AICD) or in a variable site that usually occurs 57, 59 or 61 amino acid from C-end (7). These enzymes work in combination with different outcomes (Fig. 1.3).

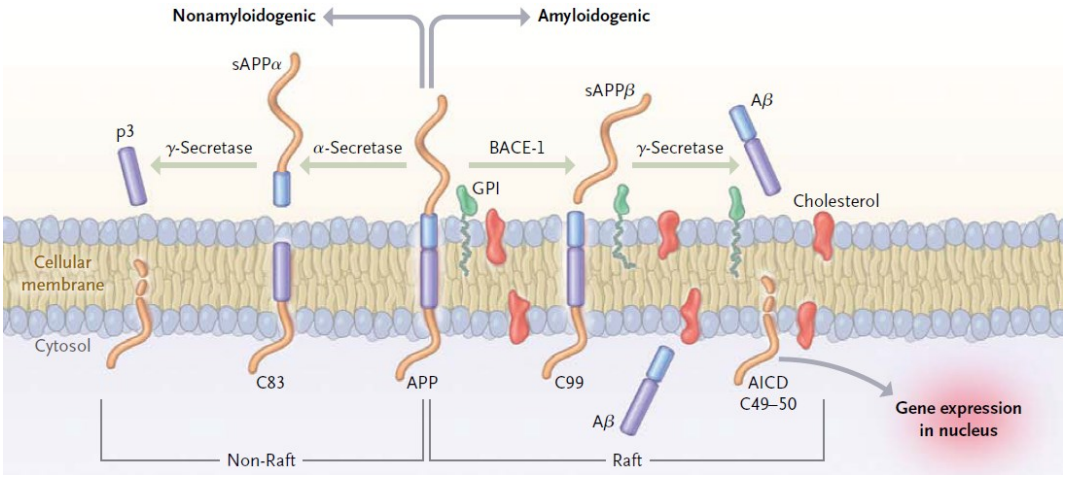

Fig. 1.3: APP processing.
Reprint from Querfurth et al., 2010 (8).

Sequential cleavage by α- and γ- secretase releases a N-terminal soluble APP (sAPPα) and a 3kDa peptide called P3 (non-amyloidgenic pathway). By contrast, proteolysis by β- and γ-secretase (amyloid pathway) produces a N-terminal soluble APP (sAPPβ) and Aβ peptide (38-43 amino acid) (Fig. 1.3) (7). Aβ peptides are a heterogeneous mixture produced by the

different γ-secretase sites of cleavages. Under physiological condition, Aβ should form a trimer-based expansion in order to prevent neuronal hyperactivity by reduction of excitatory transmission and its level is regulated by proteases neprilysin and insulin degrading enzyme. Diffuse Aβ peptides in non-fibrillar state are called "pre-amyloid" plaques and can degenerate to insoluble senile plaques. Fig. 1.4 (9,10). Intra- and extra- cellular soluble oligomers are the most neurotoxic forms of Aβ and their levels in the brain, correlate with AD cognitive impairment (3).

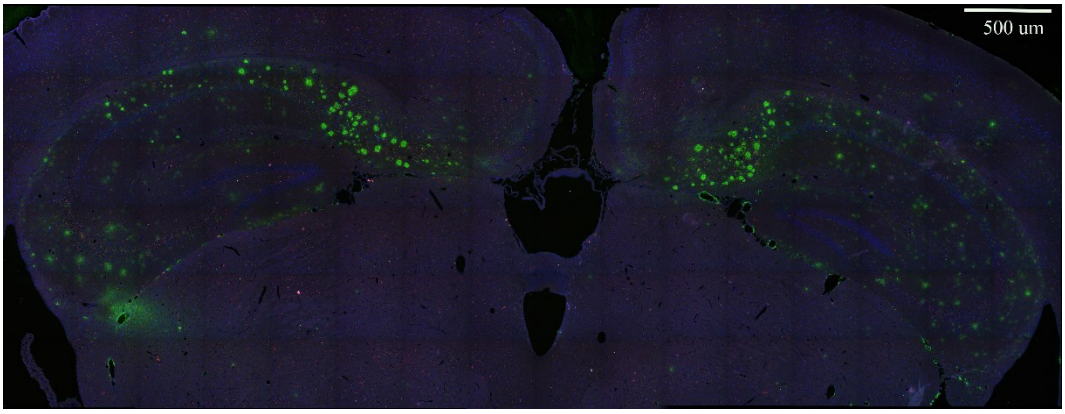

Fig. 1.4: Aβ immunostaining on 3xTg AD mice (hippocampal area).
Immunoreactivity was detected with 6E10 antibody (green) and nuclei counter-stained with DAPI (blue). Image was captured with Leica confocal microscope during exchanging period at USC, Los Angeles, CA.

1.2.1.2. *Tau*

Tau (τ) is a soluble axonal protein which allows the assembly and the stability of microtubules and vesicles transporters (3). Hyperphosphorilation that takes place during the pathological process causes a reduction in the affinity for microtubules and the hyperphosphorilated τ self-associate into pair helical filaments. The aggregation generates neurotoxic fibrils, called neurofibrillary tangles (NTF), (Fig. 1.5). Insoluble protein τ, present in the filaments, forms a complex with ubiquitin, but this complex is not efficiently degraded by proteasome (9). A wealth of experimental evidence showed that Aβ might drive the tau aggregation (3)

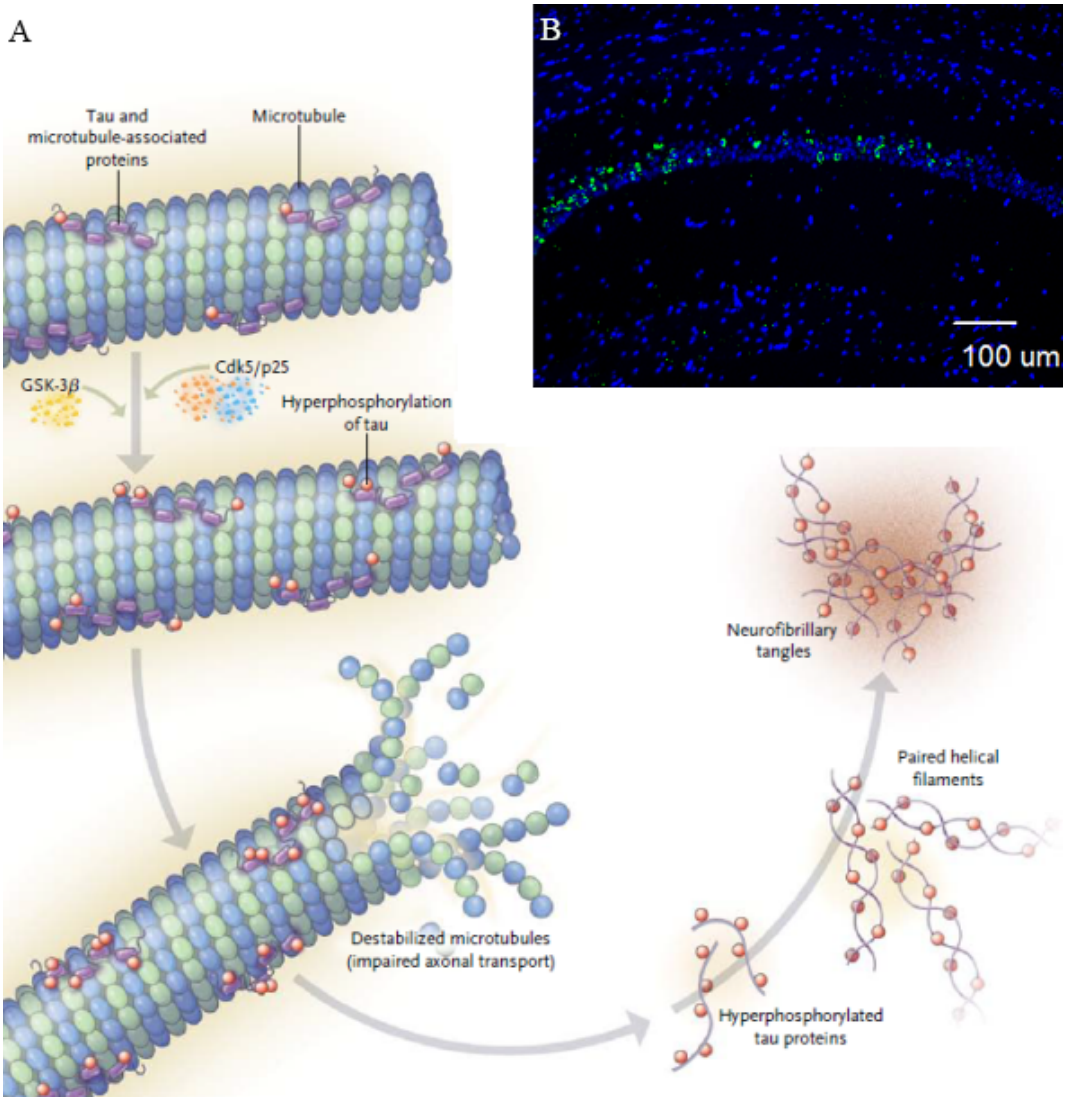

Fig. 1.5: NFT formation.
A)Normal and Pathological protein tau. Reprint from Querfurth et al., 2010 (8); B) τ immunostaining on 3xTg AD mice (CA1, hippocampal area). Immunoreactivity was detected with AT8 antibody (green) and nuclei counter-stained with DAPI (blue). Image was captured with Zeiss florescence microscope during exchanging period at USC, Los Angeles, CA.

1.2.1.3. *Mitochondrial impairment*

During physiological processes, mitochondria release free radical species, but the amount of these harmful substances significantly increases during AD. Mitochondrial impairment was observed in several patients tissues like brain, platelet and fibroblasts. The evaluation of mitochondrial function and structure showed a reduced activity of pyruvate dehydrogenase, alpha ketoglutarate dehydrogenase and cytochrome oxidase (7). A decrease in enzymatic activity causes an impairment of electron transport, ATP production, oxygen consumption and mitochondrial membrane potential which, in turn, leads to an enhancement of free oxygen radicals (ROS) production. The increase of free radicals promotes apoptosis via

cytochrome c release (8). Interestingly, there are epidemiological data showing a correlation between maternal AD status and offspring with AD and this connection seems to be due to mitochondrial DNA polymorphisms (7).

1.2.2. **Pathogenesis**

Despite years of intense research, a complete understanding of the pathogenic mechanisms underlying AD has not been achieved. Experimental and epidemiological data have resulted in the generation of several hypotheses regarding AD pathophysiology. Alois Alzheimer believed that the histopathological modifications (NFT and Aβ) observed were a consequence of an upstream dysregulation. Since then, evidence have been accumulated in support of Prof. Alzheimer's hypothesis, suggesting that mitochondria impairment, vascular disorders and oxidative stress might contribute to triggering theamylogenic process. By contrast, some data support amyloidosis in AD as primary event especially for early-onset AD with genetic components involved (7). The most commonly hold hypotheses on AD pathogenesis are the following:

- *Amyloid cascade hypothesis*: the accumulation of Aβ in the brain is the primary pathogenic event, which then triggers the entire array of subsequent brain lesions (23). This hypothesis seems to explain mostly the pathogenesis of early AD, that, however, is associated to less than 1% of AD;
- *Tau hypothesis*: τ hyperphosphorylation is considered the first event in AD;
- *Mitochondrial cascade hypothesis:* proposed by Swerdlow and Khan in 2004 (11), suggests that the basal ROS rate production from mitochondrial transport chain causes mitochondrial damages. Possible accumulation of mtDNA mutation becomes the primary cause of oxidative stress and subsequently energy failure along with amyloid beta formation can start a positive feedback cycle that ultimately leads to neuronal degeneration (12);
- *Vascular hypothesis:* AD develops first as a vascular disorder. Abnormal reduction of cerebral blood flow leads primarily to oxygen and nutrient deficiency and then to neuronal energy crisis that, in turn, promotes the cellular damages (13).

1.2.3. **Risk factors**

The greatest risk factor for dementia is aging, especially over 65 years old, followed by female gender. Moreover, several pathological conditions are associated with AD, including type II diabetes, hypercholesterolemia, hypertension or environmental factors such as smoke, diet and life-style. Another strong risk factor is familial history, with the likelihood of having the disease that considerably increases when more than one family members is affected by dementia. Finally, besides the genes mutations strictly linked to the early form of AD (APP, PSEN-1 and PSEN-2) there are also genes variants associated with LOAD risk, in particular the E4 isoform of apolipoprotein E.

1.2.3.1. *ApoE*

Apolipoprotein E (ApoE) is a component of the lipoproteins and it is expressed by hepatocytes. Hepatic ApoE does not cross the BBB (14), thus the isoform present in the brain, as HDL component, is synthesized in situ (astrocytes, microglia and "stressed" neurons). ApoE facilitates cholesterol and phospholipids mobilization in CNS by binding to low- density lipoprotein receptor family (15). In humans, ApoE gene is polymorphic for three alleles: ε2, ε3, and ε4, which code for three ApoE isoforms E2, E3 and E4, respectively, leading to six different phenotypes: E2/2, E3/3, E4/4, E2/3, E2/4, and E3/4. ε3 isoform is the most frequent in Caucasian population (65-70%), followed by ε4 (15-20%) and ε2, which is a rare isoform present only in 5-10% of subjects (14,16).

The isoforms differ from each other by amino acid substitution at residue 112 and 158. ApoE2 contains two cysteines, ApoE4 contains two arginines, instead ApoE3 contains a cysteine at position 112 and an arginine at residue 158 (15). Owing the fact that the receptor-binding site of ApoE is located between residue 136 and 158, the different isoforms mostly influence the affinity of lipoproteins to the LDL-receptor. Arginine at position 158 does not change the ability of E3 and E4 to bind ApoB and ApoE receptor, while E2 shows a significant reduction of affinity (<1%). On the other hand, substitution with arginine at position 112, reduces E4 binding with other sulfhydryl-containing proteins. As consequence, in periphery, E4 prefers to bind triglyceride rich lipoprotein (chylomicron an VLDL), resulting in enhancing receptor-mediated clearance of LDL, accumulation of hepatic cholesterol, down-regulation of LDL receptors, which, in turn, leads to elevated levels of circulating cholesterol (15,17).

In CNS, ApoE plays an important role in the delivery of cholesterol to neurons, promoting synaptogenesis and neuronal health. ApoE has also a pivotal role in the repair process after

neuronal injury. Brain lesions lead to ApoE up-regulation, in order to recycle membrane components from damaged cells and deliver cholesterol to neurons, helping in cells recovery (18). Moreover, it has been suggested that ApoE4 is associated with a "loss of function". In support of this hypothesis, the presence of the ε4 allele was found to correlate with a reduced number of dendritic spines in transgenic mice and AD patients (18). Several studies also showed how ApoE4 is associated with a more rapid impairment memory performance and an increased risk of memory decline in people affected by MCI (19). Thus, ApoE4 might be used to predict disease progression in combination with AD biomarkers and neuroimaging approaches.

1.2.3.2. *Homocysteines*

Homocysteines (HCYS) are intermediate products of physiologic biosynthesis of methionine and cysteine, from dietary methionine (from animal proteins), Fig. 1.6. HCYS are present in plasma in four different forms: 1% as free thiols; 70-80% disulphide bound plasma proteins (mainly albumin); dimer of HCYS or dimer with other thiols.

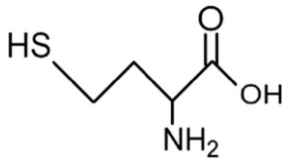

Fig. 1.6: Structure of homocysteine

HCYS levels in healthy human plasma is between 5.0 and 15.0 μmol/L, and increased levels can have adverse effect on vascular endothelium and smooth cells, most likely because of induced alteration of arterial structure and function (20). Indeed, hyperhomocysteinemia (above 15 μmol/L) is well known as risk factor for CVD and atherosclerosis. Furthermore, in the last decade, several studies have demonstrated that HCYS can mediate oxidative damage on neuronal cells (21).

1.3. **Vascular dementia**

Vascular dementia (VaD) is an heterogeneous group of disorders related to cerebrovascular disease (22). Nevertheless, several studies have revealed that a large proportion of dementia cases have mixed pathology, with both AD features and ischemic lesions (23).

Stroke doubles the risk for dementia and approximately 30% of patients with stroke develops dementia within 3 years. Owing to the pivotal role of cerebral blood supply for the structural and functional integrity of brain, it is not surprising the profound impact of cerebral blood vessels on cognitive function. Indeed, reduction of systemic perfusion caused by cardiac arrest, arrhythmias, cardiac failure or hypotension can produce impaired cognition transiently or permanently (23). Cerebral vascular pathology consists of atherosclerotic processes affecting small vessels, fibrotic change in the vessels wall resulting in thickening and microvascular distortion. Cortical microbleeds (MBs) are observed in 17% of demented patients; it is often associates to cerebral amyloid antipathy (CAA) which consists of Aβ deposit on small vessel walls of cortical and leptomeningeal arteries. MBs are small deposits of blood degradation products detectable on T2*-weighted magnetic resonance imaging (MRI). These pathological abnormalities are mainly found in VaD patients, but also, although less frequently, in AD (24).

Risk factors for VaD are largely shared with Alzheimer's disease. Aging is the mainly risk factor, especially after 65 years-old, followed by heart problems, atherosclerosis, hypertension, lifestyle, inflammation and genetic predisposition.

Genetic risk factors play an important role in VaD, mostly because of their association with large and small vessels-stroke. Familial CAAs, caused by mutations or duplication of APP gene can lead either to AD or to VaD. Moreover, Apo ε4 allele has been associated to CAAs, and both ApoE4 and E2 appear to be associated with increased lobar haemorrhages, even if a definitive link between ApoE polymorphisms and sporadic vascular cognition impairment has not been established yet (23).

1.4. **Diagnosis**

Dementia is not a specific disease, thus the diagnosis is very difficult to be drawn with absolute certainty. *Even more complicated is the diagnostic evaluation of AD, which can be made with 100% accuracy only post-mortem*.

In 1984, a task force established by the National Institute of Neurological and Communicative Disorders (NINCDS) and Stroke the Alzheimer's disease and Related Disorders Association (ADRDA) established clinical criteria for diagnosis of dementia-related disease (25). The criteria have been widely used in clinical trials and clinical research, showing a sensitivity of 80% and specificity of 70% for probable AD diagnosis.

However, 26 years later the vast expansion in the knowledge of dementia physiopathology has led to a considerable updating of these criteria. These revisited criteria took in consideration the recent data gathered in the field of genetic mutation, CSF/serum markers as well as neuroimaging investigation. Overall, the diagnostic approach presently adopted for AD, is based on a battery of different examinations that allow a "diagnosis of exclusion":

- *Medical history*: in order to establish a progressive deterioration and to identify abnormalities in the regular life, such as impaired memory, alteration mood, inability to find the way to a usual destination, inability to use money, difficulties in dressing, reading, and writing etc.
- *Clinical examination:* mainly mental status tests 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 and Hachinsky Scale (HS) for estimating the likelihood of multi-infarct dementia.
- *Neuropsycological testing:* there are no population standards for many of these test, so clinicians consider as abnormal the scores results in the lowest fifth percentile of an individual's normal control group (matched for sex, age and education). These tests can provide additional information for dementia diagnosis in addition to information regarding progression of disease or efficacy of drug's treatment.
- *Laboratory assessments*: to date, there are no specific diagnostic laboratory tests for different types of dementia, but there are laboratory and imaging technologies that can be helpful to exclude diseases different from AD. The main used techniques are as follows:

- Computerized tomography (CT), estimates tissue densities, ventricular size, CSF volume and brain mass. CT is useful for excluding other disorders such as vascular dementia or brain tumor;
- Positron emission tomography (PET) allows quantitative measurement of rate of glucose utilization, oxygen consumption and regional cerebral blood flow. It can recognize hypometabolic areas common in AD patients using the 2-[^{18}]Fluoro-2-deoxy-D-glucose (FDG). The rate of glucose utilization is expressed as mg/min or µmol/min/100g (Fig. 1.7) (26).
- Magnetic resonance imaging (MRI) reveals the separation of gray and white matter of the brain. It may discriminate AD from VaD and other causes of dementia linked to demyelinating disease like multiple sclerosis.

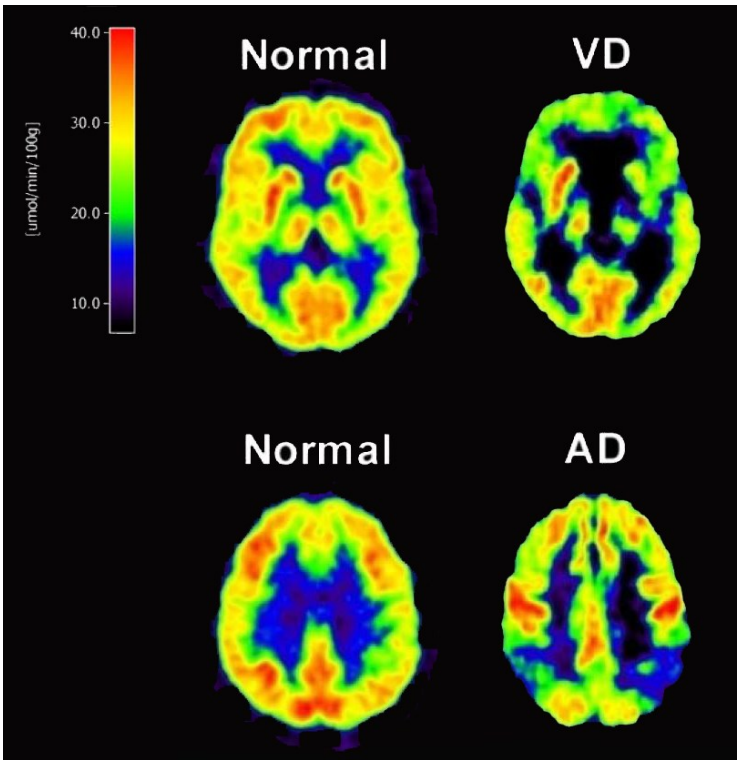

Fig. 1.7: Brain hypometabolism in dementia patients showed by 18F-FDG PET.
AD, Alzheimer's patients; VD, vascular dementia. Reprint from Jacobset al., 2005 (26).

- *Examination of body fluids*: to date, there are no specific peripheral body fluid markers for the diagnosis of AD. However, blood tests are useful in ruling out other factors that can contribute to dementia, for example: hypothyrodism and B12 deficiencies (the latter affects homocysteine metabolism) are associated with irreversible neurological damage (27,28). Moreover, several recent studies suggest

that CSF biomarkers may be used to identify prodromal AD. Indeed, CSF reflects brain amyloid β aggregation and hyperphoshorylation of tau protein. In addition, serum concentrations of Aβ42 may be predictive for AD in patients with MCI, but these measurements have not been officially included in clinical practice guidelines yet (28,29).

Based on different parameters considered above, clinicians classify AD into: probable, possible and definite.

Diagnosis of *probable* AD can be made when at least one of the cognitive domains is impaired and does not require the presence of other pathological biomarkers. Other elements in support of *probable* AD include progressive deterioration of language and visual recognition with impaired activities of daily living, family history, even with normal results on 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. *Definite* AD is assigned only after histopathological confirmation (biopsy or autopsy).

Similarly, VaD is classified into *probable, possible* and *definite* (30). The criteria for the diagnosis of *probable* VaD include decline in memory, impairment in daily activities concomitant with cerebrovascular diseases. A clinical diagnosis of *possible* VaD may be made in presence of dementia but not clear link with cerebrovascular diseases. The *definite* VaD diagnosis follows the same criteria of "*probable*" VaD, in addition to:

- histopathologic evidence of cardiovascular disease obtained post-mortem;
- absence of neuritic plaques and NFT exceeding expected for age
- absence of other clinical disorders capable to develop dementia.

1.5. **Therapy**

Currently, there is no cure for Alzheimer's, but drug and non-drug treatment helps in delaying the progression, and improving both cognitive and behavioural symptoms. Several research groups have been working on new treatment strategies. At present the most promising –modifying treatment seems to be based on a "cocktail" of medications addressed to several different targets. In particular, the ongoing pharmacological experimentations are focused on: modulators of beta-secretase activity, inhibitors of beta-amyloid plaques formation, antibodies against beta-amyloid. Moreover, research is also focusing on brain inflammatory processes, and estrogen related therapies.

No VaD-treatments have been approved yet, but it has been documented that some medications acting on risk factors could attenuate damages to brain's blood vessels and thus, the clinical progression of the diseases.

CHAPTER II

Redox disturbance in Alzheimer's disease

2.1. Introduction

Oxidative stress (OxS) is defined as an imbalance between oxidants and antioxidants, in favour of formers, which can lead to a biological damage (13). This definition implies the following fundamental concept: oxidants are physiological components of cell's life and become harmful only when overcome the cellular antioxidant defence system. OxS is an aging-related condition and is chiefly associated with some diseases such as cardiovascular diseases (CVD) and neurodegenerative disorders (31).

Brain is susceptible to OxS for its high lipid content, low antioxidant defenses in addition to high metabolic rate, and presence of transition metals, thus the preservation of a correct redox homeostasis seems to have a pivotal role in CNS heath.

2.1.1. Oxidant Species

Oxidants are species that subtract electrons from other molecules. They are mostly represented by free radicals, molecules with one or more unpaired elections in molecular or orbitals. The most harmful oxidants for biological systems are reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive aldehyde species (RAS) (32). All of these oxidants are physiologically products of normal cellular metabolism.

2.1.2. Reactive oxygen species (ROS)

Due to the high availability of oxygen in the organism, ROS (Table 2.1) are the most abundant oxidizing agents in the body.

Table 2.1: ROS species

Chemical species	***Formula***
Hydroxyl radical	$OH^{\cdot}$
Hydrohyl anion	OH^{-}
Single oxigen	$^{1}O_2$
Superoxide anion	$O_2^{\cdot -}$
Hydrogen Peroxide	H_2O_2
Hydroperoxide	ROOH

Superoxide anion is the first generated ROS after oxygen enters living cells. Thanks to the action of superoxide dismutase (SOD) this anion is converted in the more stable and less reactive hydrogen peroxide (H_2O_2). H_2O_2 can react with transition metals (i.e. iron and copper), to produce hydroxyl radical and hydroxyl anion through the Fenton reaction (reaction a) (33).

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\cdot}$ (reaction a)
$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot}$

Iron and copper can also catalyse the Haber-Weiss (reaction b) (34):

$O_2^{-} + H_2O_2 \rightarrow O_2 + HO^{\cdot} + OH^{-}$ (reazion b)

Furthermore, superoxide radical can enhance Fenton reaction, acting as reducing agent thereby regenerating the reducted form of metals (reaction c and d)

$Fe^{3+} + O2^{\cdot -} \rightarrow Fe^{2+} + O_2$ (reaction c)
$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\cdot}$ (reaction d)

From the sum of reaction "c" and "d" Haber-Weiss reaction (reaction "b") is obtained (35). Free radicals are unstable molecules able to react with all biomolecules by triggering an autocatalytic process, namely oxidative peroxidation, that starting from organic substrates (proteins, lipids, carbohydrates) leads to the formation of new reactive species.

The most "thriving", in term of generation of novel radicals, and deleterious process triggered by ROS is lipid peroxidation, which consists in three principal steps:

1. *Initiation*: ROS attack to organic compound generating a radical ($L^{\cdot}$);
2. *Propagation*: radicals formed react with molecular oxygen, producing the peroxiradical ($LOO^{\cdot}$) which is also an instable species, able to attack a new organic compound, enhancing the pro-oxidative cascade reaction;
3. *Termination*: the radical chain reaction stops when two radical species react with each other, producing non-radical species. This happens when the radicals concentration is high enough to increase the probability of collision. The chain reaction can also terminate with the intervention of antioxidants.

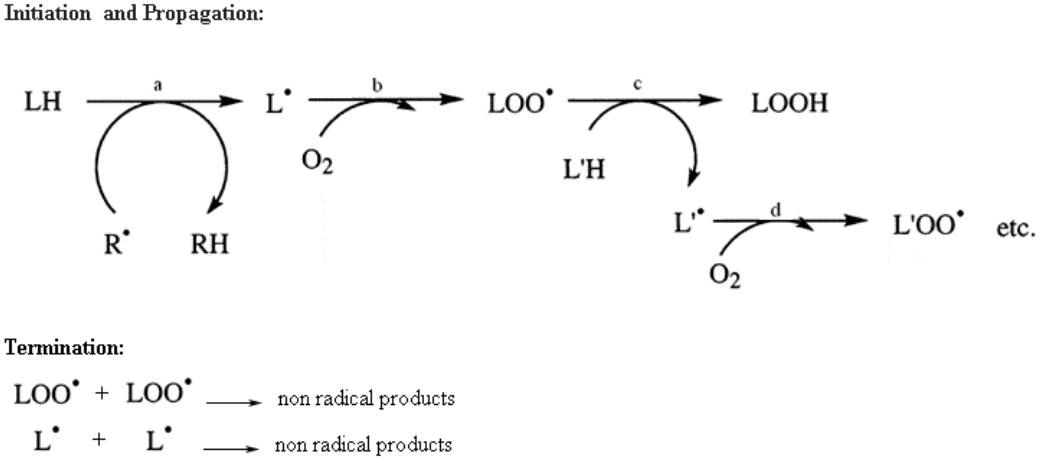

Fig. 2.1: Lipid Peroxidation.
Initiation and propagation: Radical (L·) is produced by ROS attack on organic compound (LH) (a), which react with oxygen to form an other radical (LOO·)(b) LOO· can react with a different organic compound (LOOH)(c) causing a propagation of radical chain reaction (d). Chain reaction stops (termination) for reaction of two radical compound

2.1.3. **Sources of oxidants**

Oxidants can be generated by exogenous or endogenous sources. Example of exogenous sources are represented by cigarette smoke, ultraviolet radiation, X-ray, air particles matters, etc., whereas the main endogenous sources are NADPH oxidase (NOX), xanthine oxidase (XO), and, in particular, mitochondrial respiratory chain.

NOX is a family of enzymes present in phagocytic and non-phagocytic cells. NOX-derived ROS play an important physiological role in response to stimulations of growth factors, cytokines, hormones and are implicated in many processes as endothelial dysfunction, inflammation, and apoptosis (36).

XO is the last enzyme involved in purine catabolism pathway. XO catalyses the conversion of hypoxanthine to uric acid by generating superoxide or hydrogen peroxide.

The main ROS source is represented by mitochondrial respiration chain (Fig. 2.2).

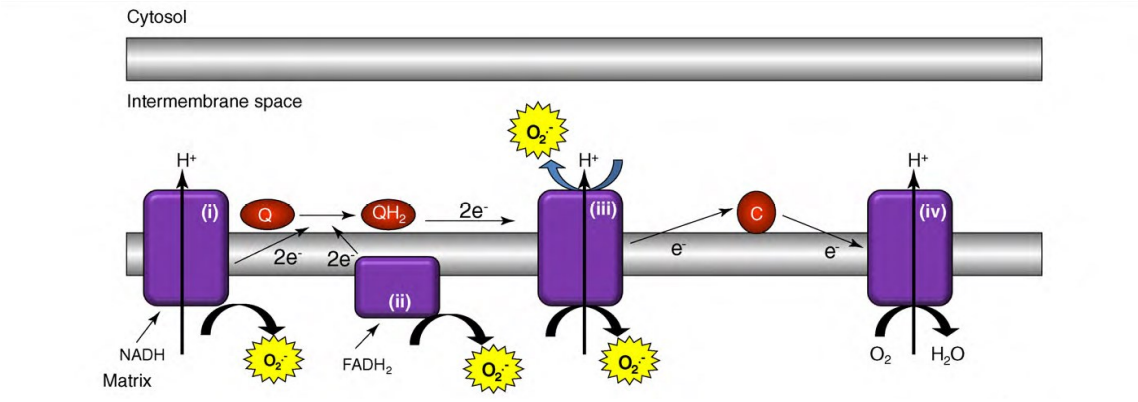

Fig. 2.2: ROS production in mitochondrial electron transport chain.
Mitochondrial complexes I and II use electrons donated from NADH and $FADH_2$ to reduce coenzyme Q, which shuttles these electrons to complex III, where they are transferred to cytochrome c. Finally, complex IV uses electrons from cytochrome c to reduce molecular oxygen to water. Complexes I, II, and III produce superoxide through the incomplete reduction of oxygen to superoxide. Reprint from Hamanaka et al, 2010; (37).

In vitro studies have estimated that mitochondrial superoxide production rate ranges between 0.15 and 2% of cellular oxygen consumption (38,39). ROS are produced by mitochondria by one-electron reduction of molecular oxygen (O_2), generating superoxide anion ($O_2^{\cdot-}$). Superoxide Dismutase (SOD) rapidly converts superoxide in the less reactive hydrogen peroxide, both within the mitochondria and in the cytosol. Complex III accepts two electrons donated to coenzyme Q by mitochondrial complexes I and II and transfers them to cytochrome c, but, cytochrome c can accept only one electron at the time. Thus complex III must sequentially remove each electron from reduced coenzyme Q (ubiquinol, QH_2)

resulting in the formation of the radical ubisemiquinone ($QH^{\cdot}$). Generally, the unpaired electron of ubisemiquinone is transferred to the cytochrome b of complex III; but it can also react with molecular oxygen to produce superoxide (39,40). Complex I and II produce ROS only into matrix, whereas complex III released ROS on both side of inner membrane (37).

2.1.4. **Antioxidants**

Antioxidant was defined as "any substances that delay, prevents or removes oxidative damage to a target molecule"; thus antioxidants are molecules able to inhibit the oxidation of other molecules, thereby interrupting oxidative chain reaction (41). Antioxidant defence are afforded by compounds of non-enzymatic as well as enzymatic nature.

Oxidative defence mechanisms involves preventive mechanisms; repair mechanisms; and scavenging actions (42). The preventive mechanisms are the first defence against free radicals, avoiding oxidants formation. Metal-binding proteins such as *ferritin*, *transferrin* and *ceruloplasmin*, are examples in this frame, since prevent the potential radical-generating reactions triggered by transition metals. However, when oxidants rate increases, cellular damage raises too, activating a multiple enzyme system involved in DNA repair (43).

Scavenging action is performed by enzymatic and non-enzymatic mechanisms. Non-enzymatic antioxidant consists on chain-braking antioxidants as *α-tocoferol* and *glutathione*. They are efficient in presence of radicals with a lifetime not too short as peroxyradical (lifetime around seconds) (44). Moreover, the three major scavenging antioxidant enzymes are SOD, catalase (CAT) and glutathione peroxidase (GPx). SOD represents the most effective cell's antioxidant, since catalyses the dismutation of superoxide into oxygen and the low reactive hydrogen peroxide, which, in turn, can be neutralized through other enzymes, such as CAT or GPx, but also converted into hydroxyl radical ($^{\cdot}OH$), through the metal-catalysed Fenton reaction (Fig. 2.3) (13,44).

Some other of antioxidant systems will be discuss in details in the following sections.

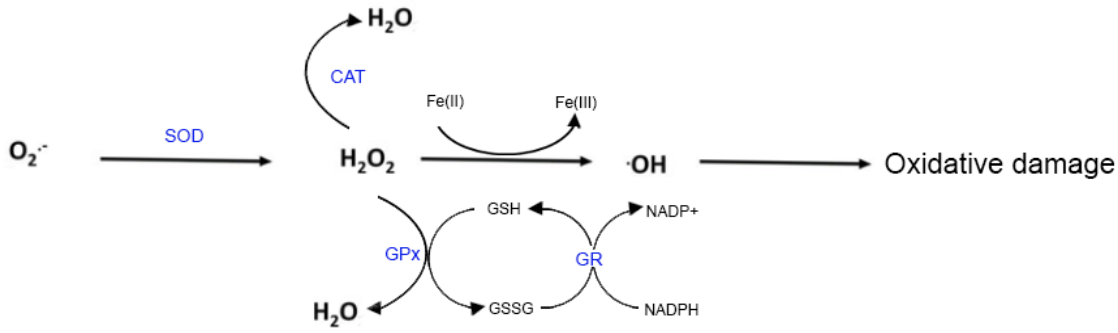

Fig. 2.3: Main enzymatic antioxidant mechanisms.
Superoxide dismutase (SOD) catalyze the conversion of superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2), which can be converted to water by catalase (CAT) or Glutathione peroxidase (GPx). Reduction of H_2O_2 by GPx causes oxidation of glutathione (GSH: reduced formed, GSSG: oxidized form). Glutathione can be regenerated by glutathione reductase (GR)that uses NADPH as cofactor. In case of deficient antioxidant mechanisms, H_2O_2 can be trasfromed into hydroxyl radical (·OH) via Fenton reaction, starting oxidative chain reaction and oxidative damage. Reactions are not stechiometrically balanced.

2.1.4.1. *Thiols*

Thiols act as antioxidants by directly scavenging free radicals and by acting as cofactors enzymatic reactions.

The most important thiols are represented by *glutathione* (GSH). GSH (Fig. 2.4) is a tripeptide, γ-L-glutamyl-L-cysteinylglycine, present in all mammalian tissues at 1-10 mM concentrations (highest concentration in liver).

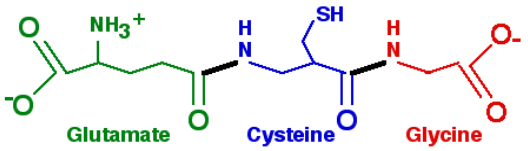

Fig. 2.4: Chemical structure of Glutathione

GSH mainly exists in the reduced form (80-85%), but it is also present as disulphide-oxidized forms (GSSG). Antioxidant functions of thiols are exerted by : (I) participating as substrate in reactions ctatlyzed by transferases, peroxidases and other antioxidant enzymes as GPx or Gluthatione tranferase; (II) acting as in amino acid transporter through the membrane; (III) scavenging hydroxyl radical and single oxygen (42).

Plasma GSH originates from the liver and plays a pivotal role in the inter-organ homeostasis of this compound, thus the dysregulation of hepatic GSH synthesis has impact on systemica GSH homeostasis (45). However, the major plasma thiols are represented by Cys-34 of

albumin (46). About one-third of the plasma albumin carries disulphide-bonded thiols at this Cys-34 residue, while the remaining part exists as thiolate anion (thiol group of Cys-34; pKa = 5), which is highly reactive with metals, thiols, and disulphides (47).

2.1.4.2. *Uric acid*

Uric acid is one of the most abundant antioxidants in human blood and acts as free radical scavanger and chelator of transitional metal ions, thereby providing above 60% of the total antioxidant capacity. Uric acid reacts with peroxynitrite, peroxide and hypoclorous acid but it cannot scavenge superoxide. This compound is produced from exogenous or endogenous purines in liver, muscles and intestine, by xanthine oxidase (XO) and xanthine dehydrogenase activity (XDH), Fig. 2.5. In physiological condition, XDH is predominant over XO in the synthesis of uric acid. On the contrary, stress conditions, in primis hypoxia, leads to the activation of XO, which, using oxygen instead of NAD as cofactor, brings about the inevitable production of superoxide anion and other reactive oxygen species. Thus, high concentration of uric acid might also exert, although indirectly, proxidant effects as proved by the documented association between elevated levels of this compound and CVD risk. Normal serum uric acid levels is considered between 6.0 and 6.5 mg/dL, instead hyperuricemia has defined above 7.0mg/dL in men and 6.5mg/dL in women.

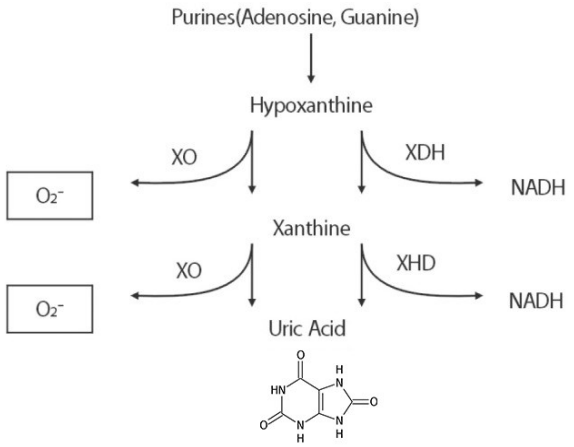

Fig. 2.5: Production and metabolism of uric acid.
XO; xanthine oxidase, XDH; xanthine dehydrogenase, NAD; nicotinamice-adenine dinucleotide, O_2; superoxide.

2.1.4.3. *Ferroxidase activity*

Keeping free Fe^{2+} levels low in blood is a very effective way to contrast the generation of ROS. This preventive action is prevalently afforded by the ferroxidase (FeOx) enzyme activity of Cp (FeOx I) and, to a less extent (10% – 20% of the tota l serum FeOx), by a second copper-containing non-ceruloplasmin iron oxidase, FeOx II (48). Cp is a multi-functional copper carrier of hepatic origin, which plays a prominent role in iron metabolism. Indeed, this protein catalyses the oxidation of Fe 2+ to Fe 3+ and promotes iron loading into apo-transferrin (Fig. 2.6), thus facilitating the efflux of the metal ion from the liver to the other organs and tissues (49).

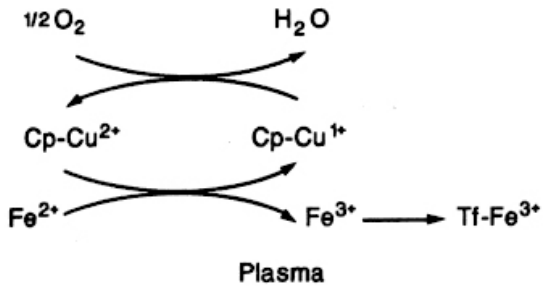

Fig. 2.6: Role of ceruloplasmin as a plasma ferroxidase.
Cp, ceruloplasmin; Tf, transferrin

2.1.4.4. *Paraoxonase*

Paroxonases family includes three enzymes: PON-1, PON-2 and PON-3, expressed by three different genes located on chromosome 7. PON-2 is ubiquitously expressed as intracellular membrane bound enzyme, PON-1 and PON-3 are expressed in the liver. Both PON-1 and -3 are bound to circulatory (50). All PONs possess antioxidant proprieties based on three different enzymatic activities: arylesterase (hydrolysis of non-phosphorous arylesters), lactonase activity (hydrolisis of peroxides and lactones) and paraoxonase (hydrolysis of organophosphates and pesticides). Importantly, paraoxonase activity is exclusively exerted by PON-1 , Table 2.2 (51).

Table 2.2: Activities carried out by the three PON enzymes

	Paraoxonase	Arylesterase	Lactonase
PON-1	+++	+++	++
PON-2	-	+	+++
PON-3	-	+	++

Human paraoxonase-1 (PON-1), the most studied PON, is a serum calcium dependent esterase with molecular weight of 43–45 kDa. PON-1 exerts multiple functions such as antioxidant (52), anti-inflammatory and antiatherosclerotic (53,54). PON-1 has also been demonstrated to stimulate HDL-dependent nitric oxide (NO) production by endothelial nitric oxide synthase.

PON-1 is usually named paraoxonase/aryleserase, but lactonase seems to be the real physiological activity. Natural substrates of lactonase are lactones derived from food hepatic catabolism, drugs metabolism and fatty acid oxidation (55). The bond of PON-1 to both lipid and protein moiety of HDL is essential for its stability and catalytic activity.

Scientific community agrees about the concept that PON-1 protects HDL and low-density lipoprotein (LDL) from oxidation and destroys the biologically active oxidized lipids on lipoproteins and in arterial cells (56). Accordingly, dysregulation of serum PON-1 activity has been associated with several human diseases such as CVD, diabetes mellitus, cancer, obesity, metabolic syndrome, and neurological disorders (52,57). Huang et al (53) showed that PON-1 inhibits initiation of lipid peroxidation by forming functional ternary complex with HDL and myeloperoxidase (MPO), a pro-oxidant enzyme linked to development of atherosclerosis. Each member of the ternary complex was shown to affect the function of the others *in vivo*. Furthermore, some studies have proposed that the lactonase activity of PON-1 may contribute to its antioxidant activity by hydrolyzing specific lipid peroxidation products (58,59). Thus, impaired PON-1 activities may increase the risk related to oxidative damage (48).

Several PON-1 polymorphisms have been identified and some of these are known to affect its activity. One of the most studied are Q192R, a coding region polymorphism that changes the codon CAA with CGA of exon 6 causing substitution of glutamine (Q) with arginine (R). The two alloenzyme have different catalytic activity, in particular R192 catalyzed paraoxon hydrolysis six times faster than Q192. Moreover R192 hydrolyzes faster thiolactones and γ-butyrolactone than the other alloenzyme does, while no differences were identified for other substrates as phenylacetate (60). Furthermore, some studies indicated that the paraoxon-hydrolysing activity of QQ/QR and RR alloenzymes is activated by high concentration of NaCl (1M) at different rate.These distinct behaviors are currently used as a useful tool for identifying PON-1 phenotypes (61). In fact, salt-stimulated paraoxonase/arylesterase activity ratio (we referred as "Ratio" for matter of simplicity)

exhibits a three modal frequencies distribution which reflects genotype distribution (60–62): low Ratio,QQ; intermediate Ratio, QR; high Ratio (the least frequent), RR.

2.2. **Physiological and pathological effects of ROS in the living systems**

The well-acknowledged fact that redox processes are part of cell's homeostasis implies that some radicals such as nitric oxide or superoxide anion play an important role in many regulatory processes (42,63). These radicals, indeed, can act as secondary massagers to regulate physiological responses as vascular smooth relaxation, respiratory ventilation and erythropoietin production. The main pathway responsive to redox regulation involves JNK/ p38 MAPK/AP-1 and NFkB (63). JNK, firstly identified as stress-activated protein kinase, phosphorylates the DNA binding protein c-Jun which, in turn, is part of the AP-1 transcription complex, a major regulator of many genes. A wide range of MAP kinases are implicated in pathways activation, leading to various outcomes. However, generally, JNK are implied in controlling cell death or apoptosis (64).

Furthermore, excess of ROS can modify genes transcription by NF-*k*B activation. Indeed even a mild pro-oxidative shift in glutathione redox state promotes NF-*k*B activation via post-translational modifications of I*k*B and IKKα.

A further well-known redox-related signalling pathway is represented by oxidation of cysteine as shown in Fig. 2.7. Oxidation of sulfhydryl groups of proteins can alter their catalytic activity and the interaction between proteins and DNA (37).

In contrast, the overproduction of oxidants leads to negative effects on cell's life. Aging, and several diseases like diabetes mellitus, CVD and neurodegenerative diseases are associated with a derangement of oxidative balance. Over-stimulation of NAD(P)H oxidase by different cytokines, dysregulation of xanthine oxidase and mitochondrial respiration chain can be possible causes of OxS onset, Fig. 2.8 (63,65).

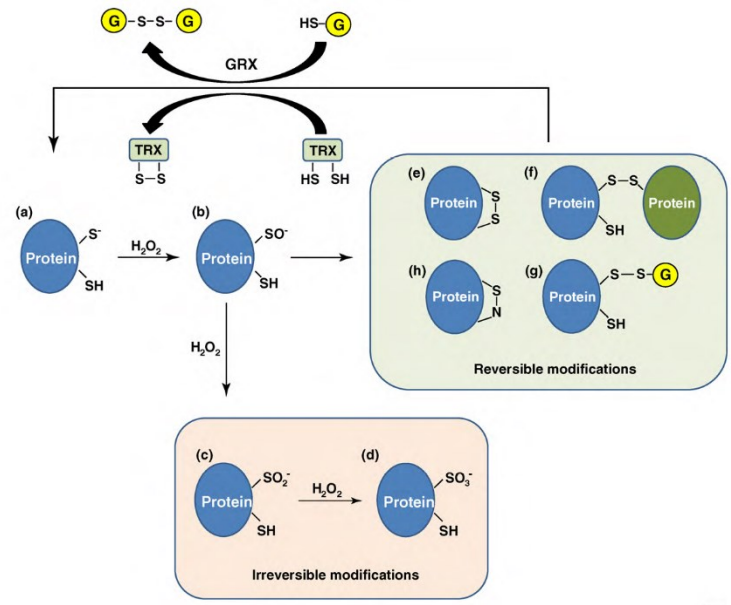

Fig. 2.7: Regulation of phosphatases through cysteine residue oxidation.
The catalytic cysteine of phosphatases can exist as a thiolate anion (a). Hydrogen peroxide (H_2O_2) oxidizes thiolate (b) inhibiting enzyme activity. Under high concentrations of H_2O_2, SO^- can undergo further oxidation to generate (c) sulfinic (SO_2^-) and (d) sulfonic (SO_3^-)acids (irreversible oxidative modifications, preventing reactivation of the phosphatase). In order to prevent irreversible oxidation of catalytic cysteine, SO^- can incorporate into a disulfide (S–S) bond with inter or intra-molecular cysteine (f); or into a sulfenic–amide (S-N. Then glutathione reductase or thioredoxin restores the oxidized enzymes back to their reduced state. Reprint from Hamanaka et al, 2010 (37).

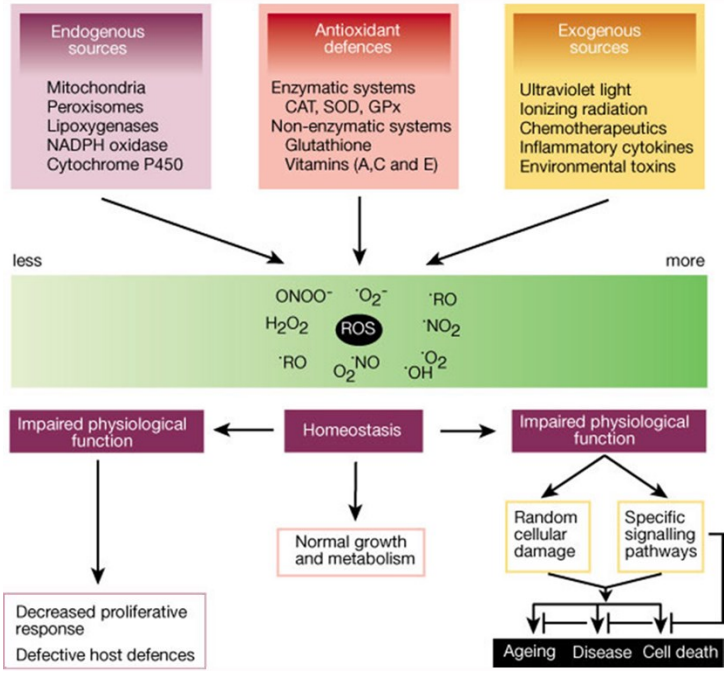

Fig. 2.8: Phisio-pathologic effects of reactive oxygen species (ROS) in living organisms.
Reprint from Finkel et al, 2000 (66).

2.3. **Biomarkers of Oxidative stress**

Biomarkers are defined as "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathological processes or pharmacological responses to a therapeutic intervention" (67).

At present, the gold-standard marker for OxS in vivo assessment is not available. High reactivity and short half-life of reactive species do not allow a direct measurement *in vivo*, consequently the measurement of OxS are made by the measurement of more stable by-products derived from oxidative bimolecular damage.

Detection of high levels of OxS in the blood/urine of patients with a given disease (eg, AD) may not be the definitive evidence of the implication of reactive species in its pathogenesis, since it might reflect the presence of concomitant diseases (eg, CVD, diabetes, and liver diseases) or subclinical pathological conditions associated to low-grade inflammation (eg, atherosclerosis and arthritis). Moreover, some large epidemiological studies have identified a number of demographic and physical lifestyles and dietary factors that affect the systemic redox balance, even in the absence of a full-blown disease. The most common examples of these factors are older age, gender, race, as well as overall and central obesity, smoking, alcohol abuse, and nutrition. The failure to identify these factors (through anamnesis, detection of disease markers, assessment of anthropometrics, etc.) or an underestimation of the impact of these factors in data analysis could considerably affect the results (and their reproducibility). In light of these consideration, the development and application of various potential OxS markers in biological fluid are currently evolving research area (13).

Traditionally, OxS markers reflect ROS/RAS/RNS attack on polyinsatured fatty acids, proteins and DNA.

2.3.1. **Markers of lipid peroxidation**

In biological systems, the most favourable substrate for peroxidation is represented by polyinsatured fatty acids (PUFA), components of cellular and subcellular membranes. The main primary products of lipid peroxidation are lipid hydroperoxides (HY), while secondary products include *malondialdehyde* (MDA), *4-hydroxynonenal* (4-HNE) and *F2-isoprostanes* (F2-iso).

HY are prominent non-radical intermediates of the oxidative modification of all unsaturated phospholipids and glycolipids, cholesterol, amino acids and carbohydrates (68). ROOH o

HY are primarily formed during the propagation phase in the lipid peroxidation cascade (Fig. 2.1) (42). HY reach high concentrations in the blood, and monitoring them does not require highly sensitive analytical procedures. The most commonly used assays for these determinations are based on the spectrophotometric assessment of the products formed through reactions between a chromogenic substrate and HY. Although positive features (low cost, simplicity, etc.) of these methods, none of them afford a highly specific *in vivo* assessment of these lipid peroxidation by-products.

The most studied index of lipid peroxidation in blood and urine is thiobarbituric acid-reactive substances (TBARS), extensively used because of the simplicity of their assessment (68). The TBARS method has been frequently employed to measure MDA, but this simple assay lacks specificity and precision (13,69), thus readable MDA levels should be assessed by mass spectrometry (MS) and techniques of chromatographic separation (70).

MDA appears to be the most mutagenic product of lipid peroxidation whereas 4-HNE is the most toxic for its fast reactions with thiols and amino groups. Reactive aldehydes, especially 4-HNE, act both as signalling molecules and as cytotoxic products of lipid peroxidation causing long-lasting biological consequences, in particular by covalent modification of macromolecules (71). 4-HNE, derived from the oxidation of ω-6 fatty acid and efficiently binds covalently to proteins, peptides, nucleic acids, etc. (72). At present, gas chromatography (GC)-MS is the highest sensitivity methods to analyse 4-HNE adducts compared with other quantification techniques, such as western blotting and high-pressure liquid chromatography (HPLC) and ELISA (13,68).

Other lipid peroxidation markers are F2-iso, a family of prostaglandin F2α compounds generated *in vivo* by non-enzymatic ROS-catalysed peroxidation of esterified arachidonic acid (68,73,74). F2-iso and other isomers (derived from different PUFAs), are considered as the most reliable serum and urinary markers of lipid peroxidation (75). The specificity and sensitivity is strictly dependent on the method used 67 68. In this regard, the most reliable analytical method (but also the least applicable in clinical routine) is GC/MS, while the easiest and most accessible method (although less accurate and occasionally misleading) is an ELISA approach (68,76).

2.3.2. **Markers of protein and nucleic acid oxidation**

Protein abundance in biological systems makes them a sensitive target of oxidative damage (13,68).The primary markers derived from the oxidation of these macromolecules are *carbonyls*, *nitrotyrosine* and *advanced protein products* (AOPP) (77,78). Among these, carbonyls are now considered the most reliable markers, but only when they are measured through MS or HPLC (13,44,79). Simpler, but less reliable, spectrophotometric or ELISA-based assays are also now available for large-scale determination (80). Conversely, 3-nitrotyrosine (formed from a reaction with peroxynitrite or nitrogen dioxide) is widely considered as a promising early marker of protein oxidation in AD-related neurodegenerative processes. It is extremely difficult to measure in serum or urine because of the high instability of this compound in these fluids (7,13). Instead, AOPP is easily detectable in serum and urine. AOPP are mainly derived from albumin-modification, but also fibrinogen and lipoproteins. Approximately 50 % of AOPP are formed by MPO-dependent activities, thus hypochlorous acid, primarily derived by MPO activity may represent one of pathways for AOPP production (81). Small quantity of oxidized proteins are produced physiologically, but the production rate increases with aging and with pathological condition (82).

Oxidative injury of DNA and RNA causes modification of bases, for this reason the most used markers are modification of guanosine of DNA (*hydroxy-2'-deoxyguanosine*, acronym: 8-OHdG) and RNA (*hydroxy-2'-guanosine*, acronym: 8-OHG) (73), which can be accurately measured through ELISA (particularly in urine, where these molecules are more stable compared with other fluids), although MS technology is considered the gold standard. Permanent modification of genetic material resulting from these oxidative damages may represent the first step of carcinogenesis and ageing (13).

2.3.3. **Involvement of oxidative stress dementia**

Brain is the most metabolically active organ of the body with the highest consumption rate of oxygen and glucose (20% and 60% respectively of total uptake in the resting state). This characteristic in combination with others such as low antioxidants defence, high PUFA and metals concentration, makes the brain tissue extremely sensitive to modification of redox balance. Mitochondrial hypothesis suggests that mitochondrial dysfunction could be the primary cause of AD onset (83,84). Experiments on transgenic mice support the concept that

oxidative burden temporally precedes the deposition of Aβ (79). The precocity of oxidative damage in AD development are supported by the finding of oxidatively modified lipids and proteins in MCIs (84,85). According with evidence OxS could precede and contribute to the formation of Aβ and NFT. On the other hand, it has been proposed that oxidative-related damage to the brain of late onset AD patients might also be the effect of Aβ aggregates, mitochondrial dysfunction, neuro-inflammation and other disease hallmarks (Fig. 2.9).

Independently of the classification of OxS as downstream or upstream pathogenic event, the experimental evidence collected so far suggest that this condition is intimately involved in AD physiopathology. Oxidative stress can compromise the BBB integrity directly or indirectly, by activation of metalloproteinases, as reported in human post-mortem analysis (86). As a consequence of an alteration in BBB permeability, Aβ clearance from the brain might be delayed, and Aβ influx from cerebrospinal fluid (CSF) might be increased. The factors that contribute to elevated ROS levels most likely include aging, genetic factors, environmental exposure to contaminants (eg, pesticides), metal dyshomeostasis, etc.(87–89). This deleterious increase in reactive species involves impaired neuronal mitochondria, which have been consistently observed as an early and prominent feature of AD (7,11). Mitochondrial dysfunction could reflect reduced energetic substrates or oxygen delivery to the highly active neurons following chronic brain hypoperfusion (90) or the defective expression of glucose receptors in the BBB. The ensuing reduction of ATP synthesis is the precursor of a biochemical cascade (altered protein and lipid synthesis, signal transduction breakdown, etc), eventually leading to OxS (32) and cellular hypometabolism, with the latter preceding any clinical manifestations of AD (13). Excess ROS generation might also influence the clinical progression of AD. Indeed, it has been suggested that these noxious species mediate the neurotoxicity of the Aβ soluble oligomers (91) and hyperphosphorylated τ (92). Specifically, experimental data have shown that Aβ oligomers, (mainly Aβ1-42 isoform), generate H_2O_2 by copper-dependent SOD-like activity (42,91,93). Copper present in high levels in amyloid plaques reacts with hydrogen peroxide to form highly reactive hydroxyl free radicals, which may subsequently activate NOX, increasing superoxide anion production.

Furthermore, aberrant oligomers could also induce the release of pro-inflammatory and pro-oxidant cytokines from astrocytes and microglia and impair mitochondria respiratory chain complex which in turn increases ROS production by alterating activity of enzyme, like Aβ-

binding alcohol dehydrogenase (13,94), or impairing the activity of respiratory chain complexes (eg, cytochromeoxidase) (94–96).

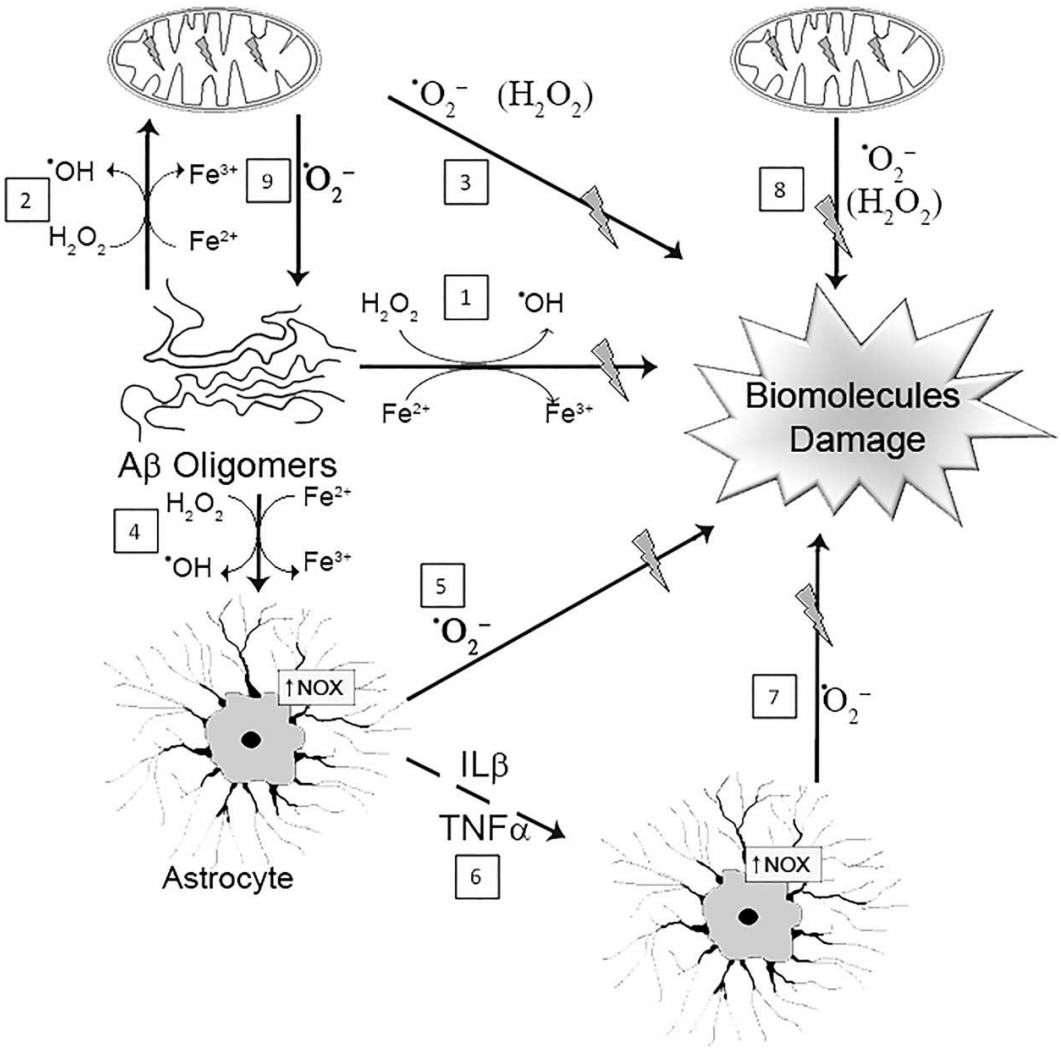

Fig. 2.9: Sources of ROS in the Alzheimer's disease brain.
Soluble amyloid-β (Aβ) oligomers and mitochondrial dysfunction are the main sources of ROS and related biomolecular damage in the brain tissue of patients affected by AD. Once formed, the Aβ oligomers are able to generate oxygen peroxide (H_2O_2), which acts with the reduced transition metals ($Fe2^+$ or $Cu^{+)}$ and produces hydroxyl radicals (˙OH). In turn, this noxious radical is able to: directly damage biomolecules (1); cause damage to neuronal mitochondria (2) and, consequently, a large leak of superoxide anion (O^-) and oxygen peroxide from the respiratory chain (3); directly activate microglial NOX (NADPH oxidase) (4) (and promote production of superoxide anion (5)) and/or induce the release of pro-inflammatory cytokines from astrocytes and microglia (6), which in turn can promote the release of ROS from other glial cells (7). Mitochondrial dysfunction, and consequent emanation of ROS from the organelle to cellular constituents (8), may also occur prior to Aβ oligomer accumulation (and be one of the causes of their formation (9)) (IL, interleukin; TNF, tumour necrosis factor).Reprint from Cervellati et al., 2015 (13).

Converging evidence suggests that OxS could be also associated with the second most frequent form of dementia: VAD. In this pathology, ROS could originate from vascular abnormalities and associated consequences, such as endothelial dysfunction, brain hypoperfusion etc., that strictly characterize VAD. Indeed, vascular endothelium is able to synthesize, store, and release free radicals in response to stimuli such as injury and hypoxia/hypoperfusion (97). Vascular OxS is also implicated in the onset of several well-recognized risk factors for VAD including diabetes, stroke, atherosclerosis, which reduce the cerebral circulation. In addition to hypoperfusion, a critical consequence of endothelial dysfunction is represented by the increased BBB permeability, which leads to extravasation of plasma proteins and production of radicals and pro-inflammatory cytokines as well as MMPs from activated microglia, and astrocytes (23). In turn, the activation of glial and endothelial cells is accompanied by the up-regulation of NOXs and thus a further increase in ROS production.(23).

It is fair to underscore that, despite the intriguing evidence described above, a connection between dementia and OxS has not been definitely proven yet. This "lack of certainty" is mainly due the scarcity of epidemiological-clinical data on the topic (97).

2.4. **Objectives**

Growing *in vitro* and animal evidence suggests a crucial role of oxidative stress (OxS) in the neurodegenerative process occurring in late Alzheimer's disease (LOAD) and Vascular Dementia (VAD). However, thus far, *in vivo* studies conducted on living humans failed to definitely prove the implication of oxidative mechanisms in the onset and progression of these two major forms of dementia. In the attempt to address this issue we evaluated a pattern of multiple peripheral markers of OxS in large sample including subjects with mild cognitive impairment (MCI), LOAD, VaD and cognitively healthy controls.

Furthermore, we longitudinally followed-up a subgroup of MCI in order to investigate whether baseline serum level of oxidants and/or serum antioxidants might be predictive of the clinical progression from MCI to LOAD.

2.5. Methods

2.5.1. Design of the study

Six hundred ninety-eight patients referred to the Day Service for Cognitive Decline (University of Ferrara, Italy) or to the Geriatric Unit of the IRCCS 'Casa Sollievo della Sofferenza (San Giovanni Rotondo, Italy) were enrolled in the study from 2009 to 2015. The study was conducted according to the Declaration of Helsinki (98), the guidelines for Good Clinical Practice (99) , and the guidelines Strengthening the Reporting of Observational Studies in Epidemiology guidelines (100). A written informed consent for research was obtained from each patient or from relatives.

Personal data and medical history were collected by a structured interview from patients and caregivers. All patients underwent a general and neurological examination. 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 nonsteroidal anti-inflammatory drugs (NSAIDS), antibiotics or steroids at the time of recruitment.

Neuropsychological test and criteria used for the diagnosis of diabetes, arterial hypertension, and CVD were previously described (101). Smokers were defined as patients with present or previous significant history of smoking (> 180 packs/years).

The diagnosis of MCI or LOAD and VaD was made according to the NINCDS-ADRDA criteria (25) by trained geriatricians. Only patients with "probable" AD or VaD (Global Deterioration Scale (GDS) ranged from stage 4 to stage 6) were selected in order to increase specificity. 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 (102). 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) was excluded.

Finally, the study was carried on 143 non demented subjects, 223 MCI, 65 VaD and 162 AD. A group of 141 MCI patients were followed-up for a mean period of 2 years (2.0 ± 0.6

years) and divided into 3 sub-groups on the basis of clinical evolution at follow-up: i) patients whose cognitive performance remained stable or slightly improved (MCI/MCI); ii) patients converted to LOAD (MCI/LOAD); iii) patients converted to VaD (MCI/VaD).

2.5.2. **Biochemical assays**

2.5.2.1. *Samples collection*

Fresh blood 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 96-well microplate spectrophotometer Tecan infinite M200 (Tecan group Ltd., UK).

2.5.2.2. *Hydroperoxides*

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) (103). For each subject, 5 µl of serum or standard (H_2O_2) was added to a solution containing 190 µl of 200mM acetate buffer (pH 4.8) and 5 µl of chromogen (N,N-diethyl-p-phenyl-enediamine 2.8 mM). 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 (103). The intra-assay and inter-assay coefficient of variation was 2.5 and 3.5%, respectively and the limit of detection was 40 CU.

2.5.2.3. *Advanced Oxidation Protein Products (AOPP)*

The concentration of advanced oxidation protein products (AOPP) was quantified in according to Capeillère-Blandin (104) with minor modifications. Sample preparation procedure needs to precipitate triglycerides (3000 xg for 10 minutes in presence of 25 mM/L $MgCl_2$ and 0.5 mM/L phosphotungstic acid) which strongly interfere with the determination final concentration. Subsequently, 230µL solution containing: 30 µl of supernatant serum, 50mM potassium iodide and 1.52M acetic acid in phosphate buffer; were used for measuring

AOPP concentration at at 340 nm. Calibration curve with chloramine-T ranged between 100 and 1.5nM (Sigma-Aldrich) were used for calculate AOPP as chloramine T μmol/L of equivalents (104). The intra-assay CV was 5.1%, whereas the inter-assay CV was 9.5%. The limit of detection was 1.5 μmol/L.

2.5.2.4. *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 (105). 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. Serum (10 μL), or standard ($FeSO_4$), was added to 190 μL of working solution (250mM acetate buffer, pH 3.6, 0.8mM TPTZ in 40mM HCl and 1.6mM $FeCl_3$ in water. 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.

2.5.2.5. *Uric Acid (UA) assay*

Serum uric acid was assessed by the direct enzymatic oxidation by uricase coupled with peroxidase (106). Final concentration (μmoles/L) was determined colorimetrically at 520 nm. 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 (in healthy subjects) (107). The constant stoichiometric 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. RAP is more sensitive index of antioxidant status in uric acid-rich fluids such as serum.

2.5.2.6. *Thiols (TH)*

Total concentration of thiols was determined by the colorimetric 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB)-based assay described by Hu et al. (108). Serum (20 μL) 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, than 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 using cysteine as reference standard (CV intra-assay 6.5%; CV inter-assay 8.5%).

2.5.2.7. *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 (CV intra-assay 1.5%; CV inter-assay 2.6%).

2.5.2.8. *Ferroxidase activity*

Enzymatic activity of FeOx was measured in serum samples according to Erel's method (109) with minor modifications as previous described (56). Briefly, 5 µl of serum with 238 µl of acetate buffer (0.4M, pH 5.8) and 67 mM $Fe(NH_4)_2SO_4$ was incubated for 3.8 min at 37˚C. At the end of the incubation, 20 µl of chromogen {3-(2-pyridyl)-5,6-bis-[2-(5-furylsulfonic acid)]- 1,2,4-triazine} was added. Colored complex formed by the chromogen and ferrous ions complex was recorded at 600 nm. The difference in the ferrous ion concentration before and after the enzymatic reaction indicated the amount of oxidized ferrous ion. The amount of enzyme that converted 1µmol of Fe^{2+} into Fe^{3+} per minute in one litre of sample was defined as 1 U/l. The detection limit and intra-assay coefficient of variation were 25.8 U/l and 2.5%, respectively.

2.5.2.9. *PON-1 activities: paraoxonase and arylesterase*

Paraoxonase activity assays was determined by monitoring the rate of formation of para-nitrophenol at 405nm. Five microliters of serum were added in 245 µL of reaction mixtures composed of 1.5mM paraoxon, 10mM Tris-HCl, pH 8, 0.9 M NaCl, and 2 mM $CaCl_2$. An extinction coefficient of 17000/M/cm was used for calculating units of paraoxonase activity,

which are expressed as the nanomoles of para-nitrophenol generated per minute per mL of serum.

The arylesterase activity was measured by adding 10 µL of pre-diluited serum (1:24), to 240 µL of reactions mixture composed by 1 mM phenylacetate and 0.9 mM $CaCl_2$ dissolved in 9 mM Tris-HCl, pH 8. A molar extinction coefficient of 1310/M/cm was used for the calculation of enzyme activity, expressed in terms of kilo unit per liter.

One enzymatic unit of paraoxonase or arylesterase is defined as 1 µmol of reaction product (4-nitophenol or phenol, respectively) produced in a minute under the conditions of the assays.

PON-1 192 polymorphism frequencies, was assessed using a dual-substrate method as pervious described by Haagen et al (61,110), based on similar average of arylesterase, but very different paraoxonase activity between the three phenotypes . The genetic polymorphism at codon Q192R is responsible for the presence of three phenotypes: QQ (wild type, low paraoxonase activity), QR (intermediate) and RR (high paraoxonase activity) (111). The ratio of the paraoxonase activity, in the presence of 1 M of NaCl, to the arylesterase activity was used to assign individuals to one of the three possible phenotypes. Cut-off values between phenotypes were as follows: type QQ, ratio < 2.95; type QR, ratio 2.95 – 6.91 and type RR, ratio > 6.91.

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

The C-reactive Protein hs-CRP serum concentration was measured by using a commercially available non-competitive ELISA kit (dbc, Diagnostic Biochem Canada, Canada) according to the guidelines. Detection ELISA limit:10mg/ml; CV intra-assay 9.5; CV inter-assay 9.0

2.5.3. **Laboratory assessment**

2.5.3.1. *Brain computer tomography scan*

All patients underwent a brain Computer Tomography (CT), using a third generation SIEMENS SOMATON HQ (slice thickness was 10 mm). Radiograms were evaluated by blinded-trained radiologists. The CT scan information was used to support the clinical diagnosis, and identify possible brain pathologies associated with secondary cognitive impairment.

2.5.4. **Statistical analysis**

Normal distributions of the variables examined were checked by the Saphiro Wilk Test and, in case of non-normal distribution the variables were transformed into base-10 logarithm value before entering in the statistical 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 independent of potential confounders.

Adjusted Odds Ratio (O.R.; 95% confidence interval – 95% CI) were calculated by multivariate logistic regression analysis. A two-tailed probability value <0.05 was considered statistically significant.

SPSS 17.00 for Windows (Chicago, Illinois, USA) was used for statistical analysis.

2.6. **Results**

2.6.1. **Principal characteristics of samples**

The main characteristics of total subjects enrolled in the study are presented in Table 2.3.

Table 2.3: Principal characteristics of MCI, LOAD, VaD and controls

	CONTROLS (n= 169)	**MCI (n=311)**	**VaD (n=60)**	**LOAD (n=158)**
Age (years)	72.0 ± 1.0	76.4 ± 0.4	77.6 ± 1.1	78.4 ± 0.5
Gender (females, %)	76.9	56.8	55	71
Smoking (%)	9.2	10.3	9.1	7.1
Education (years)	9.1 ± 0.5	6.2 ± 0.2	5.78 ± 0.5	5.4 ± 0.3
MMSE score	27.1 ±0.3	24.4 ± 0.2	21.2 ± 0.5	20.5 ± 0.4

Data presented as median ± standard error; MMSE: Mini Mental State Examination

Four subsets of this sample have been examined in four different studies that was conducted between 2011 and 2014. The studies are summarized as follows and described in details in the next sections:

- ✓ Redox balance in patients with LOAD or MCI
- ✓ OxS and CVD markers profiles in patients with LOAD or VAD
- ✓ Serum PON-1 and FeOx activities in patients with VAD, LOAD, or MCI.

2.6.2. **Redox balance in patients with LOAD or MCI**

2.6.2.1. *Cross-sectional study*

At the beginning of this study, we considered a battery of systemic markers in order to verify the presence of a redox imbalance in the considered groups. As reported

Table 2.4 and in the published paper (112), we worked on 99 not demented subjects (controls), 134 MCI, and 101 LOAD. ANCOVA analysis were performed, considering age, CVD, hypertension, smoking, gender, and hsCRP as covariate. Controls were younger than MCI and LOAD patients and hypertension showed higher prevalence in MCI than in both other groups. Other comorbidity considered did not exhibit significant differences. Among redox markers examined only HY and RAP showed significant differences between groups

after adjustment for confounding factors. In particular HY were higher in MCI and in LOAD than in controls, instead RAP was reduced considering the same comparison ($p<0.05$).

Table 2.4: Main samples characteristics and mean levels (mean ± SEM) of serum OxS markers in non-demented healthy Controls, MCI, LOAD and VaD patients.

	CONTROLS (n= 99)	**MCI (n= 134)**	**LOAD (n= 101)**
Age (years)	65.7 ± 9.0	75.3 ± 6.7[a]	77.9 ±5.6
Gender (females, %)	83.5	59.8	74.0
MMSE score	27.1 ± 2.2	24.3 ± 3.3[a,b]	20.6 ± 4.4
Hypertension (%)	36.5	62.5[a]	66.5[a]
Smoking (%)	26.9	51.9[a]	38.6
Diabetes (%)	8.6	15.0	15.2
CVD (%)	10.3	17.3	16.3
Oxidative stress markers:			
HY(CU) #	272.9 ± 19.7	305.1 ± 12.4	352.8 ± 16.4[a]
AOPP (µmoles/L)	76.8 ± 3.7	74.4 ± 2.4	70.1 ± 2.9
TH (µmoles/L)	247.7 ± 18.4	209.3 ± 11.6	206.9 ± 15.7
TAP (FRAP units)	663.8 ± 21.0	667.1 ± 13.4	693.8 ± 16.6
RAP (FRAP units)	161.4 ± 32.1	125.6 ± 21.2[a]	134.9 ± 26.6

ANCOVA: [a] $p<0.05$ vs controls;[b] $p<0.01$ vs LOAD # $p<0.05$ for overall ANCOVA, 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; TAP, total antioxidant power; FRAP, Ferric reduction antioxidant capacity; RAP, residual antioxidant power.

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 by using the median values of OxS markers as cut-off (310.97 CU for HY, and 153.7 FRAP units for RAP). 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 whether MCI or LOAD condition was associated to a derangement of redox balance, 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 Table 2.5 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. 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 whether MCI or LOAD condition was associated to a derangement of redox balance, 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.

Table 2.5: Likelihood of association between high HY or low RAP and MCI or LOAD diagnosis.

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)

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

In Fig. 2.10 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) (Fig. 2.10-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 (Fig. 2.10-B).

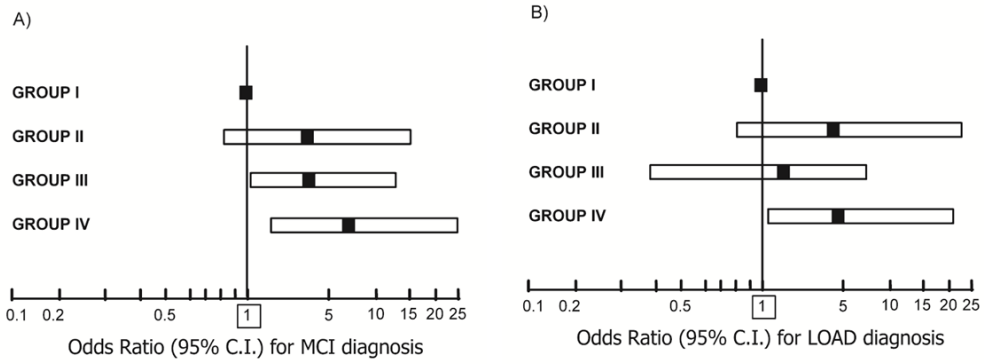

Fig. 2.10: Likelihood of the association between oxidative balance and MCI, LOAD or VaD diagnosis. *Odds Ratios (95% C.I.) for: A) MCI or B) LOAD diagnosis in subjects with: low HY and high RAP values (group I); high HY and high RAP (group II); low HY and low RAP (group III); high HY and low RAP (group IV). Abbreviations: HY, hydroperoxides; RAP, residual antioxidant power.*

1.1.1.2 Longitudinal study

A randomly selected subsample (n= 111) of MCI were longitudinally followed-up for a mean period of 2 years (2.0 ± 0.6 years), to investigate whether baseline levels of HY and RAP might be predictive of 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).

ANCOVA analysis, after adjustment for confounding factor (age, gender, smoking and comorbidities) did not show any difference in either OxS markers between these two MCI subgroups (Fig. 2.11).

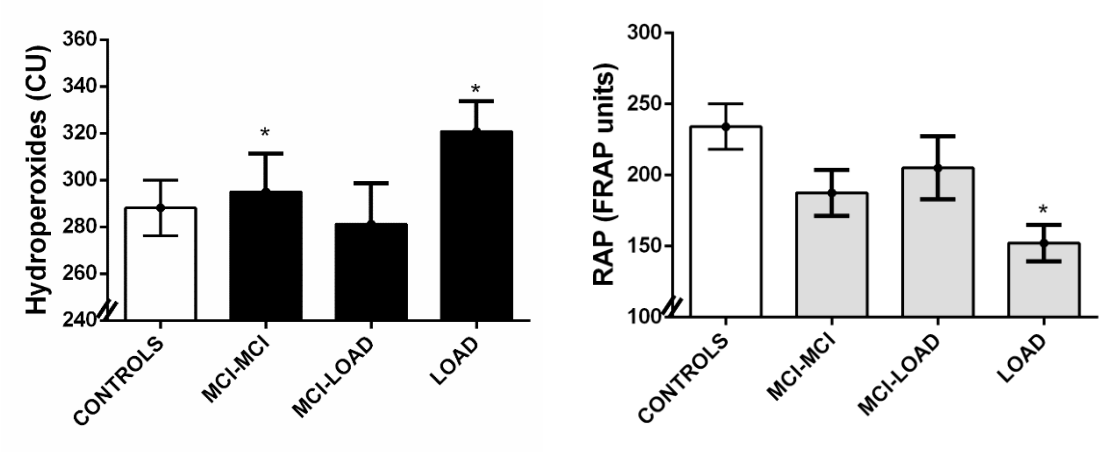

Fig. 2.11:Levels of serum hydroperoxides and residual antioxidant power (RAP) in non-demented healthy controls, MCI/MCI, MCI/LOAD, and LOAD patients.
*Data are expressed as mean ± standard error of the mean, SEM. MCI/MCI: stable MCI patients; MCI/LOAD: MCI patients converted to LOAD. CU = Carratelli Units; FRAP = Ferric reduction antioxidant capacity. In the ANCOVA model: age, gender, hypertension, cardiovascular diseases, diabetes, and smoking habit. * $p<0.05$ vs Controls*

Based on the median values of OxS markers from the statistic sample used for this battery of analyses (i.e., 305.0 CU for HY, and 208.8 FRAP units for RAP) three subgroups of individuals were identified:

- favourable redox balance: low HY and high RAP;
- intermediate OxS: high HY and high RAP or low HY and low RAP;
- full blown OxS: high HY and low RAP.

Fig. 2.12 reported the within sample group (Controls, MCI/MCI, MCI/LOAD, and LOAD) percentages of subjects with different degree of OxS. According to ANCOVA results, controls and LOAD exhibited opposite proportions as regards the two extreme states of oxidative balance. Notably, the relative percentages of favourable redox balance and full blown OxS in MCI/dementia were markedly different (about three times for both) from those of LOAD patients.

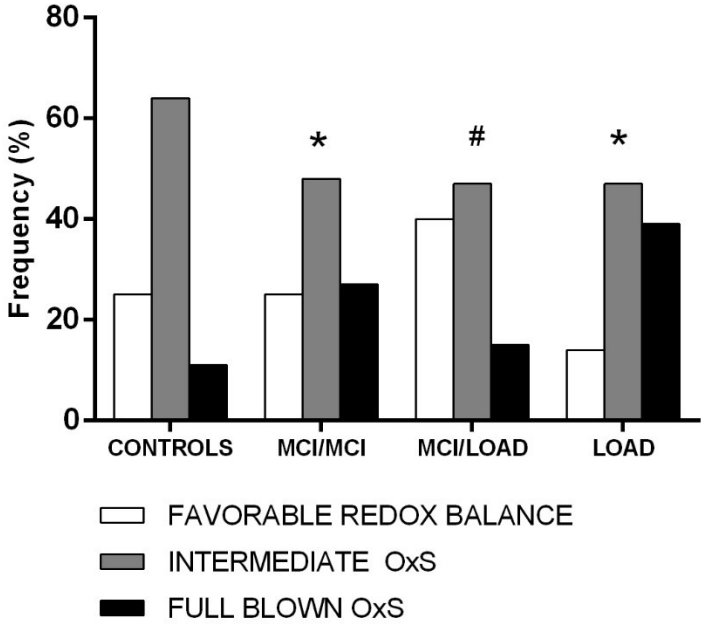

Fig. 2.12: Within group percentages of subjects with Favorable Oxidative Balance, Intermediate OxS, or Full Blown OxS. (for definitions see text).
*CU = Carratelli Units; FRAP = Ferric reduction antioxidant capacity. MCI/MCI: stable MCI patients. MCI/dementia: MCI patients converted to LOAD. * $p<0.01$ vs Controls; # $p<0.01$ vs LOAD.*

2.6.3. **OxS and CVD markers profiles in patients with LOAD or VAD**

To better understand the cardiovascular implication in dementia we took in consideration homocysteine and uric acid in 48 controls, 103 MCI, 89 LOAD in addition to 54 VaD, (97). The main characteristics of subjects considered in this study are reported in Table 2.6.

Table 2.6:Levels of serum OxS markers in non-demented healthy controls, MCI, LOAD, and VAD patients.

	CONTROLS (n= 48)	**MCI (n= 103)**	**LOAD (n= 89)**	**VAD (n= 54)**
Age (years)	77.8 ± 0.7	77.8 ± 0.4	78.8 ± 0.8	79.3 ± 058
Gender (females, %)	68.0	64.1	74.1	56.1[a]
MMSE score	27.1 ± 2.2	24.3 ± 3.3[a,b]	20.6 ± 4.4	218.2 ± 19.2[b]
Oxidative stress markers:				
Hydroperoxides (CU)	199.2 ± 15.9	261.2 ± 14.2[a]	283.1 ±14.3[a]	218.2 ± 19.2[b]
Homocysteine (µmoles/L)	14.6 ± 3.4	14.3 ± 1.1	16.8 ± 1.7	18.1 ± 2.2[c]
Thiols (µmoles/L)	165.2 ± 15.9	170.2 ± 19.0	155.0 ± 15.6	154.0 ± 14.2
Uric acid (µmoles/L)	317.0 ± 17.1	337.9 ± 10.8	344.0 ± 10.5	363.2 ± 12.9
Total antioxidant power (FRAP units)	654.5 ± 26.2	645.7 ± 16.6	665.2 ± 20.2	683.2 ± 31.2
RAP (FRAP units)	185.8 ± 27.7	130.2 ± 8.8[a]	140.8 ± 11.5[a]	140.2 ± 12.4[a]

Data are expressed as mean ± standard error of the mean, SEM.CU = Carratelli Units; FRAP = Ferric reduction antioxidant capacity. ANCOVA: [a] p<0.05 or p<0.01 vs controls; [b] p<0.05 vs LOAD; [c] p<0.05 vs MCI; adjusted for CVD, hypertension, diabetes, smoking, and gender.

VaD and LOAD patients were compared with controls, in order to evaluate whether these parameters might be associated with different probability to develop differ type of dementia after correction for confounding factors. As shown in Fig. 2.13-C, higher homocysteine levels (≥17.0 µmol/L) increase the 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). No association were found in relation to higher risk of VAD compared with Controls or MCI, in subjects with high uric acid levels (men: 400 µmol/L; women: 360 µmol/L.), after correction for confounders (Fig. 2.13-D). The risk of LOAD diagnosis was not associated with hyperuricemia or hyperhomocysteinemia Fig. 2.13-A, -B.

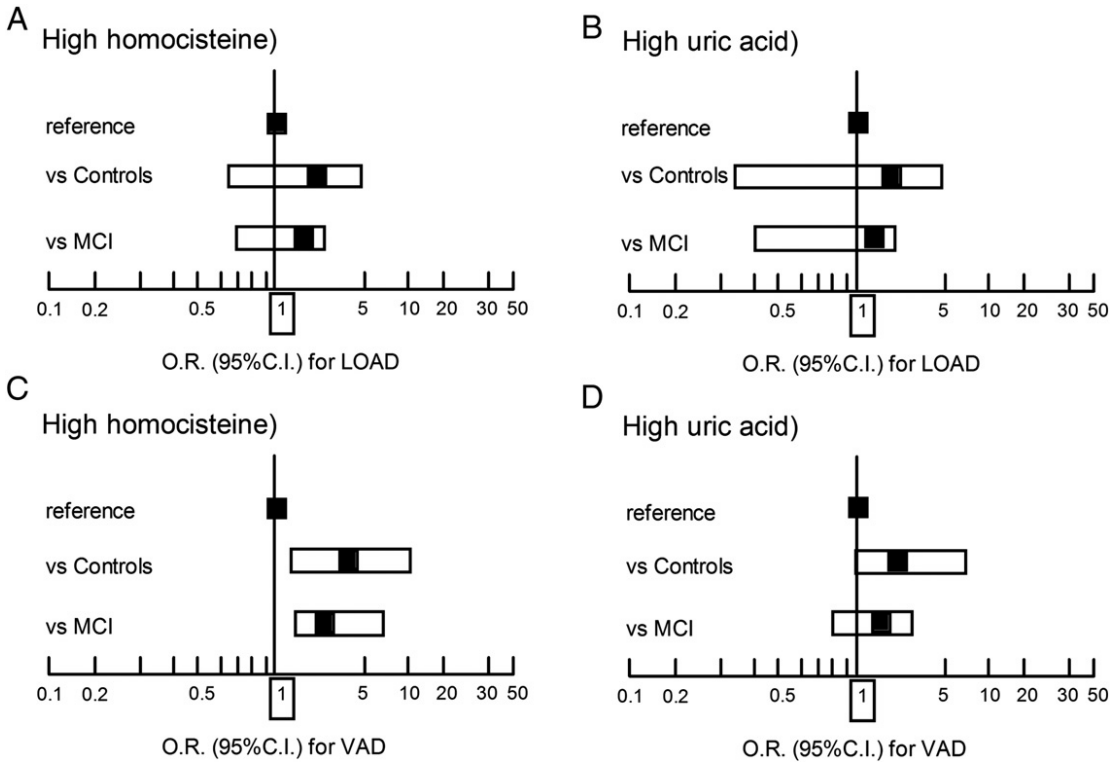

Fig. 2.13: Likelihood of the association between high levels of homocysteine or uric acid and LOAD or VaD diagnosis.
Adjusted odds ratios (95% C.I.) for diagnosis of LOAD or VAD (compared with Controls or MCI subjects) in individuals with high levels of homocysteine (≥17.0 µmol/L) or uric acid level (men: 400 µmol/L; women: 360 µmol/L). Adjusting covariates: CVD, hypertension, diabetes, smoking, and gender.

To further check the relation between neurodegenerative disorders and uric acid and homocysteine, we calculated the likelihood for LOAD or VaD after subdividing the sample in three sub-groups:

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 (Fig. 2.14-A) and MCI (Fig. 2.14-B) 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 (Fig. 2.14-C, -D).

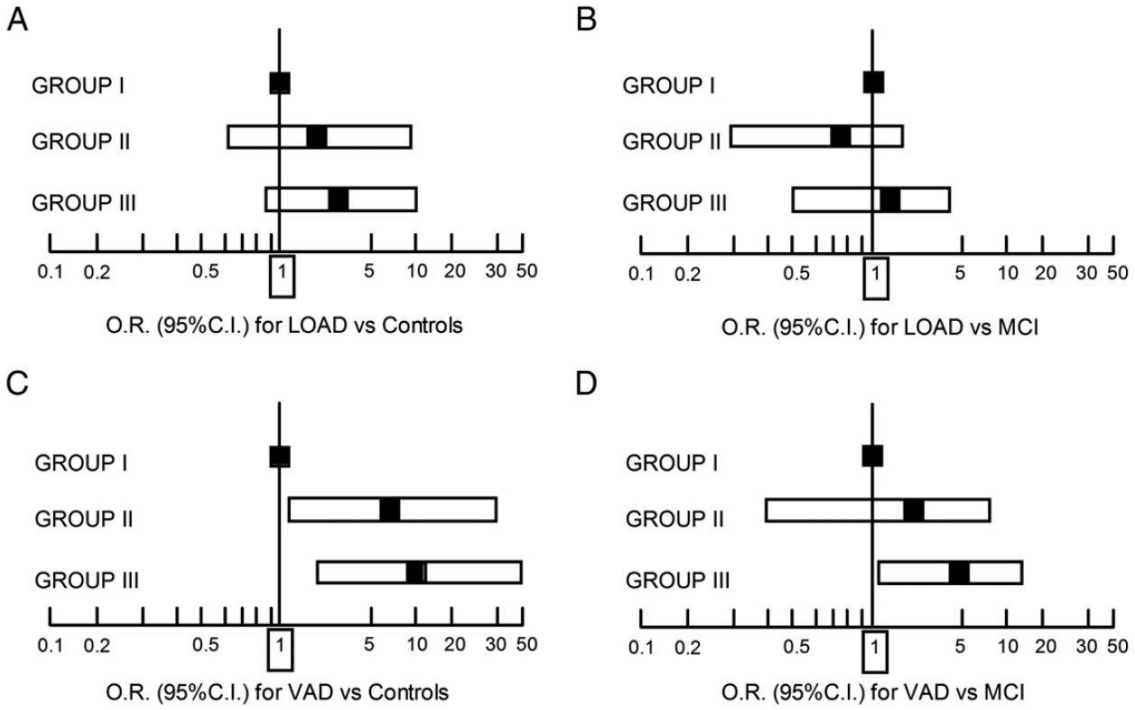

Fig. 2.14: Likelihood of the association between the combination of high levels of homocysteine and uric acid and LOAD or VaD diagnosis.
Adjusted odds ratio (95% C.I.) for the diagnosis of LOAD or VAD compared with Controls or MCI in subjects with: low homocysteine and uric acid (reference; Group I); high uric acid or homocysteine (Group II); high homocysteine and uric acid (Group III). Adjusting covariates: CVD, hypertension, diabetes, smoking, and gender.

2.6.4. **Serum PON-1 and FeOx activities in patients with VAD, LOAD, or MCI**

2.6.4.1. *Observation data*

In this study, it was investigated if Paraoxonase 1 (PON-1) and ferroxidase (FeOx) activities, well acknowledged plasma antioxidants, were associated with dementia or MCI. The study was carried out in 143 older normal cognitive controls, 223 MCI, 162 LOAD and 65 VaD (48), with the main characteristics of subject examined are reported in Table 2.7.

Table 2.7: Principal characteristics of older patients with Late Onset Alzheimer Disease (LOAD), Vascular Dementia (VAD), Mild Cognitive Impairment (MCI), and non-demented healthy controls.

	CONTROLS (n= 223)	**MCI (n= 223)**	**VAD (n=65)**	**LOAD (n=162)**
Age (years)	68.8 ± 9.3	76.4 ± 6.3[b]	77.7 ± 7.0[b]	78.6 ± 5.6[b]
Gender (females, %)[a]	76.9	60.0[b]	54.2[b]	72.3[c]
Education (years)	8 (5-13)	5 (5-8)	5 (4-8)	5 (3-5)
MMSE score[e]	26.7 (25.2-28.3)	24.4 (22.2-26.7)	24.45 (18.7-23.4)	21.0 (18.4-23.7)
Hypertension (%)[a]	43.3	62.8[b]	62.5[b]	63.5[b]
Smoking (%)	7.1	8.8	8.5	6.0
Diabetes (%)[a]	9.2	16.1[b]	32.1[b,c]	13.2[d]
CVD (%)[a]	10.2	19.3[b]	35.6[b]	13.3[d]

Mean ± standard deviation for normally distributed variables; median (interquartile range) for not normally distributed variables; percentage for discrete variables. [a] $p < 0.05$ ANOVA or χ 2 -test (post hoc test: [b] $p < 0.05$ vs. Controls; [c] $p < 0.05$ vs. MCI; [d] $p < 0.05$ vs. VAD); e $p < 0.05$ Kruskal-Wallis. CVD, cardiovascular disease; MMSE, Mini Mental State Examination.

In Fig. 2.15 are displayed median levels of Ferroxidase I and II, while in Fig. 2.16 are displayed paraoxonase and arylesterse activities of PON-1 in the different groups. Serum arylesterase, paraoxonase activities of PON-1was significantly (ANOVA: $p < 0.001$) and similarly lower in MCI, LOAD, and VAD compared to controls. Conversely, the activity of paraoxonase, FeOx I and II did not show any significant change across the groups.

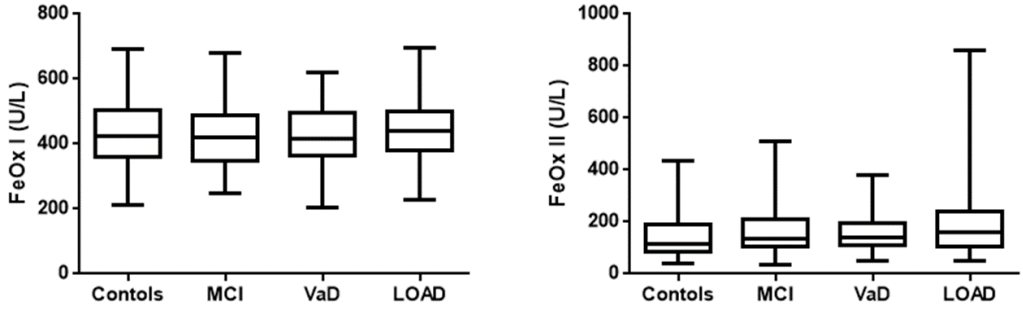

Fig. 2.15: Ferroxidase I and II activity levels in control, MCI, VaD and LOAD.
No significant levels differences were found compared with controls.

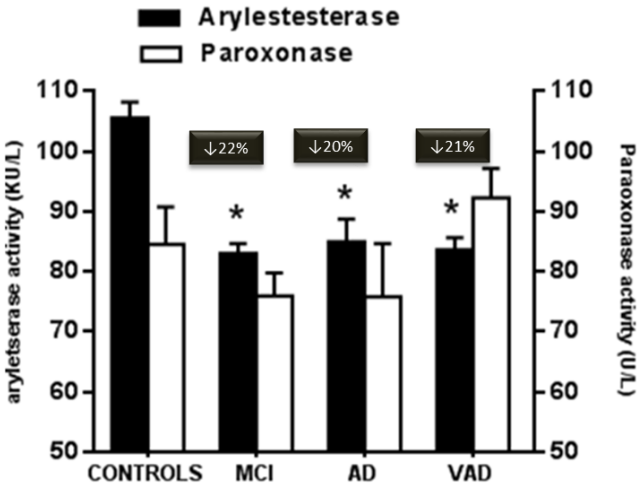

Fig. 2.16: Arylesterase and Paraoxonase activities in MCI, LOAD, VaD.
[*] *Significant depression of Arylesterase activity in MCI, LOAD, VaD compared to controls ($p<0.05$).*

In order to check whether the decrease in serum arylesterase activity observed in MCI, LOAD and VAD was due only to aging, we stratified the sample in two subsamples of younger or older subjects (by using as age cut-off the value of 77 years, i.e., the median age of the sample). As shown by Table 2.8, arylesterase activity exhibited a similar trend in both groups, with lower level in all three groups of patients compared to controls (Kruskall-Wallis: $p < 0.01$).

Table 2.8: Serum arylesterase activity in younger or older patients with LOAD, VAD, MCI and non-demented controls.

	Age >77 years			
	CONTROLS (n=41)	**MCI (n=114)**	**VAD (n=35)**	**LOAD (n=105)**
Age (years)	80.9 ± 3.6	80.9 ± 3.1	82.1 ± 3.6	81.7 ± 3.2
Arylesterase Activity (KU/L)**	99 (69-99)	86 (59-86)	83 (68-109)	86 (66-108)
	Age ≤77 years			
	CONTROLS (n=102)	**MCI (n=109)**	**VAD (n=30)**	**LOAD (n=57)**
Age (years)*	65.8 ± 6.6	71.0 ± 4.7[a]	71.2 ± 5.5[a]	72.1 ± 3.3[a]
Arylesterase Activity (KU/L)**	104 (84-130)	88 (74-106)	86 (65-103)	82 (70-108)

*Mean ± standard deviation for normally distributed variables; median (interquartile range) for not-normally distributed variables. 77 years= median age of total sample. ** Kruskal-Wallis p<0.01; * p<0.05 ANOVA (post-hoc test: a: p<0.05 vs Controls).*

Multivariate logistic analysis was run to check whether decreased serum levels of arylesterase activity modify the risk of MCI, LOAD, or VaD, regardless of potential confounding factors, such as age, gender, hypertension, CVD, diabetes, smoking status, and HDL-C. In Fig. 2.17, is shown how low levels of arylesterase were independently associated with higher likelihood of being affected by MCI (OR 2.3, 95% CI 1.3 – 3.8), LOAD (OR 2.8, 95% CI 1.5 – 5.0), and VAD (OR 2.7, 95% CI 1.2 – 6.2) compared to controls.

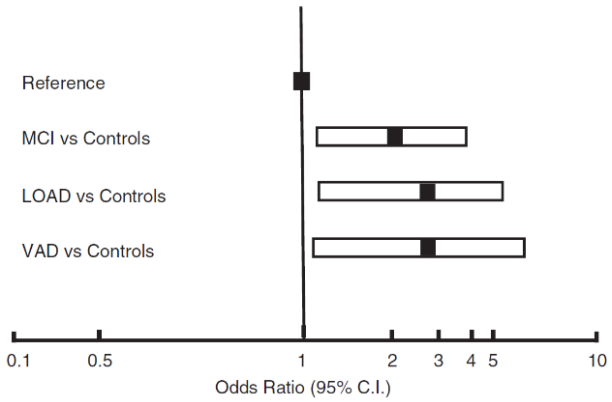

Fig. 2.17: Multi adjusted odds ratio (95% confidence interval) for the diagnosis of LOAD, VAD, MCI in subjects with low levels of serum arylesterase.
Median value = 104 KU/L. Covariates: age, gender, hypertension, CVD, diabetes, smoking status, and HDL-C.

The multi-adjusted OR only slightly differed from those obtained in the respective univariate models (MCI, OR 2.5, 95% CI 1.6 – 4.0; LOAD, OR 2.6, 95% CI 1.5 – 4.3; VAD, OR 2.9, 95% CI 1.4 – 6.0); this observation suggests a negligible interference of potential confounders on the observed association (Table 2.9).

Table 2.9: Crude and adjusted Odds Ratio (95% Confidence Interval) for the diagnosis of MCI, LOAD and VaD in subjects with low levels of serum arylesterase

O.R. (95% C.I.) for diagnosis of:	MCI	LOAD	VaD
Crude model	2.5 (1.6-4.0)	2.9 (1.4-6.0)	2.9 (1.4-6.0)
Adjusted model 1	2.4 (1.5-3.8)	3.1 (1.6-6.5)	3.1 (1.6-6.5)
Adjusted model 2	2.4 (1.5-3.8)	2.9 (1.3-6.0)	2.8 (1.3-5.8)
Adjusted model 3	2.3 (1.4-3.8)	2.4 2.7 (1.2-6.2)	2.6 (1.2-5.9)

Median value = 104 KU/L. Multivariate logistic analysis adjusted for confounding factors: Model 1: hypertension; Model 2: hypertension, CVD and diabetes; model 3: hypertension, CVD, diabetes, age, sex, HDL-C, sex, smoking.CVD: cardiovascular disease

In literature, PON-1 activity is affected by severe gene mutations and polymorphisms. We took in consideration possible differences on 192 polymorphism frequencies between the groups of patients and controls. This polymorphism can be revealed by genetic analysis as well as by ratio between salt-enhanced paraoxonase activity and arylesterase activity, with the latter approach generating a three modal distribution reflecting the 3 different phenotypes named as QQ (low ratio), QR (intermediate ratio) and RR (high ratio). In Fig. 2.18 are represented the phenotypes frequencies that resulted by the analysis of our sample. The distribution of PON-1 192 phenotypes was at the Hardy-Weinberg equilibrium in both total sample and each subsamples (Table 2.10). Particularly, in control group the distribution of the three phenotypes was not significantly different from those observed in MCI, LOAD, or VAD groups. The same negative results emerged by comparing the phenotypic frequency between MCI and the two groups with demented patients. This result is not surprising since QQ, QR, and RR alloenzymes exhibit different hydrolysis rates using paraoxon as substrate, whereas their ability to hydrolyze phenylacetate is unmodified (61).

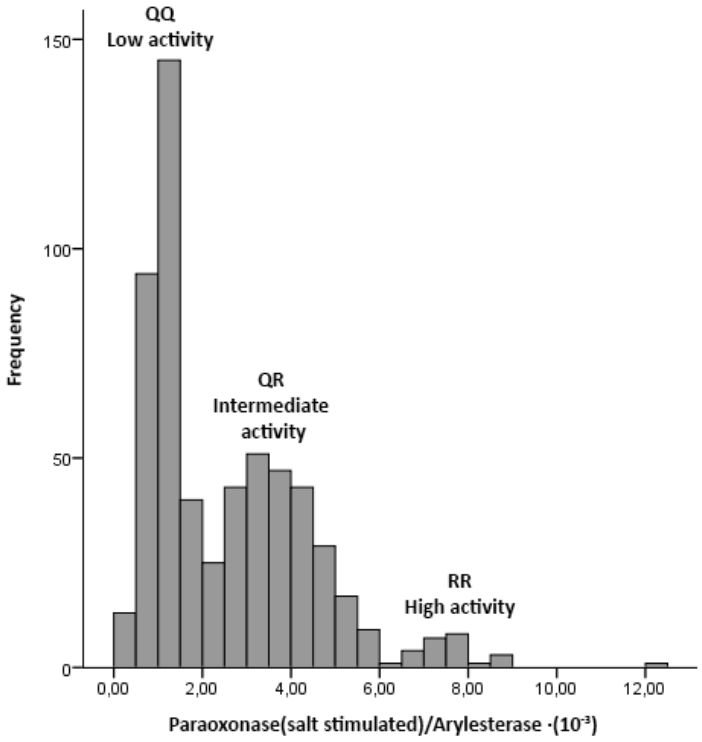

Fig. 2.18: Three modal population distribution of PON-1 192 phenotype by using paroxonase/arylesterase ratio.

Table 2.10: PON-1$_{192}$ phenotype and allele frequencies in LOAD, VAD, MCI, and non-demented controls.

	PON1 Phenotypes		
Groups	**QQ**	**QR**	**RR**
	(n= 388)	**(n= 192)**	**(n= 13)**
CONTROLS	0.678 (97)	0.294 (42)	0.028 (4)
MCI	0.628 (140)	0.354 (79)	0.018 (4)
VAD	0.723 (47)	0.231 (15)	0.046 (3)
LOAD	0.642 (104)	0.346(56)	0.012 (2)

χ^2= 1.71, p=0.425 for comparison of CONTROLS vs. MCI; χ^2= 1.21, p=0.545 for comparison of CONTROLS vs. VAD; χ^2=1.73, p=0.420 for comparison of CONTROLS vs. LOAD; χ^2=4.70, p=0.09 for comparison of MCI vs. VAD; χ^2=1.24, p=0.888 for comparison of MCI vs. LOAD; χ^2=4.82, p=0.089 for comparison of VAD vs. LOAD.

2.6.4.2. *Longitudinal study*

A subsample of MCI were longitudinally followed up for mean period of 2 years (2.0 ± 0.6 years), to investigate possible modification in baseline levels of Paraoxonase/Arylesterase activity in relation to progression of MCI to LOAD (MCI/LOAD) or to VaD (MCI/VaD)

within 2 years of follow-up. The main clinical characteristics of subsamples considered were reported in Table 2.11. In this subgroup, there were no differences in age, gender, MMSE score, prevalence of hypertension, smoking habits, diabetes and CVD. MCI patients had lower median arylesterase and paraoxonase levels compared with controls, but the difference was only significant for arylesterase ($p < 0.0001$). Taking into account only the MCI subgroups, lower levels of paraoxonase were found only in MCI/VAD compared with controls (Mann–Whitney $p < 0.05$), whereas lower levels of arylesterase activity were observed in MCI/MCI, MCI/LOAD, and MCI/VAD compared with controls (Mann–Whitney all $p < 0.001$), Table 2.11.

Table 2.11: Principal characteristics of older patients with LOAD, VAD, MCI, and non-demented healthy controls.

	CONTROLS (n=78)	**MCI-MCI (n=86)**	**MCI-LOAD (n=34)**	**MCI-VAD (n=21)**
Age (years)	76 (73-79)	77 (71-81)	78 (74-83)	79 (75-82)
Gender (females, %)	64.1	54.7	55.9	47.6
Education (years)[c]	8 (5-13)	5 (4-8)	5 (5-8)	5 (3-8)
MMSE score	26.4 (25.0-28.0)	26.3 (25.0-27.0)	24.5 (22.2-25.6)	24.5 (23.7-25.9)
Hypertension (%)	64.4	57.6	55.9	61.9
Smoking (%)	5.1	4.7	8.8	0
Diabetes (%)	16.4	18.8	20.6	33.3
CVD (%)[a]	15.4	32.6	20.6	33.3
Paraoxonase (U/L)[b]	77.2 (46.3-161.9)	92.5 (46.8-148.5)	75.0 (42.24-145.9)	48.7 (28.3-75)
Arylesterase (KU/L)[c]	104.1 (84.9-122.8)	74.4 (57.8-102.7)	82.7 (61.1-106.1)	57.8 (44.7-88.7)

Not-normally distributed variables are expressed as median (interquartile range), discrete variables as percentage. MCI, mild cognitive impairment; LOAD, late-onset Alzheimer's disease; VAD, vascular dementia; MCI/MCI, stable MCI patients; MCI/LOAD, MCI patients converted to LOAD; MCI/VAD, MCI patients converted to VAD; CVD, cardiovascular disease; MMSE, mini mental state examination. [a] $p < 0.05$, χ^2, controls versus MCI. [b] $p < 0.05$, Kruskal–Wallis between controls, MCI/MCI, MCI/LOAD, and MCI/VAD. [c] $p < 0.001$, Kruskal–Wallis between controls, MCI/MCI, MCI/LOAD, and MCI/VAD.

When we analysed possible differences in PON-1 activities between MCI subgroups, we found a significant overall difference for paraoxonase (Kruskal–Wallis $p < 0.05$), and a trend toward a statistical difference for arylesterase (Kruskal–Wallis $p = 0.06$). As shown in Fig. 2.19-A, paraoxonase activity was significantly lower only in MCI/VAD compared with

patients who did not convert (MCI/MCI) (p = 0.009), whereas no differences emerged regarding arylesterase (Fig. 2.19-B).

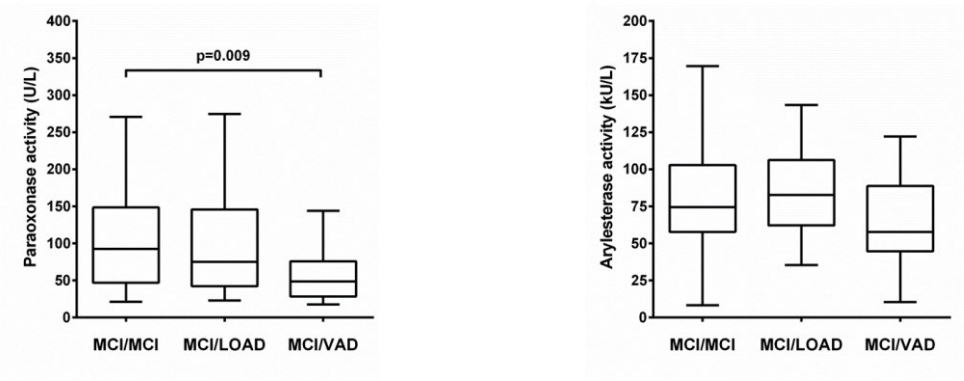

Fig. 2.19: Paraoxonase and arylesterase activities in MCI not converted and converted to LOAD or VaD.
Box plot analysis for serum paraoxonase (A) and arylesterase (B) activity in 86 MCI not converting to dementia (MCI/MCI), in 34 MCI converting to LOAD (MCI/LOAD) and in 21 MCI converting to VAD (MCI/ VAD) after the 2-year follow-up. The boundaries of the box represent the 25th–75th percentile. The line within the box indicates the median. The whiskers above and below the box correspond to the highest and lowest values, excluding outliers.

We then analysed the frequencies of low arylesterase levels within groups (< 56.44 kU/L: I quartile) and, as reported in Fig. 2.20, MCI/VAD patients had higher frequencies of low arylesterase activity compared with MCI/MCI and MCI/LOAD (47.6% vs. 22.4% and 12.1%, respectively; p < 0.05). Paraoxonase activity, showed insignificant trend toward a higher prevalence of low activity levels (< 44.16 U/L: I quartile) in MCI/VAD compared with MCI/MCI (45.0% vs. 20.9%).

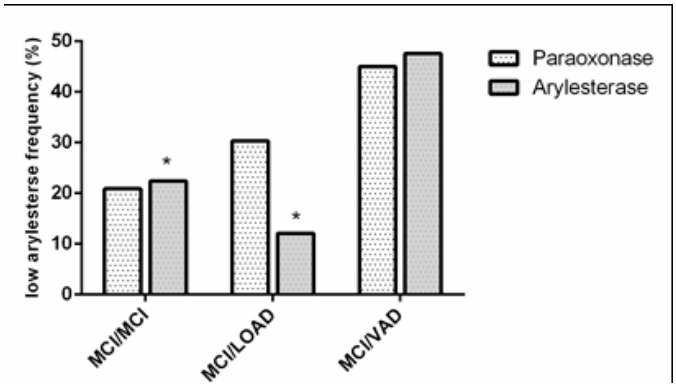

Fig. 2.20: **Prevalence of low levels (first quartile) of paraoxonase and arylesterase activity in MCI/MCI, MCI/LOAD, and MCI/VAD patients.**
*p < 0.05 versus MCI/VAD. Cut-off value for Paraoxonase activity: 44.16 U/L. Cut-off value for Arylesterase activity: 56.44 kU/L.MCI/MCI, stable MCI; MCI/LOAD, MCI to LOAD; MCI/VAD, MCI to VAD.

Finally, by multinomial logistic regression we tested whether low baseline levels of serum paraoxonase and arylesterase activity were useful in predicting the 2-year risk of conversion from MCI to LOAD or VAD. As reported in Fig. 2.21, after multivariate adjustment for age, gender, smoking habit, CVD, hypertension, and diabetes, low levels of paraoxonase activity were significantly associated with an increased likelihood of converting to VAD (MCI/VAD vs. MCI/MCI, odds ratio (OR): 3.74, 95% confidence interval (CI): 1.37–10.25) but not LOAD (MCI/LOAD vs. MCI/MCI, odds ratio (OR): 1.63, 95% confidence interval (CI): 0.64–4.19). Similarly, low levels of arylesterase were associated with the risk of conversion to VAD (MCI/ VAD vs. MCI/MCI, OR: 3.16, 95% CI: 1.17–8.56), but not LOAD (MCI/LOAD vs. MCI/MCI, odds ratio (OR): 0.42, 95% confidence interval (CI): 0.12–1.46).

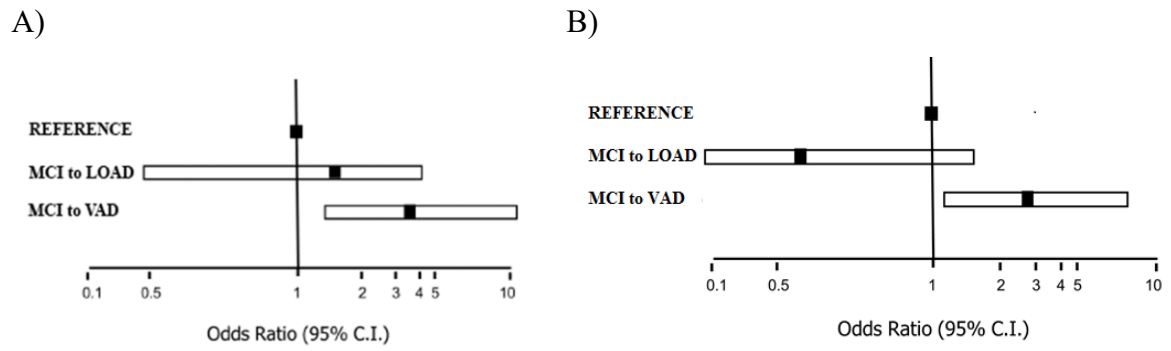

Fig. 2.21: Likelihood of association between low levels of paraoxonase or arylesterase and conversion from MCI to LOAD or VaD.
Adjusted odds ratio (95% C.I.) for (A) low paraoxonase or (B) low arylesterase activity and likelihood of conversion from MCI to LOAD or VAD. (A)Low levels of paraoxonase activity were significantly, associated with an increasing odd of becoming VAD, but not LOAD; B) Low levels of arylesterase activity were significantly, associated with an increasing odd of becoming VAD, but not LOAD.

2.7. **Discussion**

Alzheimer's disease is a neurodegenerative disorder with still unknown etiopathogenesis. (113). Scientific community agrees on the existence of a link between AD and derangement of redox state, as demonstrated by the accumulation of products of oxidative damage in *post-mortem* brain tissue from patients affected by different forms and stages of dementia (114). According with these findings, growing evidence from transgenic animal models (115) has shown that intraneuronal deposition of amyloid-β (Aβ) correlates with modification induced by OxS, which, in turn, can lead to impaired cellular function and death. In particular, the experimental evidence collected so far suggest a possible dual role of OxS in AD pathogenesis: trigger factor of pathophysiological events and/or "armed hand" of the pathological hallmarks (Aβ-senile plaques and NFT) of the disease. (116). In contrast with the consistency of animal and in vitro data, findings from human studies are still conflicting. The most important reason of these discordant results might be the lack of gold standard markers for in vivo OxS determination. At present, the best way to quantify redox state in a biological fluid is based on the measurement of a battery of different indirect markers of oxidative damage, i.e. by-products derived by biomolecules oxidation, and antioxidants. Therefore, in this study we applied this methodological approach to evaluate the possible association between dementia and OxS in a large sample of elderly individuals.

The main finding of our work was that dementia-related disorders, AD and VaD, as well as the phase that precedes the clinical symptoms of dementia, MCI, are characterized by depression antioxidant defence accompanied by increase of oxidative damage markers. Indeed, LOAD and MCI patients showed significant, independent and comparable drop of RAP (a parameter that reflects the collective contribute of the main non-enzymatic antioxidants) and increase in HY (one of the by-product of lipid peroxidation). Moreover, the risk for both MCI and LOAD greatly, and similarly, increased when antioxidants and oxidative products changed together from a favourable balance (high RAP-low HY) to a "full blown" OxS state (low RAP-high HY) (Fig. 2.10). These data suggest that OxS might represent an early event in LOAD pathogenesis, and that the redox dyshomeostasis may be already present in the prodromal phase. In line of the hypothesis, a wealth of studies showed a similar oxidative and inflammatory [38] pattern in MCI and LOAD. Considering this proposed "oxidative equivalence" between pre- and clinical stage [39] of dementia, we were

not surprises to observe no significant differences s in peripheral OxS between stable MCI and those that converted to LOAD within 2 years (longitudinal data table….). Moreover, these results are consistent with prospective study conducted in a sample of 70 MCI subjects, by Baldeiras et al. (117). It is, however, fair to say that literature also reports data in contrast with ours. Indeed, a recent study found that cerebrospinal fluid (CSF) F2-isoprostanes were significantly higher in MCI patients who later converted to LOAD compared to stable patients (118). The inconsistencies between these data and ours might be mainly due to the differences regarding the markers and biological fluids that were used for OxS detection as well as regarding the general characteristics (e.g. age and lifestyle habits) of the population-samples.

With the regard to the second form of dementia considered in our research: patients affected by VAD showed a less evident "redox imbalance" compared to LOAD or MCI. The published studies on this topic have obtained controversial results, since several, but not all, showed a similar increase of OxS in VAD in LOAD compared to controls (48,71,119,120). However, these studies differed from ours, mostly in relation to the methodology, the sample composition/size and confounders considered in the statistical analyses.

More intriguing outcomes emerged from the determination of homocysteine (HCYS) and uric (UA), two well-recognized CVD risk factors that are also described to have opposite functions in systemic redox regulation (121,122). Homocysteine works as a strong pro-oxidant while uric acid is regarded as the most abundant non-enzymatic circulatory antioxidant. However, high levels of urate can also negatively affect Redox homestasis, leading to OxS (123). We found that high HCYS levels by themselves or in combination with high levels of UA, increased the likelihood to be affected by VaD but not LOAD and MCI (Fig. 2.13, Fig. 2.14). These results suggest that, regardless of a possible common OxS-related route in their pathogenesis, VAD and LOAD maintain distinctive features, with hyperhomocysteinemia and hyperuricemia that were synergically associated with the risk of the former, but the latter form of dementia.

The prevention and protection against insult by free radicals can be afforded by low molecular antioxidants (especially vitamin C, E, uric acid, etc.) and some enzymes. Notably, compared to the non-enzymatic antioxidants, the biological catalysts are, by definition, faster and more efficient, even at low concentration, in scavenging reactive species. There is a corollary of evidence demonstrating that paraoxonase and arylesterase activity, elicited by

PON-1, and ferroxidase activity of Cp (and other still obscure molecules) exert a potent protective role in vivo against OxS-induced damage.

Given the aforementioned multiple roles of PON-1 and Cp-related FeOx activity, is conceivable to hypothesize that an alteration of these two enzymes might be associated with the risk of developing dementia in older population. Nevertheless, this thesis has not been definitely confirmed by solid epidemiological data, mainly because of important methodological limitations (i.e. small sample size, lack of consideration of important confounding factors, etc.) affecting the available current literature [25-30 da articolo su pon-1 trasversale]. In the attempt to shed light upon this still confusing and uncertain scenario, we conducted the present study dealing with the evaluation of serum paraoxonase, arylesterse, and FeOxs activity in a large sample of older individuals (n= 593) including patients affected by VAD, LOAD, mild cognitive impairment (MCI), and cognitively healthy Controls.

FeOx activity exerts a preventive protection against iron (II), the main trigger factor for hydroxyl radical generation. This ROS is the most aggressive radical and is able to trigger the lipid peroxidation cascade, alter the biological properties of membranes (e.g. fluidity and inactivation of receptors), induce oxidative damage to proteins, nucleic acids and carbohydrates (13). A possible involvement of ferroxidase activity in AD development was first suggested by the finding that APP ability to oxidize iron is inhibited in AD brain. To the best of our knowledge, we have been among the first to investigate if also systemic ferroxidase activity was associated with the risk of LOAD or VAD. The result of an unmodified FeOx in these patients compared to healthy controls, suggest that this could not be a factor to blame for the increase of systemic OxS observed in these diseases.

More compelling results were obtained with PON-1 activities. Indeed, in line with previous pilot studies (124,125), we found that arylesterase activity was depressed in all patients (MCI, LOAD, VaD) compared with controls. Furthermore, the probability of being affected by MCI, LOAD, VaD was higher in patients with low levels of arylesterse activity, independently of confounding factor (age, smoke, CVD, hypertension, diabetes, total cholesterol). Thus, depression in arylesterasy activity might occur precociously in dementia, and might contribute to the derangement of systemic redox status observed in MCI patients.

The possible involvement of PON-1 in dementia has also been also highlighted by our longitudinal-like investigation. Indeed, the gathered data showed that low arylesterase activity was associated with a higher likelihood of developing VAD, but not LOAD, while

paraoxonase even if reduced in those MCI patients that converted to VAD during the 2-year follow-up, cannot predict any type of progression (Fig. 2.21). We found an overall consistency of the results with other large studies dealing with this topic (124,126). Although arylesterase was frequently associated with a LOAD diagnosis, while paraoxonase turned out to be related with VAD in other two works (127,128).

Notably, the single nucleotide polymorphism Q192R, that we determined by dual substrate methods as suggested by Haangen et al (110), did not highlight susceptibility for either dementia or prodromal condition. This result, in agreement with previous studies, may be ascribed, as started by Erlich et al. (126) and Camps et al. (129), to the other polymorphisms in PON-1 gene that influence the catalytic function of the enzymes.

Finally, the main findings of the evaluation of OxS markers in patients with LOAD, VAD and MCI can be summarized as follows:

- Redox imbalance is an early feature in LOAD progression;
- Regardless of a possible common OxS-related route in their pathogenesis, VAD and LOAD maintain distinctive features. Indeed, risk of VAD, but not LOAD, significantly increased in older individuals when hyperhomocysteinemia and hyperuricemia were simultaneously present;
- PON-1 could be precociously involved in dementia (both LOAD and VAD) pathogenesis;
- PON-1 could be a reliable early marker to predict the conversion of MCI to VAD.

CHAPTER III

Involvement of lipid metabolism in Alzheimer's disease during perimenopause transition

3.1. Introduction

Almost two-thirds of Alzheimer's patients are women and the prevalence of neurodegenerative diseases is greater in post-menopausal women than in pre-menopausal ones. In particular, AD-related risk appears to markedly increase during the transition between these two reproductive phases. This period of women's life, classically defined as perimenopause, is characterized by irregularity in the menstrual cycle caused by fluctuations of hypothalamic, pituitary, and ovarian hormones levels. Furthermore, during perimenopause the 80% of women experiences several climacteric symptoms such as insomnia, hot flushes, depression, functional and memory impairment.

Published data showed that glucose metabolism in brain declines during perimenopausal transition leading to the generation of a hypometabolic phenotype. More recently, it has been proposed that increased utilization of endogenous brain lipid (from white matter) might compensate the loss of carbohydrates-derived energy (130). These evidences suggest a considerable impairment of lipid metabolism that could become a risk factor for AD development.

3.1.1. The role of estrogens in regulation of lipid metabolism

The main estrogen in women's body is 17-beta-estradiol (E2). Its action is mediated by two estrogen receptors (ERs), ERα and ERβ, which are expressed at different levels (131).

E2 seems to play a beneficial role for the organism. The estrogen is able to ameliorate endothelial function and inflammatory homeostasis, increase neuronal survival, etc…. (131). E2 also influences the levels of plasma lipids by regulating lipid metabolism in adipocytes and hepatocytes.

The decline of E2 in women after menopause leads to a change in plasma lipoproteins concentration, with levels of LDL and VLDL that increase and those of HDL that diminish. These metabolic changes chiefly increase the risk of cardiovascular as well as neurodegenerative diseases. Epidemiological studies have already shown a dysregulation of systemic lipid metabolism in AD (132), that could be linked, at least partially, to mutation of ApoE.

ApoE gene is regulated by estrogens, which can bind their estogen-response elements (ERE) on promoter, intron 1 and exon 4 (which contain the sites that discern the three phenotypes:ε2, ε3, ε4). ApoE can be modulated by differential estrogen binding to ERα or

ERβ, as showed by Wang et al. ERα either by 17β-estradiol or aspecific-agonist, up-regulated ApoE mRNA and protein expression. In contrast, the ERβ-selective agonist, down-regulated ApoE mRNA and protein expression (133).
Furthermore, several published paper indicated that hormone replacement therapy improve plasma lipoprotein-lipid profiles in ApoE3 and APOE2 perimenopause women but not in APOE4 ones (134).

3.1.2. **Cholesterol Metabolism**

3.1.2.1. *Synthesis*

Cholesterol is an essential biological molecule, owing its role in membrane composition and synthesis of vitamin D, steroid hormones and bile acids (14).
Cholesterol balance is maintained by the regulation of synthesis, uptake, and catabolism. In mammals, cholesterol can be obtained from the diet through gut absorption or it can be synthetized *de novo* in liver and brain, which represent the only two organs designed for this function. Cholesterol synthesis starts with the condensation of acetil-CoA with acetoacetil-CoA to form HMG-CoA, and overall involves more than 20 reactions in cytoplasm and microsomes (ER) (135). The major rate-limiting enzyme ,hydroxymethyl-CoA reductase (HMGCR), converts HMG-CoA to mevalonate as schematized in Fig. 3.1. In Golgi reticulum, cholesterol is used for build-up lipid raft, which are specialized micro domains involved in several biological processes as assembling of signalling molecules, modification of membrane fluidity and regulation of neurotransmission (136).

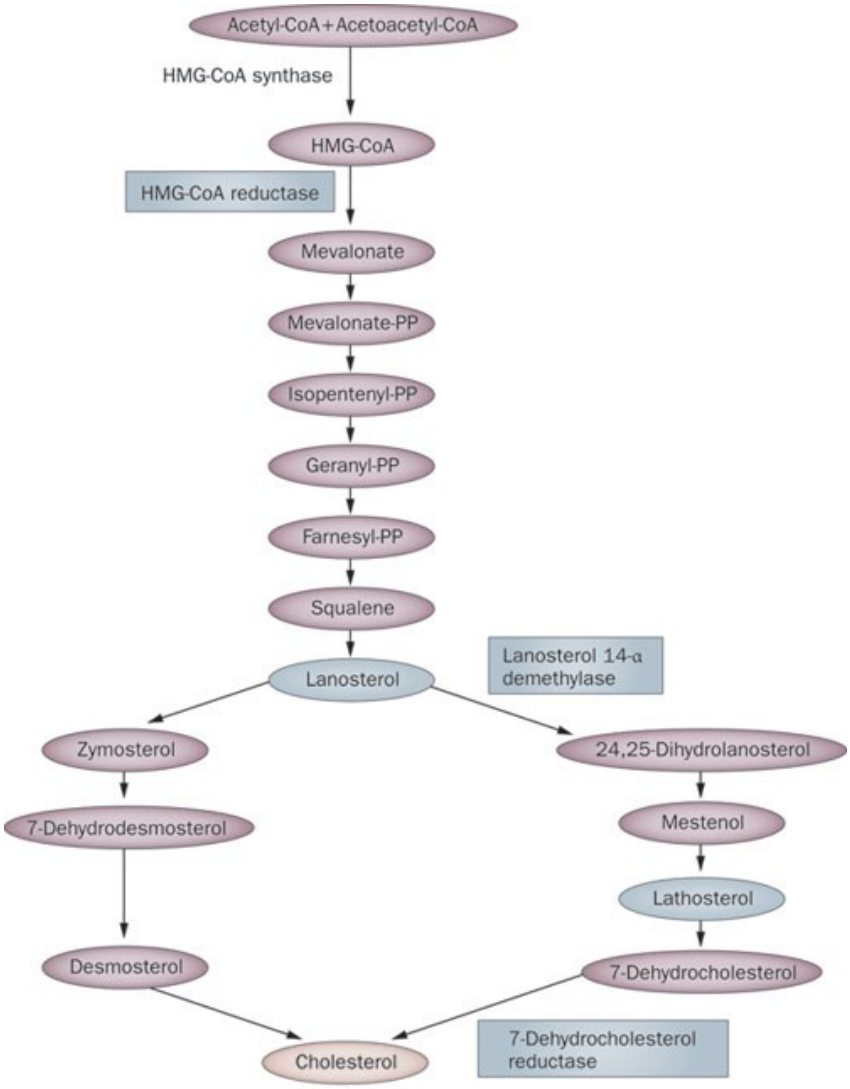

Fig. 3.1: Main steps of the endogenous cholesterol synthesis pathway.
Reprint from Karasinska JM et al, 2011 (137)

3.1.2.2. *Transport and uptake*

Cholesterol transport from synthesis and/or absorption site to the other body tissues, requires lipoprotein carriers. Dietary cholesterol is carried through blood by chylomicrons, lipoproteins formed in the intestinal cells; endogen cholesterol is transported by very-low density lipoproteins (VLDLs) that are synthetized in the liver. Both lipoproteins, contains, besides cholesterol, triglycerides, phospholipids, different types apolipoproteins (Apos).

After the release of trygliceirdes, through the interaction with lipoprotein lipase present in the surface of capillaries endothelium, and some Apos, VLDLs becomes intermediate-density lipoproteins (IDLs). One of the fate of this lipoprotein is the definitive transformation into low-density lipoproteins (LDLs). The sole protein present in LDLs is apolipoprotein B100 that serves to bind the lipoprotein particles to LDL-specific receptors on the surface of many cells (especially hepatocytes). The successful receptor-mediated internalization of LDL is followed by lipoproteins degradation by liposomal enzymes and release of cholesterol.

High-density lipoproteins (HDLs) play an essential role in the transport of cholesterol from periphery to liver (reverse cholesterol transport). Free cholesterol is removed from peripheral tissues through the action of HDL-bound lecithin: cholesterol acyltransferase. A portion of freshly formed cholesteryl esters is transferred to chylomicrons and VLDL by a transfer protein. The rest of esters are incorporated into the core of HDL particles and carried to the liver that internalize scholesterol through hepatic receptors that selectively bind ApoE (present along with other Apos on the surface of the lipoprotein). Excess of cholesterol is then converted to bile acid and excreted by bile ducts.

In the brain, HDLs are the only type of lipoproteins and the major apolipoprotein constituent is ApoE, produced in situ by astrocytes and microglia. Apolipoprotein A-I, the major protein component of plasma HDL, is not synthesized in the CNS, but can enter and become a component of CNS lipoproteins. Cholesterol uptake requires internalization via ApoE interaction with LDL-receptor-related protein (LPR). ApoE has a pivotal role in CNS for cholesterol mobilization and redistribution in order to maintain myelin formation and the integrity of neuronal membrane during growth and after injury (135).

3.1.2.3. *Storage and catabolism*

Cholesterol is stored into the cells either as free cholesterol (FC) or as cholesterol esters (CEs) (formed by acetyl coenzyme A cholesterol acyltransferase (ACAT)) (136). This

process might takes place also in brain cells, even though cholesterol storage in this tissue is still not well understood (135). ACAT is regulated by the cholesterol forms in order to maintain cell's homeostasis. Alternatively, FC can be eliminated from cells by oxidation as reported in Fig. 3.2.

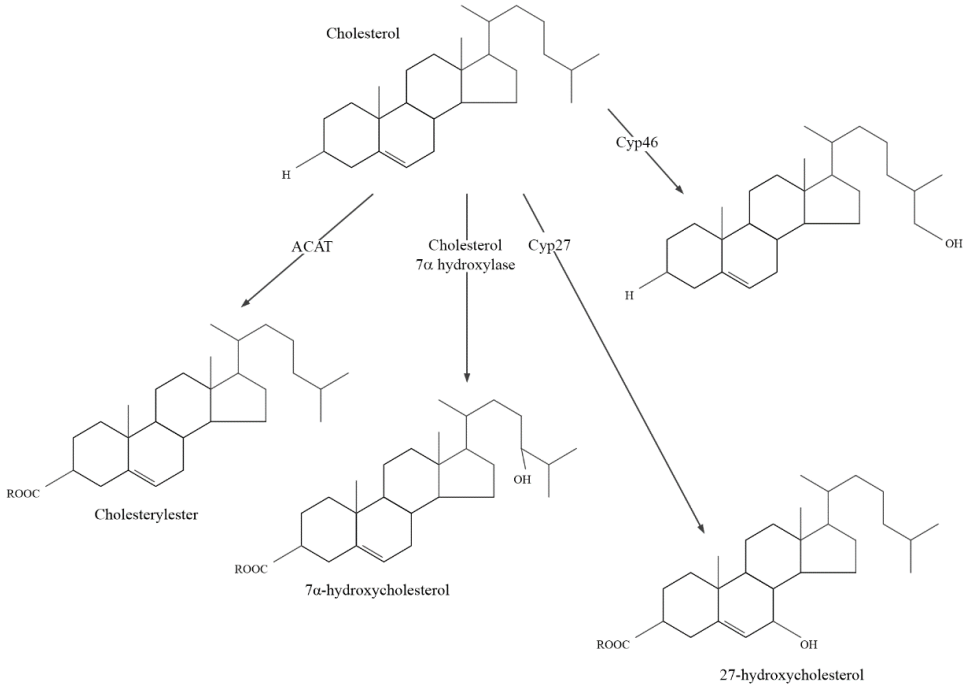

Fig. 3.2: Cholesterol catabolism.
Reprint from Barrow et al.,2006 (136).

Oxidation in position 7α produces the 7α-hydroxicolesterol, which will be secreted as bile acids. On the other hand oxidation in position 24 or 27, by mitochondrial enzyme cholesterol 24 or 27 hydroxylase (Cyp46, and Cyp27, respectively) produces oxysterols, by-products that can cross the cell membrane and act as positive transcription factor on liver X receptor (LXR) (136). LXR are sterol sensors that protect cells from cholesterol overload; after dimerization with retinoic acid receptor (known also as retinoic X receptor: RXR), they can promote the expression of enzymes and transporters involved in: (I) reverse cholesterol transport; (II) conversion of cholesterol to bile acids; (III) biliary excretion of cholesterol (14).

3.1.3. **Brain cholesterol metabolism**

Brain is the most cholesterol rich organ, containing about 20-25% total cholesterol pool of the whole body (brain per kg organ contains 10 times more cholesterol than the rest of the body) (14). The intact blood brain barrier (BBB) does not allow lipoproteins (LDL, VLDLs, chylomicrons) or cholesterol uptake from the circulation in vertebrates (14). For this reason brain cholesterol homeostasis is maintained by independent processes that include *in situ* synthesis, storage, transport, and removal (132). The de novo synthesis of cholesterol is primarily made by neurons, astrocytes, microglia cells. Excessive synthesis, plasma membrane breakdown, myelin (the main depot of cholesterol in CNS) breakdown, and neuronal loss (14,138) are the most likely causes of cholesterol excess in the brain. During embryogenesis and childhood the cholesterol synthesis activity reaches the maximal level. After the development, the general cholesterol biosynthesis yield progressively decreases. Concerning the brain, while neurons lose the ability to produce cholesterol by themselves, astrocytes and oligodendrocytes preserve their biosynthetic propriety.

Efflux of CNS-derived cholesterol through the BBB occurs as 24(S) hydroxycholesterol (24S-OH-C) and 27-hydroxycholesterol (27-OH-C) (14). Since 24S-OH-C is produced exclusively in the CNS, the systemic levels of 24S-OH-C reflect the number of metabolically active neurons and alteration of plasma levels of this by-product are associated with early phase of neurodegenerative disease. 27-OH-C is almost ubiquitously produced, but with low rate in brain. Thus, the presence of high concentration of this lipid in CNS mostly reflects increased extra-cerebral production (139).

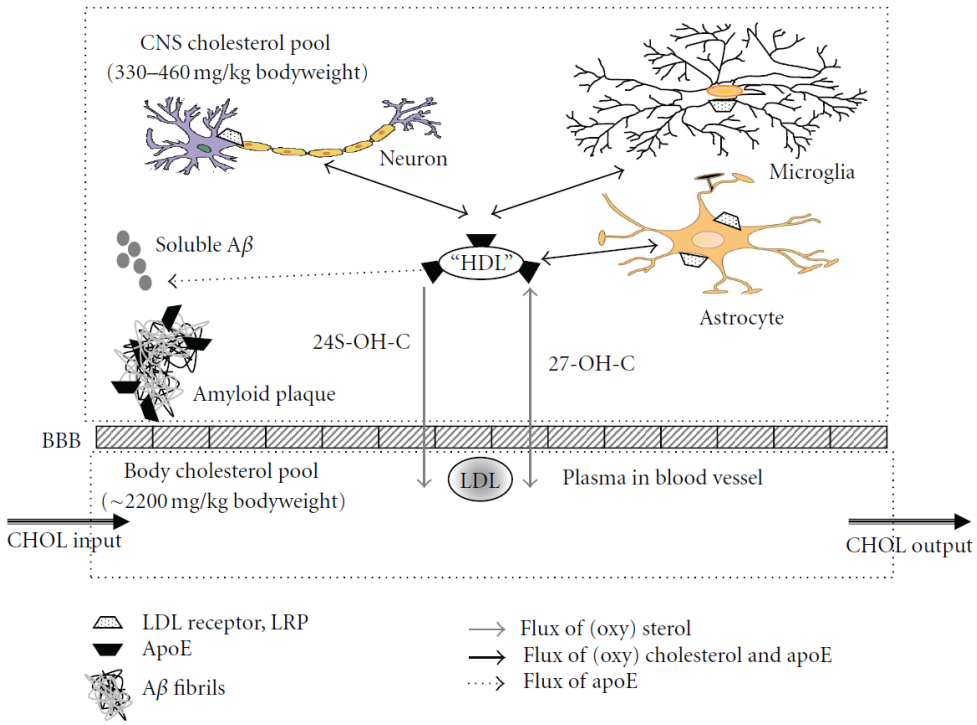

Fig. 3.3: Cholesterol pathway in CNS.
Cholesterol is synthetized by de novo in brain cells. Efflux of CNS cholesterol occurs through Blood Brain Barrier (BBB) as 24 (S) Hydroxcholesterol (24S-OH-C) and 27-hydroxycholesterol (27-OH-C). The flux of cholesterol across the whole body is ~700 mg/day. The flux across the CNS is only 0.9% of whole body (~12 mg/day). The efflux of 24(S)-hydroxycholesterol through the BBB is limited to ~6-7mg per day, the daily influx of 27-hydroxycholesterol into the brain has been estimated to be ~5mg. Reprint from Orth et al, 2012 (14).

3.1.4. **Lipid metabolism's influence on Amyloid beta clearance**

Astrocytes and microglia secrete HDL-like lipoproteins that are composed of phospholipids, cholesterol and apolipoproteins. The major protein component of these CNS-derived lipoproteins is ApoE, which is essential for the interaction with the LDL receptor family, and, thus, for the exchanges of cholesterol among brain cells. A further function of ApoE consists in the modulation of Aβ clearance. Recent *in vivo* studies, have shown that impaired clearance of Aβ, contributes to the development of Alzheimer's disease (140). In particular, availability of cholesterol and ApoE (mainly phenotype e4) promote the formation of amyloid fibrils from soluble Aβ in the CNS (14). Brain ability to clear Aβ diminishes with age and *Batemen et al.* (141) demonstrated that LOAD patients have approximately 30% impairment of this mechanism (5.6% per hour in AD compared to 7.6% per hour in controls). At present, the impact of diseases on Aβ clearance mechanism is not well known. Aβ is cleared from brain by two pathways: (I) endopeptidase-mediated proteolytic degradation, and (II) receptor-mediated endocytosis by cells in the brain parenchyma or through the BBB.

This second way is mediated by apoE and ATP-binding cassette A1 (ABCA1) (14) of which expression is induced by activation of liver X receptor (LXR), peroxisome proliferator-activated receptor gamma (PPARγ), and retinoid X receptor (RXR).

The lipidation of ApoE by ABCA1 stimulates the degradation of Aβ through multiple pathways (Fig. 3.4):

1) extracellular degradation by insulin-degrading enzyme (IDE);

2) uptake by microglial cells and subsequent liposomal degradation by proteases;

3) clearance from the central nervous system by Aβ binding to ApoE receptors such as low-density lipoprotein receptor (LDLR) or LDLR-related protein 1 (LRP1) that mediate clearance by glial cells or transport across the blood-brain barrier. However ApoE and Aβ do not directly interact, but they compete for LRP1 binding, changing the Aβ clearance rate (140). Consequently ApoE-Aβ complex, reduces the clearance rate because this complex is unable to bind LRP1 receptor, but only other members of LDLR family members (140). Furthermore, ApoE ε4 has much lower affinity with the peptides and its association with Aβ is weaker compare to ε2 and ε3 isoforms. These differences among the isoforms lead to a reduced ability of Apo ε4 to clear Aβ with the consequent accumulation of Aβ in senile plaques (142).

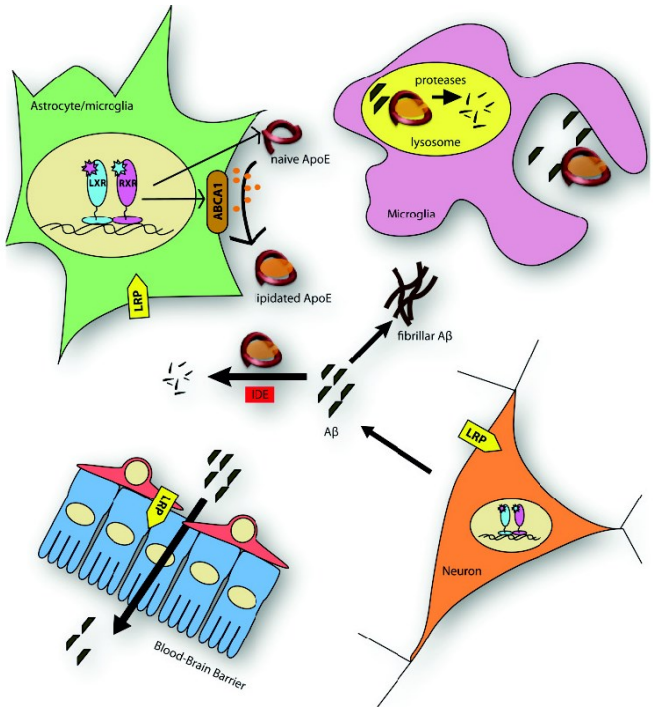

Fig. 3.4: Mechanisms of amyloid beta clearance related to lipid metabolism.

3.2. **Objectives**

Perimenopause transition is characterized by reproductive and neurological senescence that may increase the risk of neurodegenerative disease such as Alzheimer's disease. Perimenopause is also associated with modification of lipid and glucose metabolism.

To better understand the potential mechanism of cholesterol metabolism during perimenopause transition and its relation with neurological degeneration, the present study was focused on the evaluation of major genes involved in cholesterol synthesis, transportation, and clearance. Gene and protein expression were measured in rat models with principal characteristics of human perimenopause.

Furthermore, in order to investigate the concordance of brain data with the systemic metabolites, we have also assayed lipid profile in serum.

Subsequentially, rat's systemic metabolites were compared to systemic lipid levels in a cohort of women during perimenopause transition status.

3.3. Methods

3.3.1. Animal model

Sprague-Dawley female rats aged 9-10 months were ranked into groups according to the stage of ovarian senescence established on the basis of Reproductive Aging Workshop (STRAW) (143,144) bleeding criteria (that characterizes human reproductive aging through menopause) (Fig. 3.5). The endocrine aging groups included: regular cyclers (4-5 day cycles), irregular cyclers (5-8 day cycles), and acyclic (no cycling within 9 days) at 9-10 months. Chronological aging were assessed using 6-months-old regular cycling rats and 16-months-old acyclic rats (145).

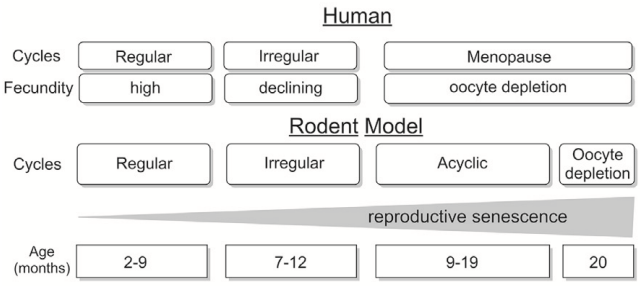

Fig. 3.5: Comparison of main stages of human and rodent reproductive senescence.
In human and rodents, ovarian senescence is associated with decreasing fecundity and increasing irregularity of estrus cycles. Reprint from Yin et al., 2015 ***(83)****.*

3.3.2. Brain tissue collection

Rats were euthanized and the brains were rapidly dissected on ice. The cortical hemisphere was fully peeled laterally, harvested, and frozen in -80 °C for subsequent analyses.

3.3.3. Lipoprotein signalling and cholesterol metabolism gene expression array

Lipoprotein signalling and cholesterol metabolism RT^2 Profiler PCR array was performed on 84 key genes involved in lipoprotein transport and cholesterol metabolism (complete list of genes are reported in Table 3.1.). Total RNA was isolated from the rat cortex (left emisfere) tissue using the PureLink Mini Kit (Invitrogen). The quality and the quantity of RNA were analysed before complementary cDNA synthesis using RT^2 First Strand kit (Quiagen). SYBER®- Green-real time and quantitative polymerase chain reactions (PCR), were performed on 384-well plate, with 10ng cDNA samples mixed with RT^2 SYBER®-Green master mix and the fluorescence was detected on an ABI 7900HT Fast Real-Time

PCR System (Applied Biosystems). Data were analysed using RT^2 Profiler PCR Array Data Analysis version 3.5 (SABioscence-Qiagen). Relative gene expression levels or fold changes relative to the reference group were calculated by the comparative Ct (ΔΔCt) method, with Ct denoting threshold cycle. For each sample, ΔCt was calculated as the difference in average Ct of the target gene and the endogenous control gene. For each group, mean $2^{-\Delta Ct}$ was calculated as the geometric. mean of $2^{-\Delta Ct}$ of all samples in the group. Fold change was then calculated as mean $2^{-\Delta Ct}$ (comparison group)/mean $2^{-\Delta Ct}$ (reference group). The $2^{-\Delta Ct}$ values for each target gene were statistically analyzed by ANOVA followed by pairwise comparisons using Student t test. The statistical significancewas indicated by $p< 0.05$; p-values were not adjusted for multiple testing as discussed previously(146).

Table 3.1: List of gene assesses on rat cortex with lipoprotein signaling and cholesterol metabolism *RT^2profiler (Qiagen)*

Symbol	Description	Gene's name
Abca1	ATP-binding cassette, subfamily A (ABC1), member 1	-
Abca2	ATP-binding cassette, subfamily A (ABC1), member 2	Abc2
Abcg1	ATP-binding cassette, subfamily G (WHITE), member 1	Abc8
Acaa2	Acetyl-Coenzyme A acyltransferase 2	-
Akr1d1	Aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase)	-
Angptl3	Angiopoietin-like 3	-
Ankra2	Ankyrin repeat, family A (RFXANK-like), 2	ANKRA
Apoa1	Apolipoprotein A-I	apoA-I
Apoa2	Apolipoprotein A-II	APOAII/Apo-AII/ApoA-II
Apoa4	Apolipoprotein A-IV	Apo-AIV/ApoA-IV/apoAIV
Apob	Apolipoprotein B	Ac1-060/Apo B-100/ApoB-100/ApoB-48
Apoc1	Apolipoprotein C-I	ALPCI/Apo-CIB/ApoC-IB/LRRG04
Apoc3	Apolipoprotein C-III	ApoC-III/apo-CIII
Apod	Apolipoprotein D	AOPDGN
Apoe	Apolipoprotein E	APOEA
Apof	Apolipoprotein F	-
Apol2	Apolipoprotein L, 2	Apol8
Cdh13	Cadherin 13	Cdht/T-cadherin/Tcad
Cel	Carboxyl ester lipase	Bal/Bssl
Cela3b	Chymotrypsin-like elastase family, member 3B	Ela3b
Cnbp	CCHC-type zinc finger, nucleic acid binding protein	Cnbp1/Znf9
Colec12	Collectin sub-family member 12	-
Cxcl16	Chemokine (C-X-C motif) ligand 16	-
Cyb5r3	Cytochrome b5 reductase 3	Dia1/Nadhcb5/RNNADHCB5
Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1	Cyp11a/Cypxia1/P450scc
Cyp39a1	Cytochrome P450, family 39, subfamily a, polypeptide 1	-

Cyp46a1	Cytochrome P450, family 46, subfamily a, polypeptide 1	-
Cyp51	Cytochrome P450, family 51	Cyp51a1/RATCP14DM
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	CHAP/CYP7/CYP7S1
Cyp7b1	Cytochrome P450, family 7, subfamily b, polypeptide 1	-
Dhcr24	24-dehydrocholesterol reductase	-
Dhcr7	7-dehydrocholesterol reductase	-
Ebp	Emopamil binding protein (sterol isomerase)	-
Fdft1	Farnesyl diphosphate farnesyl transferase 1	-
Fdps	Farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase)	Ac2-125/FPS
Hdlbp	High density lipoprotein binding protein	-
Hmgcr	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	3H3M
Hmgcs1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	-
Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (mitochondrial)	Hmgcs1/Mt3h3mg
Idi1	Isopentenyl-diphosphate delta isomerase 1	-
Il4	Interleukin 4	Il4e12
Insig1	Insulin induced gene 1	-
Insig2	Insulin induced gene 2	-
Lcat	Lecithin cholesterol acyltransferase	-
Ldlr	Low density lipoprotein receptor	LDLRA
Ldlrap1	Low density lipoprotein receptor adaptor protein 1	RGD1563417
Lep	Leptin	OB/obese
Lipe	Lipase, hormone sensitive	HSL
Lrp10	Low-density lipoprotein receptor-related protein 10	-
Lrp12	Low density lipoprotein-related protein 12	-
Lrp1b	Low density lipoprotein-related protein 1B (deleted in tumors)	-
Lrp6	Low density lipoprotein receptor-related protein 6	-
Lrpap1	Low density lipoprotein receptor-related protein associated protein 1	-
Mbtps1	Membrane-bound transcription factor peptidase, site 1	S1p/Ski-1
Mvd	Mevalonate (diphospho) decarboxylase	Mvpd
Mvk	Mevalonate kinase	-
Nr0b2	Nuclear receptor subfamily 0, group B, member 2	Shp
Nr1h2	Nuclear receptor subfamily 1, group H, member 2	OR-1
Nr1h3	Nuclear receptor subfamily 1, group H, member 3	LXRalpha
Nr1h4	Nuclear receptor subfamily 1, group H, member 4	Fxr
Nsdhl	NAD(P) dependent steroid dehydrogenase-like	-
Olr1	Oxidized low density lipoprotein (lectin-like) receptor 1	LOX-1/Oldlr1/Oldr1
Osbpl1a	Oxysterol binding protein-like 1A	-
Osbpl5	Oxysterol binding protein-like 5	-
Pcsk9	Proprotein convertase subtilisin/kexin type 9	NARC-1/Narc1/PC9
Pmvk	Phosphomevalonate kinase	-
Ppard	Peroxisome proliferator-activated receptor delta	Pparb
Prkaa1	Protein kinase, AMP-activated, alpha 1 catalytic subunit	AMPKalpha1
Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit	Ampk/Ampka2
Prkag2	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	-
RGD1564999	Similar to isopentenyl-diphosphate delta isomerase 2	-
Scap	SREBF chaperone	-
Scarf1	Scavenger receptor class F, member 1	-
Snx17	Sorting nexin 17	-
Soat1	Sterol O-acyltransferase 1	Acat-1
Soat2	Sterol O-acyltransferase 2	Acat-2

Sorl1	Sortilin-related receptor, LDLR class A repeats-containing	Lr11/RGD619914
Srebf1	Sterol regulatory element binding transcription factor 1	ADD-1/ADD1/SREBP-1/SREBP-1c/Srebp1
Srebf2	Sterol regulatory element binding transcription factor 2	SREBP-2/SREBP2/Srebf2_retired
Stab2	Stabilin 2	FEEL-2/Hare
Stard3	StAR-related lipid transfer (START) domain containing 3	-
Tm7sf2	Transmembrane 7 superfamily member 2	-
Trerf1	Transcriptional regulating factor 1	-
Vldlr	Very low density lipoprotein receptor	-
Actb	Actin, beta	Actx
B2m	Beta-2 microglobulin	-
Hprt1	Hypoxanthine phosphoribosyltransferase 1	Hgprtase/Hprt
Ldha	Lactate dehydrogenase A	Ldh1
Rplp1	Ribosomal protein, large, P1	-

3.3.4. **Bioinformatics' analysis by Ingenuity Pathway Analysis**

Expression data for genes were analysed by Ingenuity Pathway Analysis core analysis composed of a network analysis (cuts off settings: fold change1.2; p-value 0.05).

The network analysis identified biological connectivity among molecules in the data set that were relatively up-regulated or down-regulated and their interactions with other molecules present in the Ingenuity Knowledge Base. Focus molecules were combined into networks that maximized their specific connectivity (direct and indirect interactions among focus and interacting molecules). Generated networks were ranked by the network score according to their degree of relevance to the network eligible molecules from the data set. The network score was calculated with Fisher's exact test, taking into account the number of network eligible molecules in the network and the size of the network, as well as the total number of network eligible molecules analysed and the total number of molecules in the Ingenuity Knowledge Base that were included in the network. Higher network scores are associated with lower probability of finding the observed number of network eligible molecules in a given network by chance.

The Ingenuity's Upstream Regulator Analysis in Ingenuity Pathway Analysis is a tool that predicts upstream regulators from gene expression data based on the literature and compiled in the Ingenuity Knowledge Base. A Fisher's exact test p-value was calculated to assess the significance of enrichment of the gene expression data for the genes downstream of an upstream regulator. A z-score was given to indicate the degree of consistent agreement or disagreement of the actual versus the expected direction of change among the downstream

gene targets. A prediction about the state of the upstream regulator, either activated or inhibited, was made based on the z-score.

3.3.5. **Protein expression analysis**

Brain cortex tissue was stored at -80°C and it was processed for protein extraction using Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) with phosphatase and protease inhibitors (Sigma, St. Louis, MO, USA). Proteins concentration was determined with Bradford assay (Bio-Rad). Equal amount of proteins (20 µg/well) were loaded to SDS PAGE Criterion gel (Bio-Rad, Hercules, CA, USA) and electrophoresed with Tris/glycine running buffer (pH 8.3). Proteins were transferred to 0.45-mm pore size polyvinylidene difluoride membranes and probed with primary antibodies against: cholesterol 24-hydroxylase (cyp46, Millipore, Darmstadt, Germany.), Lipoprotein repector-11 (LR11, BD transduction laboratories, Franklin Lakes, USA), scavenger receptor class B member 1 and ATP binding cassette 1 (ABCA1), apolipoprotein E (ApoE), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), low density lipoprotein receptor-related protein 1 (LRP1), low density lipoprotein receptor (LDLR), 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2). All the primary antibodies mentioned above were obtained from Abcam (Cambrige, USA).

Horseradish peroxidase-conjugated anti-rabbit antibody, horseradish peroxidase-conjugated anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) and horseradish peroxidase-conjugated anti-goat (Cambrige, USA)were used as secondary antibodies. Immunoreactive bands were visualized with Pierce SuperSignal Chemiluminescent Substrates (Thermo Scientific,Waltham, MA, USA) and captured by Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories). The quantification of the bands obtained was performed using the Image Lab (Bio-Rad) software. All the proteins tested were normalized by the expression of the β-actin as a loading control.

3.3.6. **Perimenopause women cohort**

This study were undertaken within the framework of a protocol approved by the Medical School Ethics Committee, and in accordance with the Declaration of Helsinki (98), and the guidelines for Good Clinical Practice (99). Eligible participants were engaged upon signed informed consent. Participants were 139 caucasian women aged 42-55 years in different

menopausal stages (as assessed according to STRAW criteria (144)), and were enrolled by the Menopause and Osteoporosis Centre of the University of Ferrara (Ferrara, Italy). To confirm the assignment of menopausal status of each woman, blood levels of follicle stimulating hormone and E2 were assessed, by using commercially available chemiluminescent microparticle immunoassays (Architect, Abbott Park, IL, USA). Women under birth control treatment (including vaginal estrogens), and women diagnosed with chronic disease (e.g diabetes, hypertension, etc.) were excluded from the study. Significant younger pre-menopausal and post-menopausal women (40^{th} percentile of pre- and post-menopausal women respectively) were considered as comparing aging effect in pre- and post-menopause groups.

3.3.7. **Serum lipid levels determination**

Concentration of total cholesterol, triglycerides, and cholesterol-HDL were determined enzymatically using commercial kits (Randox-laboratories).

Total cholesterol was determined enzymatically after hydrolysis of cholesterol esters by Cholesterol esterase and oxidation of resulting free cholesterol by cholesterol oxidase, as reported in Fig. 3.6. The hydrogen peroxide formed during the oxidative process in presence of peroxidase and phenol reacts with 4-aminoantipyridine producing quinoneimine, which can be detected spectrophotometrically at 505nm. Final concentrations were calculated based on standard cholesterol samples provided by the kits.

$$Cholesterol\ Ester + H_2O \xrightarrow{Cholesterol\ esterase} Cholesterol + Fatty\ acids$$

$$Cholesterol + O_2 \xrightarrow{Cholesterol\ oxidase} Cholestene - 3 - one + H_2O_2$$

$$H_2O_2 + phenol + 4 - aminoantipyrine \xrightarrow{peroxidase} quinoneimine + 4\ H_2O$$

Fig. 3.6: Total cholesterol assay principle

HDL-cholesterol was assessed on serum samples pre-treated with phosfotunstic acid (0.55mM) that, in presence of magnesium ions (magnesium chloride 25mM), induce the precipitation of LDL, VLDL and chylomicrons fraction. After centrifugation 10 minutes at 8.000xg at RT, the cholesterol present in HDL fraction (supernatant) was assayed as previously described (Fig. 1,16).

Triglycerides concentration was determined after hydrolysis with lipases and spectrophometrically assessed by monitoring the quinoneimine formation as reported in Fig. 3.7.

$$Triglycerides + H_2O \xrightarrow{lipase} glycerol + fatty\ acids$$

$$Glycerol + ATP \xrightarrow{Gk} glycerol - 3 - phosphate + ADP$$

$$glycerol - 3 - phosphate + O_2 \xrightarrow{GPO} \text{dihydroxyacetone} + \text{phosphate} + H_2O_2$$

$$2H_2O_2 + phenol + 4 - aminophenazone + 4\ chloropheno \xrightarrow{POD} quinoneimine + HCl + 4H_2O$$

Fig. 3.7: Triglycerides assay principle.
Gk, Glycerol kinase; GPO, Glycerol-3-Phosphate Oxidase; POD, Peroxidase

3.3.8. **Ketone body (β-Hydroxybutyrate) assay**

β-Hydroxybutyrate (β-HB), which is a ketone body produced by liver from fatty acids as fuel source, was determined by colorimetric kit by Cayman Chemical (Ann Arbor-USA). β-Hydroxybutyrate dehydrogenase oxidized serum β-HB to acetoacetate in presence of NAD^+. The formed NADH, in presence of diaphorase, reacts with colorimetric detector (WST-1) to produce formazan dye which is detectable at 450nm (Fig. 3.8).

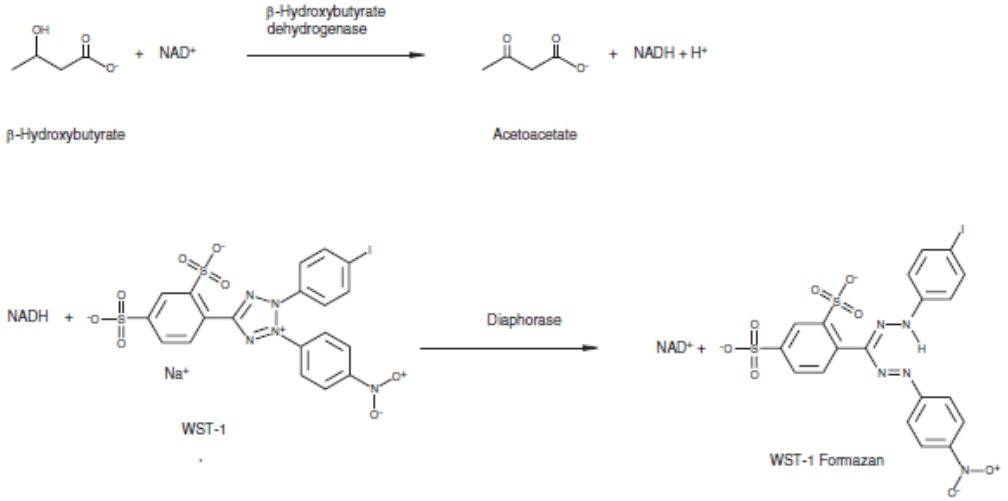

Fig. 3.8: β-Hydroxybutyrate assay principle.
NADH products from β-Hydroxybutyrate oxidation, react with the colorimetric detector colorimetric detector to produced formazan dye detectable at 450nm.

3.3.9. **Statistical analysis**

SPSS 17.00 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Unpaired Student t test (for normal variables), and Mann-Whitney U test (for non-normal variables) were used to identify significant differences in sample groups considered.

A two tailed $p < 0.05$ was considered statistically significant.

3.4. Results

3.4.1. Lipoprotein and Cholesterol metabolism related gene expression in cortex

Lipoprotein signalling and cholesterol metabolism profiles of 84 genes were determined in rats' cortex. Differences in gene expression were analysed by ANOVA followed by pairwise t-tests for each transition period. Heatmaps of gene expression clustered by their function were generated (Fig. 3.9) and significant differences (p <0.05) are summarized in Fig. 3.9.

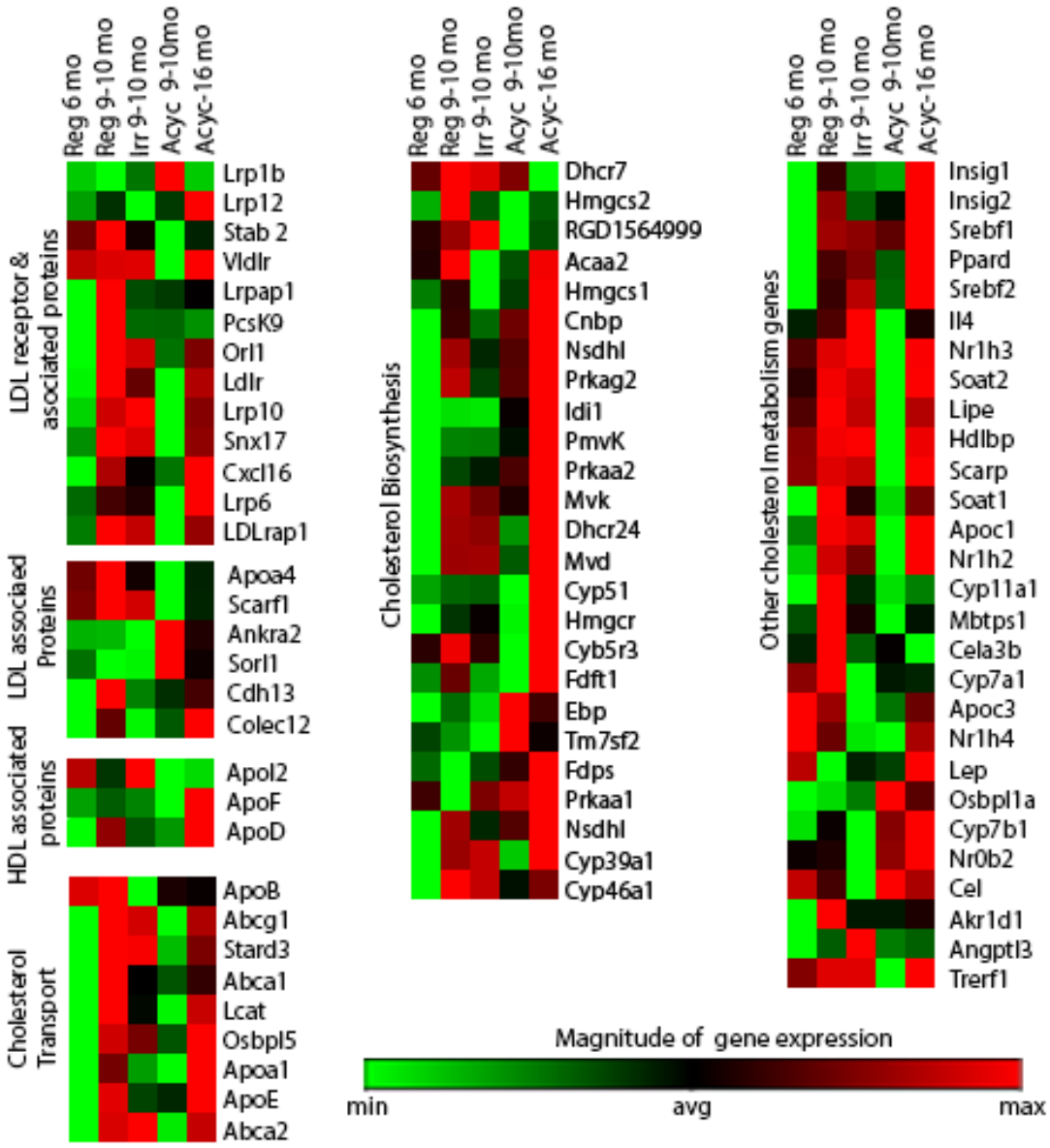

Fig. 3.9: Heat map

Age- and endocrine-related changes were analysed separately. Comparison between regular 6 months and regular 9-10 months-old, was investigated in order to evaluate the effect of chronological aging before the peri-menopausal transition. *The regular 9–10 months* showed a significant up-regulation of genes implied on synthesis of cholesterol (Cyp46a1, Dhcr24, Dhcr7, Mvd), ketogenesis (Hmgcs2), steroid hormone (Cyp11a1, Lipe), LDL receptor and LDL associated proteins (Cxcl16, LDLrap1, Lrp10), and cholesterol transporter (ABCA1, ABCA2, Stard3). *Aging after menopause transition* assessed comparing acyclic 9-10 months and acyclic 16 months, was still associated with an up-regulation of genes, although in a lower number compared to those observed in premenopuase chronological aging. The significant changes involved genes coding for HDL composition (ApoF), LDL internalization (Snx17), Ketogenesis (HMGCS2), cholesterol synthesis (Fdft1), and cholesterol metabolism (Nr1h4). Comparison among 9-10 months-old rats in different endocrine status: regular, irregular, and acyclic reflected the impact of endocrine status independently of chronological aging. Compared with regular group, irregular did not exhibit significant differences while irregular as compared to acyclic showed an up-regulation of gene coding for oxisterol binding protein (Osbpl1a) and down-regulation of LDL receptor associated protein encode by Snx17.

In contrast, considering the comparison between acyclic 9-10 months and the regular one (endocrine aging), all the significant variations consisted in genes down-regulation, with the lone exception of Osbpl1 that it was still up-regulated. In particular, the comparison showed down-regulation of genes implied in cholesterol transport (ABCA1), cholesterol biosynthesis (ABCAA2, Fdft1), cholesterol metabolism (Mbtps1), HDL associated protein (ApoF) and LDL receptor (LDLR, LDLRap1, Scarf1, SNx17), steroid hormone synthesis (Cyp11a1, Lipe), and Ketogenesis (HMGCS2).

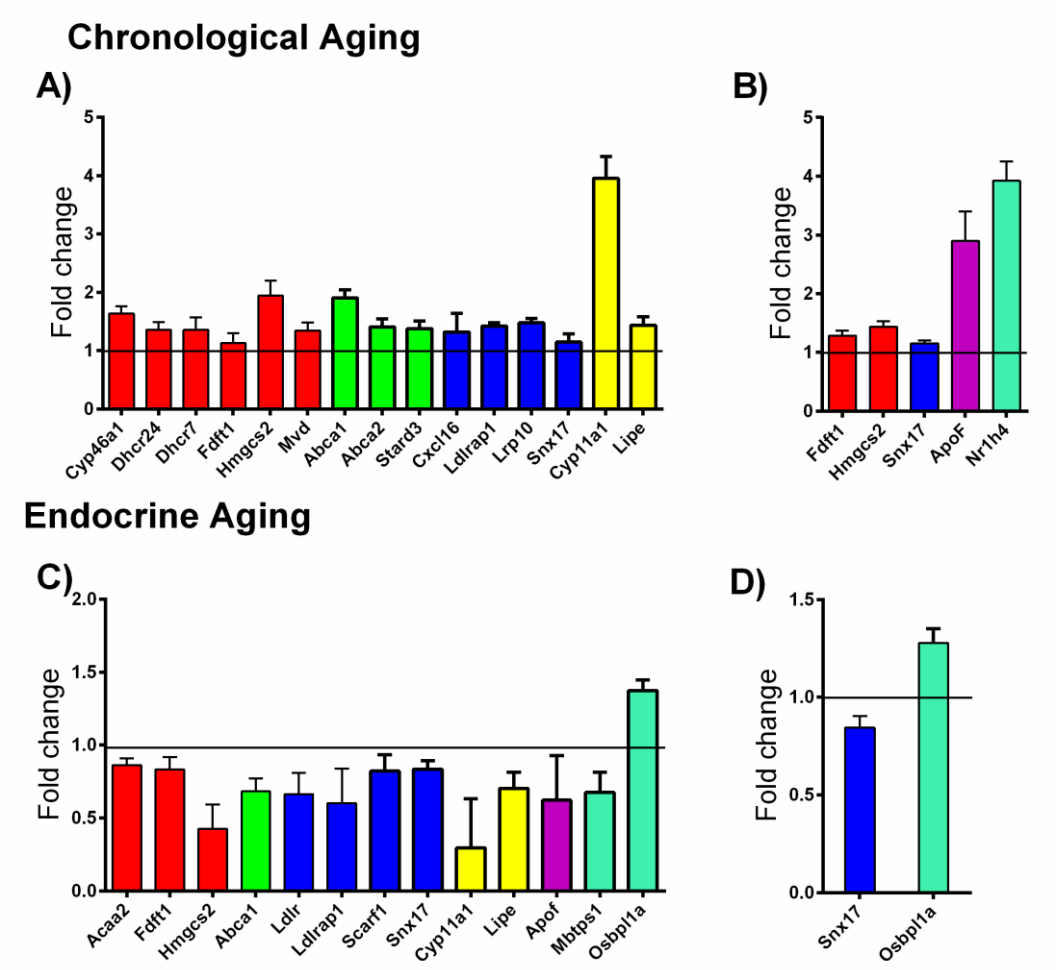

Fig. 3.10: Genes with significant fold change (p<0.05) in different chronological and endocrine transition. *A*) reg 6mo. vs reg 9-10 mo. B) Acyclic 16mo vs Acyclic 9-10mo, C) Acy 9-10mo vs Reg 9-10mo , D) Acyc 9-10mo vs Irr 9-10mo

3.4.2. **Network and pathway analyses identified gene nodes and upstream regulators**

Cortical gene expression data were analysed by the Ingenuity Pathway Analysis (IPA-Qiagen) in order to identify networks of genes and their upstream regulators (
Table 3.2).

The network analysis revealed known interactions among genes in the dataset and predicted interactions from the knowledge base. Thirteenth networks related to lipid metabolism were identified within chronological and endocrine transition. The highest-scoring for each comparison were ranked by the score and presented in

Table 3.3.

The scores, the number of focus proteins, and the size of the network were used to define the relevance of the networks. The first ranked network, which consisted of 23 focus molecules and 12 interconnected molecules (proteins that were not assayed by the array), was then subjected to "Function and Diseases" analysis by IPA software focused on cholesterol biosynthesis, uptake, efflux, and export. Subsequently, all transition analysis were overlaid on the network with disease outcome prediction and compared each other.

The functional and diseases analysis showed two different predicted effects during chronological and endocrine transition.

Aging pre- and post- menopause showed activation of cortical cholesterol synthesis, export and efflux; on the other hand, endocrine transition seemed to be associated to increase in cholesterol uptake and simultaneous decrease in cholesterol synthesis, efflux, and export (Table 3.4).

Table 3.2: Upstream regulators identified by IPA

	Upstream regulator	Predicted activation state (If predicted)
Reg 9-10 mo vs Reg 6 mo	INSIG1	Inhibited
	NR1H3	Activated
	SCAP	
	NR1H2	
	SREBF1	
	Cyp7a1	
Acyc 16 mo vs Acyc 9 mo	NR1I2	
	Isoquercitin	
	GW 4064	
	Chenodeoxycholicacis	
	INSIG11	
Irr 9 mo vs Reg 9 mo	No molecules eligible for the analysis	
Acyc 9 mo vs Irr 9 mo	INSIGN1	Activated
	Cholesterol	
	Sterol	
	Pioglitazone	
	ApoE	
Acyc 9 mo vs Reg 9 mo	SREBF2	Inhibited
	INSIG1	Activated
	SCAP	Inhibited
	PPARA	
	POR	

Table 3.3: Top three networks involved in lipid metabolism, generated by IPA analyzing chronological and endocrine transition
Networks are ordered by score.

Comparison	Score	Focus Proteins	Molecules
Reg 9-10 mo vs Acy 9-10 mo	51	23	ANGPTL3, APOA2, APOA4, APOB, Apoc3, APOD, APOF, arginase, CEL, Collagen Alpha1, CYP46A1, ERK1/2, FDFT1, FDPS, HDL-cholesterol, HMG CoA synthase, INSIG1, INSIG2, LCAT, LDL-cholesterol, lipoprotein lipase, LRP, LRPAP1, MBTPS1, NCOR-LXR-Oxysterol-RXR-9 cis RA, NR1H3, OLR1, PCSK9, SCAP, SOAT1, SOAT2, Srebp, STAB2, VLDL, VLDL-cholesterol
Irr 9-10 mo vs Acy 9-10 mo	49	20	ABCA2, ABCG1, APOA4, Apoc1, Apoc3, APOF, CYP46A1, CYP7A1, ERK1/2, HDL, HDL-cholesterol, HMG CoA synthase, LDL-cholesterol, LDLR, LDLRAP1, LXR ligand-LXR-Retinoic acid-RXRα, MBTPS1, NCOR-LXR-Oxysterol-RXR-9 cis RA, NR0B2, Nr1h, NR1H2, NR1H3, OLR1, PEPCK, Rxr, SCAP, SOAT2, sPla2, SREBF2, Srebp, STAB2, T3-TR-RXR, TM7SF2, VLDL, VLDL-cholesterol
Reg 6 mo vs Reg 9-10 mo	47	19	ABCA1, ABCA2, ABCG1, ANGPTL3, APOA2, Apoc1, APOD, CYP46A1, ERK1/2, HDL, HDL-cholesterol, HMG CoA synthase, LCAT, LDL-cholesterol, LDLR, LDLRAP1, lipoprotein lipase, LXR
Acy 9-10 mo vs acy 16 mo	12	4	Akr1b7, AMBP, APOA2, APOA5, APOC2, APOC3, APOF, APOM, BAAT, bile salt, CETP, CPT2, CYP2B6, CYP8B1, FABP, FDFT1, FXR ligand-FXR-Retinoic acid-RXRα, HMGCS2, LCAT, LDL, LIPC, MTTP, NPC1L1, NR1H4, Orm1 (includes others), PEX5L, PLTP, PON1, PPARA, pyruvate kinase, RXRG, SLC10A2, SULT2A1, taurochenodeoxycholate, UGT2B4

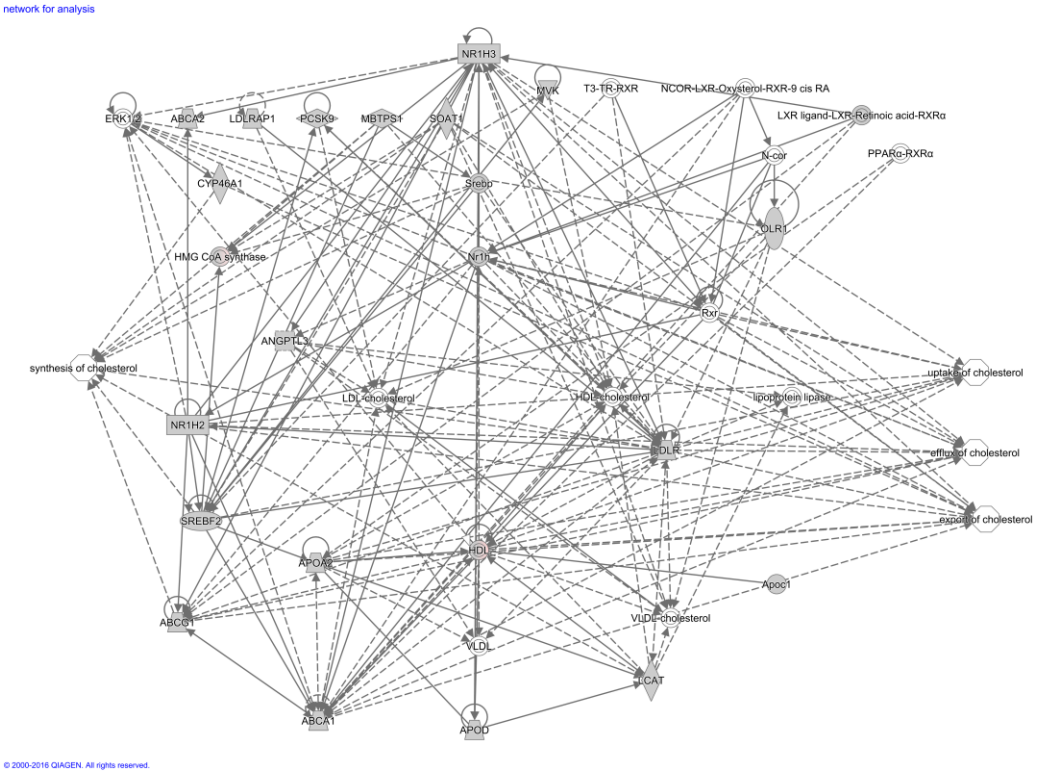

Fig. 3.11: IPA network selected

Table 3.4: Resume of predicted disease and function outcomes.
"+" activation; "-" inhibition.

	Chronological aging		Endocrine aging		
	Reg 9-10 mo vs Acy 9-10 mo	Acy 9-10 mo vs acy 16 mo	Irr 9-10 mo vs Acy 9-10 mo	Acy 9 mo vs Irr 9 mo	Reg 6 mo vs Reg 9-10 mo
Cholesterol biosynthesis	+	-	+	n.s.	-
Cholesterol uptake	-	+	-	n.s.	+
Cholesterol efflux	+	-	+	n.s.	-
Cholesterol export	+	-	+	n.s.	-

1.1.2 Modification in cholesterol synthesis

The protein levels of HMGCR, the rate limiting enzyme in cholesterol synthesis, were decreased in post-menopause aging (p=0.045, Acy 16 mo vs Acyc 9 mo), while gene expression did not show any significant differences. No variations were observed in association with endocrine transition.

According with pathway prediction gene expression, the levels of protein HMGCS2 (enzyme indirectly involved in cholesterol synthesis) exhibited significant variation in relation to *endocrine aging* (less marked differences than those detected for gene expression). The most evident difference was the decrease related to endocrine decline (comparison between irregular and acyclic 9-10 months). Taken together HMGCS2 and HMGCR, data could better explain a possible pathway modulation.

According with bioinformatics' prediction from gene array, endocrine transition overall exhibited a down-regulation of cholesterol synthesis, as shown by the significant reduction of HMGCS2 protein levels.

Moreover, during pre-menopause aging both HMGC2 and HMGCR showed an increased trend of gene and protein expression levels, in a way consistent with the prediction of cholesterol synthesis activation.

Conversely, within postmenopausal aging the results obtained for protein levels did not support the activation of cholesterol synthesis, since HMGCR protein levels were inversely correlated with aging.

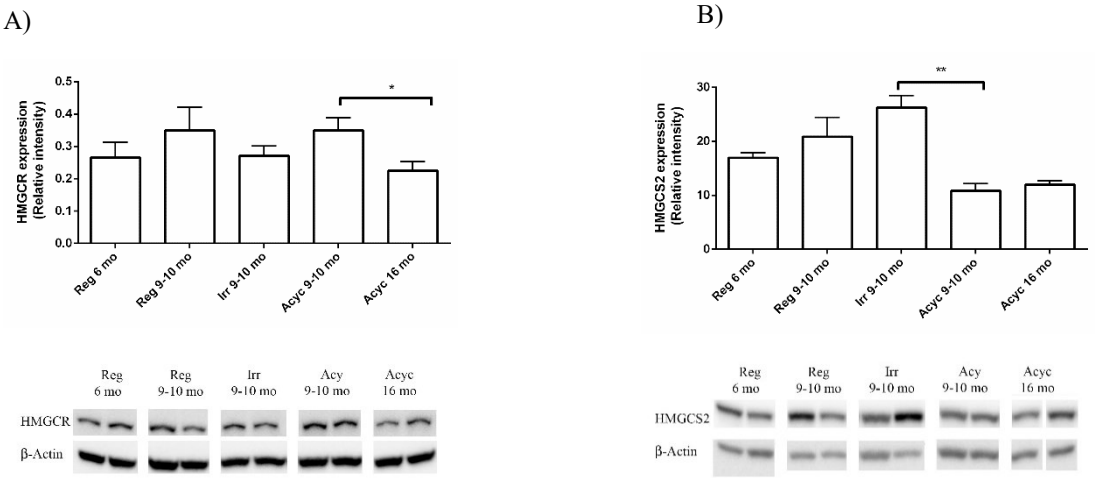

Fig. 3.12: Expression of proteins involved in cholesterol synthesis in rat cortex.
*6-months-oldr regular cycling rats, Reg 6 mo; regular 9- to 10-months-old, Reg 9-10 mo; irregular 9- to 10-months-old, Irr 9-10 mo; acyclic 9- to 10-months-old, Acyc 9-10 mo; 16-months-old acyclic rats, Acyc 16mo. (A) HMGCR 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR). (B) HMGCR 3-hydroxy-3-methylglutaryl-CoA sinthetase2 (HMGCS2). Data were presented as average± standard error of the mean, *p< 0.05, **p<0.01; n =4-5. Representative blot images were shown under the bar graphs.*

3.4.3. **Cholesterol efflux and transport**

The following step of our investigation was the analysis of proteins involved in other aspects of lipid and cholesterol metabolism: cholesterol efflux and mobilization in rat cortex. The proteins examined in this frame were ABCA1, involved in cholesterol efflux, and APOE, that plays an important role in cholesterol transport.

Although ABCA1 gene was up-regulated in older rats within chronological aging group, the protein expression showed only an increasing trend. Conversely, during endocrine transition, there was as significant decrease in both gene and protein expression.

The protein levels of ApoE, the mainly component of HDL-lipoproteins in CNS, was down-regulated only during postmenopausal chronological aging, thus suggesting a reduced efficiency of cholesterol mobilization with perimenopause transition.

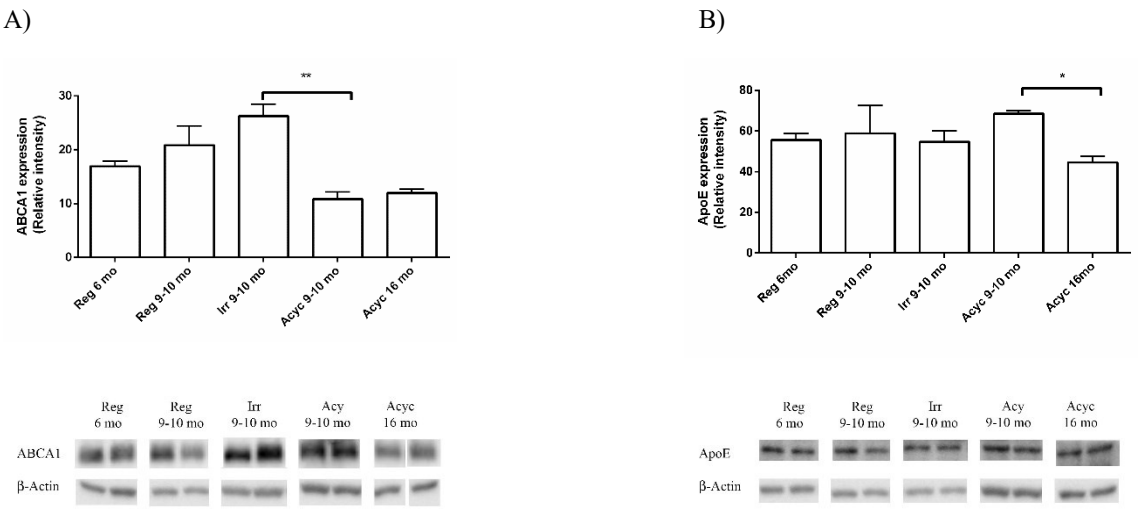

Fig. 3.13: Expression of proteins involved in cholesterol transport and efflux in rat cortex.
*6-months-oldr regular cycling rats, Reg 6 mo; regular 9- to 10-months-old, Reg 9-10 mo; irregular 9- to 10-months-old, Irr 9-10 mo; acyclic 9- to 10-months-old, Acyc 9-10 mo; 16-months-old acyclic rats, Acyc 16mo.. (A) ATP-binding cassette transporter A1 (ABCA1). (B) Apolipoprotein E (APoE). Data were presented as average± standard error of the mean, *p< 0.05, **p<0.01; n =4-5. Representative blot images were shown under the bar graphs.*

3.4.4. **Cholesterol uptake/clearance**

Four members of LDL receptor family proteins were assessed in order to investigate modulation in ApoE binding for cholesterol cells uptake. Consistently with previous results, LRP1 protein levels were lower regular 9-10 months rats than in age-matched acyclic ones. Contrariwise, ApoE receptor LR11 was down-regulated in association with aging, only within postmenopausal chronological groups (Acyclic 16mo vs acyclic 9-10mo), while LDLR did not show any protein modulation.

3.4.5. **Cholesterol clearance via oxysterol production**

The modulation of oxysterol production, as possible clearance pathway, was investigated by evaluating cholesterol 24-hydrossilase (Cyp46), enzyme that catalyzes the synthesis of 24S-OH-C. A raise in 24S-OH-C serum levels has been widely found in association to Alzheimer's disease. These data can be explained by its unique propriety to cross the BBB and the fact that 24S-OH-C is the only brain-derived cholesterol metabolite present in the systemic circulation.

Regarding Cyp46, we found no significant changes in gene expression levels, although the protein levels decreased during chronological post-menopausal aging andalso during endocrine transition.

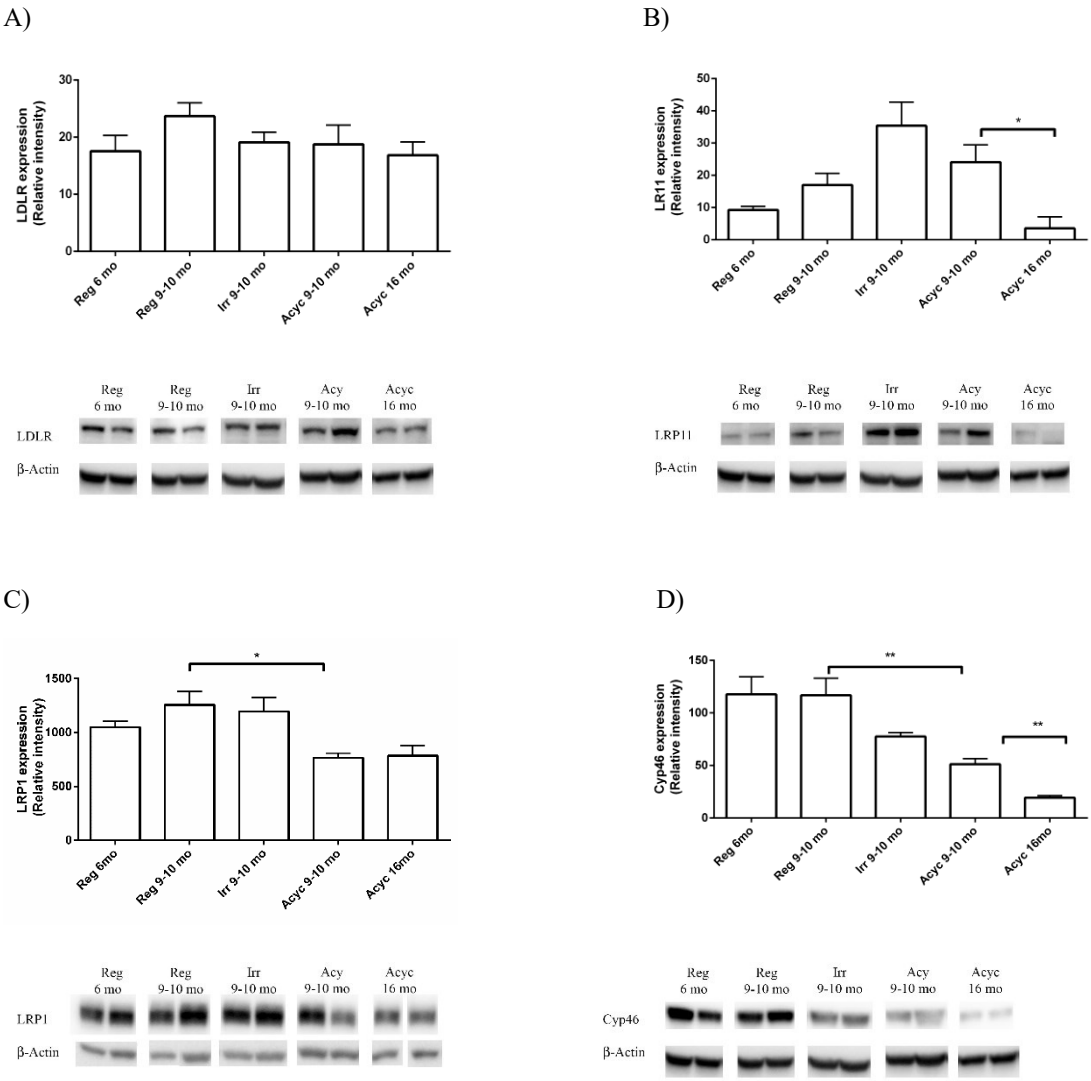

Fig. 3.14: Expression of proteins involved in cholesterol uptake and clearence in rat cortex.
*6-months-oldr regular cycling rats, Reg 6 mo; regular 9- to 10-months-old, Reg 9-10 mo; irregular 9- to 10-months-old, Irr 9-10 mo; acyclic 9- to 10-months-old, Acyc 9-10 mo; 16-months-old acyclic rats, Acyc 16mo. (A) LDL receptor (LDLR)protein expression. (B) Sortilin-related receptor SorLA/LR11 (LR11) protein expression. (C) Low density lipoprotein receptor-related protein 1 (LRP1) protein expression. (D) Cholesterol 24-hydroxylase (Cyp46)protein expression. Data were presented as average± standard error of the mean, *p< 0.05, **p<0.01; n =4-5. Representative blot images were shown under the bar graphs.*

3.4.6. **Serum lipid profile in rats**

As shown in Table 3.5, endocrine transition (9 months with different endocrine stages) was not associated with significant variations in: total cholesterol, triglycerides, HDL, LDL, and ketone bodies; on the other hand, chronological aging showed significant association involving some of the lipid parameters.

More specifically, pre-menopause aging was associated to a increasing levels of total cholesterol and LDL and a fall in ketone bodies. Furthermore, during post-menopausal aging there was an increase of triglycerides levels.

Table 3.5: The perimenopausal rodent model characteristics

Groups	Reg 6 mo	Reg 9-10 mo	Irr 9-10 mo	Acy 9-10 mo	Acy 16 mo
Cycling status	Regular	Regular	Irregular	Acyclic	Acyclic
Number of animals (n)	5	5	5	4	4
Age (months)	6	9-10	9-10	9-10	16
Body weight (g)	260 ± 4$^{\$}$	284 ± 8	302 ± 7	298 ± 10	294 ± 5
Serumlipidparameters:					
Chol-Tot (mg/dL)	101.5 ± 4.5	124.8 ± 8.6*	114.0 ± 7.4	125.6 ± 16.7	122.3 ± 7.8
Tryglicerides (mg/dL)	53.8 ± 10.5	75.8 ±11.5	63.6 ± 11.7	55.7 ± 9.6	130.7 ± 57.0$^{\#}$
Chol-HDL (mg/dL)	66.7 ± 2.5	62.8 ± 6.4	54.1 ± 5.2	60.7 ± 4.3	60.8 ± 4.3
Chol-LDL (mg/dL)	24.4 ± 5.6	46.78 ±11.9*	47.2 ± 11.5	53.7 ±18.9	53.7 ±18.9
Ketone bodies (mM)	0.24 ± 0.02	0.19 ± 0.02*	0.16 ± 0.02	0.22 ±0.03	0.22 ±0.03

*Rat model Data are presented as average ± standard error of the mean, t- test were performed between Reg6 mo –Reg 9-10 mo and between acy 9-10 mo- acy 16 mo. ANOVA analysis were performed between Regular, irregular and acyclic at 9-10 of age. Significant differences were reported as follow: $, p<0.05 vs each of the other group; *p<0.05 vs reg 6 mo; #p<0.05 vs acy9mo.*

3.4.7. **Serum lipid profile in women**

As shown in *Table 3.6*, women were stratified according to reproductive stages. According with this classification, 17-β-estradiol and follicle stimulating hormone levels decreased and increased, respectively, passing from fertile period (pre-menopause) to reproductive senescence (peri- and post-menopause). Anthropometric parameters (BMI, waist, and hip circumference) and lipid profile levels, did not exhibit significant variations in the cohort examined.

As in the rat model, endocrine transition (which was not characterized by significant differences in age between groups) was not associated with significant changes in lipid profile. Inversely, there was an increasing trend in total and LDL-cholesterol levels in pre-menopause aging group (Mann-Whitney 2-tailed p=0.053 and p=0.061 respectively) and a milder increase in triglycerides in aging after menopause (Mann-Whitney 2-tailed p=0.090).

Table 3.6: Perimenopausal women samples characteristics.

Groups	Y Pre	Pre	Peri	Y Post	Post
Number of subjects (n)	21	24	33	27	34
Age (years)	43 (42-44) $	43 (42-44) *$	48 (47-51) *$	49 (49-50) *$	53 (53-55) *
Smoking (n, %)	0 (0)	0 (0)	7 (21.2)	3 (11.1)	2 (5.9)
Hypertension (n, %)	2 (10)	3 (12.5)	4 (12.1)	6 (25)	5 (14.7)
Antropometricparameters:					
BMI (kg/m2)	23 (21-23)	23 (21-25)	24 (22-28)	24 (22-26)	24 (22-26)
waist (cm)	78 (74-82)	76 (74-84)	83 (77-90)	83 (76-92)	87 (78-93)
Hip (cm)	99 (93-104)	98 (95-101)	99 (95-108)	103 (94-108)	101 (96-105)
Waist to hip ratio	0.77 (0.75-0.86)	0.81 (0.78-0.90)	0.82 (0.79-0.86)	0.82 (0.77-0.87)	0.85 (0.81-0.93)
Sex hormones:					
E2 (pg/mL)	81.1 (43.3-278.75)	115.0 (15.0-170.0)	20.0 (8.0-64.0)	8.0 *# (8.0-18.0)	10.0 *# (8.0- 15.0)
FSH (mIU/mL)	6.2 (3.8-9.7)	6.7 (4.2-12.9)	63.3 *# (30.3-88.4)	67.7 *# (51.9-87.5)	73.9 *# (48.3-92.6)
Serumlipidparameters:					
Chol-Tot (mg/dL)	171.8 (144.4-204.7)	205.0 (168.8-234.0)	209.0 (181.1-233.9)	200.0 (176.0-252.0)	209.0 (163.7-239.3)
Tryglicerides (mg/dL)	56.5 (50.7-76.0)	61.5 (52.3-82.3)	74.0 (50.5-90.5)	65.9 (43.0-99.0)	74.7 (58.4-104.0)
Chol-HDL (mg/dL)	49.8 (41.7-64.5)	68.0 (48.8-80.3)	61.0 (54.5-73.0)	57.6 (50.0-95.6)	62.0 (50.8-73.5)
Chol-LDL (mg/dL)	97.7 (57.9-120.8)	127.5 (84.4-127.5)	123.7 (101.7-147.1)	135.6 (77.2-171.2)	130.9 (87.9-153.5)

*Data are presented as median ± interquartile range. Y Pre, young premenopausal women (40 percentile of all premenopausal group); Pre, premenopausal women; Peri, perimenopause women, Y Post, young Postmenopausal women (40 percentile of all postmenopausal group); Post, postmenopausal women; BMI, body mass index; E2, 17,β-estradiol; FSH, follicle stimulating hormones. Kruscal-Wallis or Mann Whitney were performed. The significant level is 0.05; * vs young premenopausal women; # vs premenopausal women; $ vs post menopause women*

3.5. **Discussion**

Growing epidemiological evidence has shown higher prevalence of AD in women than men. Some researchers suggest that these data could reflect gender differences in brain bioenergetics system. In support of this hypothesis, previous studies reported that the mitochondrial respiration capacity decreased with aging and during reproductive senescence in AD model mice (3xTgAD). Furthermore, this variation was accompanied by changes in brain energetic metabolism, especially during reproductive senescence; indeed, glucose consumption, the only fuel source in young brain, dropped by 96% and 66% in the brain of aged healthy controls and AD, respectively. Notably, ketonic bodies, were used as compensatory fuel source during the early phases of the disease, but their availability declined with disease progression.

The above described scenario along with previous studies reporting a reduction of cholesterol levels in cortex and hippocampal areas in AD patients prompted us to explore the modulation of lipid, in particular cholesterol, metabolism during chronological aging and endocrine perimenopause transition.

For this study the modification of brain lipid homeostasis linked to aging and perimenopausal transition was explored in rat models. The 6-months-old regular cyclers correspond to STRAW stage 4 of regular cycles and peak fecundability; the 9-10-months-old irregular cyclers with prolonged cycles correspond to declining fecundability with increased cycle irregularity (STRAW stage 3b); 9- to 10-months-old acyclic may correspond to increased anovulatory cycles (STRAW Stage 2); and the 16-months-old acyclic rodents correspond to postmenopausal STRAW stage 0 (143). Chronological aging was analysed during fertile period (reg 9mo vs reg 6mo) or after menopause (acy 16mo vs acy 9-10mo), while modification during perimenopause transition (endocrine aging) have been studied in age-matched rats (9-10 months-old) with different endocrine states (regular, irregular and acyclic).

Our preliminary data showed that cholesterol metabolism changes in rat brain cortex in association with both chronological and endocrine aging, but in opposite ways. Chronological aging appeared to be related with an increase of cholesterol synthesis accompanied by up-regulation of transporters and proteins for HDL and LDL binding. These modifications were emphasized effect during the fertile than postmenopausal period.

Conversely, estrogen decline was associated to a down-regulation of the cholesterol metabolism pathways with a significant reduction in ABCA1. In a previous study, ABCA1 deletion was associated with increased Aβ deposition (140) and knock-out mice exhibited reduced ApoE and cholesterol levels in neurons and glia cells compared with ABCA1 (+/+) (142). In apparent contradiction with this reported evidence, our data did not show a connection between endocrine transition and ApoE, but only a change (decrease) associated to aging during postmenopausal period. This result in combination with the observed reduction in HMGCR expression suggest that the impairment of both synthesis and delivery cholesterol is not related to endocrine change but only to aging.

Histological evidence, in animal model, showed an inverse association between the activity of Cyp46 enzyme and Aβ deposition in the medial temporal lobe (147). Cyp46 is the main enzyme involved in the conversion from cholesterol to 24S-OH-C, which represents the only form of the lipid able to directly cross the BBB. Our data, showing a decrease Cyp46 levels during perimenopause endocrine transition and post-menopausal chronological aging, suggest a progressive accumulation of cholesterol in brain tissue.

Several experimental evidences clearly suggest that the processes underlying the clearance of Aβ could represent an important therapeutic target. It is well recognized that Aβ clearance is tightly related to lipid catabolism in the brain. Our data showed that endocrine transition may be associated with an impaired Aβ clearance caused by significant lower levels of LRP1, APOE receptor involved in Aβ degradation.

Although the metabolism of cholesterol in the brain is different from that of the rest of the body, it is true that the two pathways influence each other. Indeed, any modifications in lipid profile composition alter oxygen and nutrients supply to the brain. Thus, the high levels of triglycerides, total, and LDL cholesterol that was observed to be associated with aging, but not with endocrine transition, may have important repercussions in brain health. Alteration in lipid profile are, indeed, well recognized risk factors for atherosclerosis and strokes, that, in turn, are also closely linked to AD (and VAD) risk. (136,150). We found that increase of serum cholesterol due to chronological aging appears not to be influenced by estrogen decline in both humans and rat models.

In conclusion, although the limitations, our study show that estogens decline could be associated to a primary modification of cholesterol metabolism in rats' cortex and this decline could increase the risk for neurodegenerative diseases. Moreover, the increase of

serum cholesterol levels due to chronological aging appears to be influenced by aging but not by estrogen decline in both humans and rat models.

General Conclusion

Taken together the data collected in our two separate studies on humans and animal model suggest that:

- ✓ Redox imbalance is an early event in AD;
- ✓ A reduction in PON-1 activity is precociously associated with LOAD, VaD and with the risk of clinical progression from pre-clinical to clinical phase of dementia;
- ✓ Aging greatly influence lipid metabolism in both systemic and brain compartment.

In our view, these results are only apparently disconnected to each other, mostly if the multifactorial nature of PON-1 is considered.

PON-1 is the main responsible of the HDL ability to act as antioxidant, anti-aterosclerotic and anti-inflammatory agent as well as to regulate cholesterol efflux from macrophages (148). Thus, an impairment of PON-1 activity could be ascribed as possible cause of the derangement in redox balance seen in MCI and demented patients. Besides, considering the hypothesised cross-talk between systemic and CNS cholesterol, a low catalytic efficiency of PON-1 might also have negative effects on brain cholesterol homeostasis.

Furthermore, we observed that aging and estrogen decline during perimenopause transition might exacerbate cholesterol dysregulation in female brain. Since, previous i*n vitro* data demonstrated a possible modulation of PON-1 by oestrogen, future studies should be aimed to better elucidate if antioxidant activity and cholesterol efflux mediated by PON-enzymes could influence cholesterol metabolism both in plasma and in CNS.

Bibliography

1. WHO. Dementia. Media Center- Fact Sheet n.362. 2015 [cited 2016 Feb 3]. Available from: http://www.who.int/mediacentre/factsheets/fs362/en/
2. Alzheimer's Disease International. World Alzheimer Report 2015.The Global Impact of Dementia.An analysis of prevalence, incid ence, cost and trends. 2015.
3. Petersen RC, Morris JC. Mild cognitive impairment as a clinical entity and treatment target. Arch Neurol. 2005;62(7):1160–3;1167.
4. Hippius H, Neundörfer G. The discovery of Alzheimer's disease. Dialogues Clin Neurosci. 2003;5(1):101–8.
5. NIH. Alzheimer's Disease. NIH Publ. 2015;15-6423:1–8.
6. Thinakaran G, Koo EH. Amyloid precursor protein trafficking, processing, and function. J Biol Chem. 2008;283(44):29615–9.
7. Swerdlow RH. Pathogenesis of Alzheimer's disease. Clin Interv Aging. 2007;2(3):347–59.
8. Querfurth HW, Laferla FM. Alzheimer's Disease. N Engl J Med. 2010;362:329–44.
9. Moreira PI, Zhu X, Smith MA, Perry G. Alzheimer's Disease: An Overview. In: Encyclopedia of Neuroscience. Elsevier; 2009. p. 259–63.
10. Wang Z-X, Tan L, Liu J, Yu J-T. The Essential Role of Soluble Aβ Oligomers in Alzheimer's Disease. Mol Neurobiol. 2015;
11. Swerdlow RH, Khan SM. A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. Med Hypotheses. 2004;63(1):8–20.
12. Coppedè F, Migliore L. DNA damage in neurodegenerative diseases. Mutat Res Mol Mech Mutagen. Elsevier B.V.; 2014;776:84–97.
13. Cervellati C, Wood PL, Romani A, Valacchi G, Squerzanti M, Sanz JM, et al. Oxidative challenge in Alzheimer's disease: state of knowledge and future needs. J Investig Med. 2015;0:1–12.
14. Orth M, Bellosta S. Cholesterol: Its Regulation and Role in Central Nervous System Disorders. Cholesterol. 2012;2012:1–19.
15. Poirier J, Miron J, Picard C, Gormley P, Théroux L, Breitner J, et al. Apolipoprotein E and lipid homeostasis in the etiology and treatment of sporadic Alzheimer's disease. Neurobiol Aging. Elsevier Ltd; 2014;35:S3–10.
16. Mahley RW, Weisgraber KH, Huang Y. Apolipoprotein E: structure determines function, from atherosclerosis to Alzheimer's disease to AIDS. J Lipid Res.

2008;50:S183–8.

17. Larson IA, Ordovas JM, DeLuca C, Barnard JR, Feussner G, Schaefer EJ. Association of apolipoprotein (Apo)E genotype with plasma apo E levels. Atherosclerosis. 2000;148(2):327–35.
18. Riddell DR, Zhou H, Atchison K, Warwick HK, Atkinson PJ, Jefferson J, et al. Impact of Apolipoprotein E (ApoE) Polymorphism on Brain ApoE Levels. J Neurosci. 2008;28(45):11445–53.
19. Liu C-C, Liu C-C, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat Rev Neurol. 2013;9(2):106–18.
20. Zhang S, Bai Y, Luo L, Xiao W, Wu H, Ye P. Association between serum homocysteine and arterial stiffness in elderly : a community-based study. 2014;32–8.
21. Ganguly P, Alam SF. Role of homocysteine in the development of cardiovascular disease. Nutr J. 2015;14:6.
22. Flemming KD, Jones LK. Mayo Clinic Neurology Board Review: Clinical Neurology for Initial Certification and Moc. Oxford University Press; 2015.
23. Iadecola C. The Pathobiology of Vascular Dementia. Neuron. Elsevier Inc.; 2013;80(4):844–66.
24. Cordonnier C, van der Flier WM. Brain microbleeds and Alzheimer's disease: innocent observation or key player? Brain. 2011;134(2):335–44.
25. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology. 1984;34(7):939–44.
26. Jacobs AH, Winkler A, Castro MG, Lowenstein P. Human gene therapy and imaging in neurological diseases. Eur J Nucl Med Mol Imaging. 2005;32(14):S358–82.
27. Yaari R, Corey-Bloom J. Alzheimer's disease. Semin Neurol. 2007;27(1):32–41.
28. Jayakar JP, Huang J. Alzheimer's disease: a review of diagnostic criteria. Univ West Ont Med J. 2010;79(2):7–12.
29. Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L. Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. Lancet Neurol. 2006;5(3):228–34.
30. Hogervorst E, Bandelow S, Combrinck M, Irani SR, Irani S, Smith AD. The validity

and reliability of 6 sets of clinical criteria to classify Alzheimer's disease and vascular dementia in cases confirmed post-mortem: added value of a decision tree approach. Dement Geriatr Cogn Disord. 2003;16(3):170–80.

31. Bergamini CM, Gambetti S, Dondi A, Cervellati C. Oxygen, Reactive Oxygen Species and Tissue Damage. Curr Pharm Des. 2004;10(14):1611–26.
32. Kosenko E, Solomadin I, Tikhonova L, Reddy V, Aliev G, Kaminsky Y. Pathogenesis of Alzheimer Disease: Role of Oxidative Stress, Amyloid beta Peptides, Systemic Ammonia and Erythrocyte Energy Metabolism. CNS Neurol Disord - Drug Targets. 2014 Feb 31;13(1):112–9.
33. Fenton HJH. Oxidation of tartaric acid in presence of iron. J Chem Soc Trans. 1894;65:899.
34. Koppenol WH. The Haber-Weiss cycle--70 years later. Redox Rep. 2001;6(4):229–34.
35. Radák Z. Free Radicals in Exercise and Aging. 2000.
36. Bedard K, Krause K-H. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev. 2007;87(1):245–313.
37. Hamanaka RB, Chandel NS. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends Biochem Sci. Elsevier Ltd; 2010;35(9):505–13.
38. Brand MD. The sites and topology of mitochondrial superoxide production. Exp Gerontol. 2011;45(7-8):466–72.
39. Murphy MP. How mitochondria produce reactive oxygen species. Biochem J. 2009;417(1):1–13.
40. Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch Biochem Biophys. 1985 Mar;237(2):408–14.
41. Halliwell B, Gutteridge J. Free radicals in biology and medicine. fourth. Bioscience Oxford; 2012.
42. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39(1):44–84.
43. Halliwell B. Tell me about free radicals, doctor: a review. J R Soc Med. 1989;82(12):747–52.

44. Sies H. Oxidative stress: oxidants and antioxidants. Exp Physiol. 1997;82(2):291–5.
45. Lu SC. Glutathione synthesis. Biochim Biophys Acta - Gen Subj. 2013;1830(5):3143–53.
46. Carter DC, Ho JX. Structure of serum albumin. Adv Protein Chem. 1994;45:153–203.
47. Prakash M, Shetty MS, Tilak P, Anwar N. Total Thiols: Biomedical importance and their alteration in various disorders. Online J Heal Allied Sci. 2009;8(2):1–9.
48. Cervellati C, Romani A, Bergamini CM, Bosi C, Sanz JM, Passaro A, et al. PON-1 and ferroxidase activities in older patients with mild cognitive impairment, late onset Alzheimer's disease or vascular dementia. Clin Chem Lab Med. 2015;53(7).
49. Osaki S, Johonson D, Frieden E. The Possible Significance of the Ferrous Oxidase Activity of Ceruloplasmin in Normal Human Serum. J Biol CHEM. 1966;241(12):2746–51.
50. Costa LG, Laat R De, Dao K, Pellacani C, Cole TB, Furlong CE. NeuroToxicology Paraoxonase-2 (PON2) in brain and its potential role in neuroprotection. Neurotoxicology. Elsevier B.V.; 2014;43:3–9.
51. Cervellati C, Romani A, Seripa D, Cremonini E, Bosi C, Magon S, et al. Systemic oxidative stress and conversion to dementia of elderly patients with mild cognitive impairment. Biomed Res Int. 2014;2014:309507.
52. Gugliucci A, Caccavello R, Kotani K, Sakane N, Kimura S. Enzymatic assessment of paraoxonase 1 activity on HDL subclasses: A practical zymogram method to assess HDL function. Clin Chim Acta. 2013;415:162–8.
53. Gu X, Huang Y, Levison BS, Gerstenecker G, DiDonato AJ, Hazen LB, et al. Form a Functional Ternary Complex. J Clin Invest. 2013;123(9):3815–28.
54. Gu X, Huang Y, Levison BS, Gerstenecker G, DiDonato AJ, Hazen LB, et al. Identification of critical paraoxonase 1 residues involved in high density lipoprotein interaction. J Biol Chem. 2015;
55. Bełtowski J, Wójcicka G, Marciniak A. Species- and substrate-specific stimulation of human plasma paraoxonase 1 (PON1) activity by high chloride concentration. Acta Biochim Pol. 2002;49(4):927–36.
56. Cervellati C, Romani A, Fainardi E, Trentini A, Squerzanti M, Baldi E, et al. Serum Ferroxidase Activity in Patients with Multiple Sclerosis: A Pilot Study. In Vivo. 2014;28(6):1197–200.
57. Martinelli N, Girelli D, Olivieri O, Guarini P, Bassi A, Trabetti E, et al. Novel serum

paraoxonase activity assays are associated with coronary artery disease. Clin Chem Lab Med. 2009 Jan;47(4):432–40.

58. Khersonsky O, Tawfik DS. The histidine 115-histidine 134 dyad mediates the lactonase activity of mammalian serum paraoxonases. J Biol Chem Biol Chem. 2006;281(11):7649–56.

59. Rosenblat M, Gaidukov L, Khersonsky O, Vaya J, Oren R, Tawfik DS, et al. The catalytic histidine dyad of high density lipoprotein-associated serum paraoxonase-1 (PON1) is essential for PON1-mediated inhibition of low density lipoprotein oxidation and stimulation of macrophage cholesterol efflux. J Biol Chem Biol Chem. 2006;281(11):7657–65.

60. Rajkovic MG, Rumora L, Barisic K. The paraoxonase 1, 2 and 3 in humans. Biochem medica. 2011;21(2):122–30.

61. La Du BN, Eckerson HW. The polymorphic paraoxonase/arylesterase isozymes of human serum. Fed Proc. 1984;43(8):2338–41.

62. Costa LG, Giordano G, Furlong CE. Pharmacological and dietary modulators of paraoxonase 1 (PON1) activity and expression: the hunt goes on. Biochem Pharmacol. 2011;81(3):337–44.

63. Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82(1):47–95.

64. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK , JNK , and p38 protein kinases. Science. 2002;298:1911–2.

65. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;408(6809):239–47.

66. Finkel T, Holbrook NJ, Alberti A, Bolognini L, Macciantelli D, Caratelli M, et al. Oxidants, oxidative stress and the biology of ageing. Nature. Elsevier Inc; 2000 9;408(6809):239–47.

67. Lesko LJ, Atkinson AJ. Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies. Annu Rev Pharmacol Toxicol. 2001;41:347–66.

68. Dalle-Donne I, Rossi R, Colombo R, Giustarini D, Milzani A. Biomarkers of oxidative damage in human disease. Clin Chem. 2006;52(4):601–23.

69. Kadiiska MB, Peddada S, Herbert RA, Basu S, Hensley K, Jones DP, et al. Biomarkers of oxidative stress study VI. Endogenous plasma antioxidants fail as

useful biomarkers of endotoxin-induced oxidative stress. Free Radic Biol Med. 2015;81:100–6.

70. Moselhy HF, Reid RG, Yousef S, Boyle SP. A specific, accurate, and sensitive measure of total plasma malondialdehyde by HPLC. J Lipid Res. 2013;54(3):852–8.
71. Ayala A, Muñoz MF, Argüelles S. Lipid Peroxidation : Production , Metabolism , and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. oxidtive Med Cell Longev. 2014;2014.
72. Weber D, Milkovic L, Bennett SJ, Griffiths HR, Zarkovic N, Grune T. Measurement of HNE-protein adducts in human plasma and serum by ELISA-Comparison of two primary antibodies. Redox Biol. 2013;1(1):226–33.
73. Petersen RC. Mild cognitive impairment as a diagnostic entity. J Intern Med. 2004;256(3):183–94.
74. Milne GL, Gao B, Terry ES, Zackert WE, Sanchez SC. Measurement of F2-isoprostanes and isofurans using gas chromatography-mass spectrometry. Free Radic Biol Med. 2013;59:36–44.
75. Forman HJ, Augusto O, Brigelius-Flohe R, Dennery PA, Kalyanaraman B, Ischiropoulos H, et al. Even free radicals should follow some rules: a guide to free radical research terminology and methodology. Free Radic Biol Med. 2015;78:233–5.
76. Kadiiska MB, Gladen BC, Baird DD, Germolec D, Graham LB, Parker CE, et al. Biomarkers of oxidative stress study II: are oxidation products of lipids, proteins, and DNA markers of CCl4 poisoning? Free Radic Biol Med. 2005;38(6):698–710.
77. Ho E, Karimi Galougahi K, Liu C-C, Bhindi R, Figtree GA. Biological markers of oxidative stress: Applications to cardiovascular research and practice. Redox Biol. 2013;1:483–91.
78. de Zwart LL, Meerman JH, Commandeur JN, Vermeulen NP. Biomarkers of free radical damage applications in experimental animals and in humans. Free Radic Biol Med. 1999;26(1-2):202–26.
79. Praticò D, Uryu K, Leight S, Trojanoswki JQ, Lee VM. Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. J Neurosci. 2001;21(12):4183–7.
80. Alamdari DH, Kostidou E, Paletas K, Sarigianni M, Konstas AGP, Karapiperidou A, et al. High sensitivity enzyme-linked immunosorbent assay (ELISA) method for

measuring protein carbonyl in samples with low amounts of protein. Free Radic Biol Med. 2005;39(10):1362–7.

81. Bochi GV, Torbitz VD, Cargnin LP, de Carvalho JAM, Gomes P, Moresco RN. An Alternative Pathway Through the Fenton Reaction for the Formation of Advanced Oxidation Protein Products, a New Class of Inflammatory Mediators. Inflammation. 2014;37(2):512–21.
82. Piwowar A. [Advanced oxidation protein products. Part I. Mechanism of the formation, characteristics and property]. Pol Merkur Lekarski. 2010;28(164):166–9.
83. Yin F, Yao J, Sancheti H, Feng T, Melcangi RC, Morgan TE, et al. The perimenopausal aging transition in the female rat brain: decline in bioenergetic systems and synaptic plasticity. Neurobiol Aging. Elsevier Inc; 2015;36(7):2282–95.
84. Keller JN, Schmitt FA, Scheff SW, Ding Q, Chen Q, Butterfield DA, et al. Evidence of increased oxidative damage in subjects with mild cognitive impairment. Neurology. 2005;64(7):1152–6.
85. Markesbery WR, Schmitt FA, Kryscio RJ, Davis DG, Smith CD, Wekstein DR. Neuropathologic substrate of mild cognitive impairment. Arch Neurol. 2006;63(1):38–46.
86. Bading JR, Yamada S, Mackic JB, Kirkman L, Miller C, Calero M, et al. Brain clearance of Alzheimer's amyloid-beta40 in the squirrel monkey: a SPECT study in a primate model of cerebral amyloid angiopathy. J Drug Target. 2002;10(4):359–68.
87. Valko M, Morris H, Cronin MTD. Metals, toxicity and oxidative stress. Curr Med Chem. 2005;12(10):1161–208.
88. Golden TR, Hinerfeld DA, Melov S. Oxidative stress and aging: beyond correlation. Aging Cell. 2002;1(2):117–23.
89. Wiener HW, Perry RT, Chen Z, Harrell LE, Go RCP. A polymorphism in SOD2 is associated with development of Alzheimer's disease. Genes Brain Behav. 2007;6(8):770–5.
90. Zhu X, Smith MA, Honda K, Aliev G, Moreira PI, Nunomura A, et al. Vascular oxidative stress in Alzheimer disease. J Neurol Sci. 2007;257(1-2):240–6.
91. Cuajungco MP, Goldstein LE, Nunomura A, Smith MA, Lim JT, Atwood CS, et al. Evidence that the beta-amyloid plaques of Alzheimer's disease represent the redox-silencing and entombment of abeta by zinc. J Biol Chem. 2000;275(26):19439–42.
92. Dias-Santagata D, Fulga TA, Duttaroy A, Feany MB. Oxidative stress mediates tau-

induced neurodegeneration in Drosophila. J Clin Invest. 2007 Jan;117(1):236–45.

93. Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov. 2004;3(3):205–14.

94. Wilkinson BL, Landreth GE. The microglial NADPH oxidase complex as a source of oxidative stress in Alzheimer's disease. J Neuroinflammation. 2006;3:30.

95. Butterfield DA, Swomley AM, Sultana R. Amyloid β -Peptide (1–42)-Induced Oxidative Stress in Alzheimer Disease: Importance in Disease Pathogenesis and Progression. Antioxid Redox Signal. 2013;19(8):823–35.

96. Keisari Y, Braun L, Flescher E. The oxidative burst and related phenomena in mouse macrophages elicited by different sterile inflammatory stimuli. Immunobiology. 1983;165(1):78–89.

97. Cervellati C, Romani A, Seripa D, Cremonini E, Bosi C, Magon S, et al. Oxidative balance, homocysteine, and uric acid levels in older patients with Late Onset Alzheimer's Disease or Vascular Dementia. J Neurol Sci. 2014;337(1-2):156–61.

98. WMA. WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects. 1964. Available from: http://www.wma.net/en/30publications/10policies/b3/

99. European Medicines Agency. Principles and detailed guidelines for good clinical practice as regards investigational medicinal products for human use, as well as the requirements for authorisation of the manufacturing or importation of such products. Off J Eur Union. 2005;28(EC):1–7.

100. Strobe. Strengthening the Reporting of Observational Studies in Epidemiology guidelines. 2007.

101. Zuliani G, Ranzini M, Guerra G, Rossi L, Munari MR, Zurlo A, et al. Plasma cytokines profile in older subjects with late onset Alzheimer's disease or vascular dementia. J Psychiatr Res. 2007;41(8):686–93.

102. Petersen RC, Doody R, Kurz A, Mohs RC, Morris JC, Rabins P V, et al. Current concepts in mild cognitive impairment. Arch Neurol. 2001;58(12):1985–92.

103. Alberti A, Bolognini L, Macciantelli D, Caratelli M. The radical cation of N,N-diethyl-para-paraphenylendiamine: A possible indicator of oxidative stress in biological samples. Res Chem Intermed. 2000;26(3):253–67.

104. Capeillère-Blandin C, Gausson V, Descamps-Latscha B, Witko-Sarsat V. Biochemical and spectrophotometric significance of advanced oxidized protein

products. Biochim Biophys Acta - Mol Basis Dis. 2004;1689(2):91–102.

105. Benzie IF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol. 1999;299:15–27.
106. Fossati P, Prencipe L, Berti G. Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine. Clin Chem. 1980;26(2):227–31.
107. Duplancic D, Kukoc-Modun L, Modun D, Radic N. Simple and rapid method for the determination of uric acid-independent antioxidant capacity. Molecules. 2011;16(8):7058–68.
108. Hu ML. Measurement of protein thiol groups and glutathione in plasma. Methods Enzymol. 1994;233:380–5.
109. Erel O. Automated measurement of serum ferroxidase activity. Clin Chem. 1998;44(97):2313–9.
110. Haagen L, Brock A. A new automated method for phenotyping arylesterase (EC 3.1.1.2) based upon inhibition of enzymatic hydrolysis of 4-nitrophenyl acetate by phenyl acetate. Eur J Clin Chem Clin Biochem. 1992;30(7):391–5.
111. Costa LG, Furlong CE. Paraoxonase (PON1) in Health and Disease. Costa LG, Furlong CE, editors. Boston, MA: Springer US; 2002.
112. Cervellati C, Cremonini E, Bosi C, Magon S, Zurlo A, Bergamini CM, et al. Systemic oxidative stress in older patients with mild cognitive impairment or late onset Alzheimer's disease. Curr Alzheimer Res. 2013;10(4):365–72.
113. Squitti R, Salustri C, Siotto M, Ventriglia M, Vernieri F, Lupoi D, et al. Ceruloplasmin/Transferrin ratio changes in Alzheimer's disease. Int J Alzheimers Dis. 2010;2011:231595.
114. Barone E, Di Domenico F, Cenini G, Sultana R, Coccia R, Preziosi P, et al. Oxidative and nitrosative modifications of biliverdin reductase-A in the brain of subjects with Alzheimer's disease and amnestic mild cognitive impairment. J Alzheimers Dis. 2011;25(4):623–33.
115. Mtsuokaa Y, Picciano M, La Francois J, Duff K. Fibrillar b-amyloid evokes oxidative damage in a transgenic mouse model of Alzheimer's disease. Neuroscience. 2001;104(3):609–13.

116. Sultana R, Butterfield DA. Oxidatively modified, mitochondria-relevant brain proteins in subjects with Alzheimer disease and mild cognitive impairment. J Bioenerg Biomembr. 2009;41(5):441–6.
117. Baldeiras I, Santana I, Proença MT, Garrucho MH, Pascoal R, Rodrigues A, et al. Oxidative damage and progression to Alzheimer's disease in patients with mild cognitive impairment. J Alzheimers Dis. 2010;21(4):1165–77.
118. Brys M, Pirraglia E, Rich K, Rolstad S, Mosconi L, Switalski R, et al. Prediction and longitudinal study of CSF biomarkers in mild cognitive impairment. Neurobiol Aging. 2009;30(5):682–90.
119. Guidi I, Galimberti D, Lonati S, Novembrino C, Bamonti F, Tiriticco M, et al. Oxidative imbalance in patients with mild cognitive impairment and Alzheimer's disease. Neurobiol Aging. 2006;27(2):262–9.
120. Serra JA, Domínguez RO, Marschoff ER, Guareschi EM, Famulari AL, Boveris A. Systemic oxidative stress associated with the neurological diseases of aging. Neurochem Res. 2009;34(12):2122–32.
121. Glantzounis GK, Tsimoyiannis EC, Kappas AM, Galaris DA. Uric acid and oxidative stress. Curr Pharm Des. 2005;11(32):4145–51.
122. Perna AF, Ingrosso D, De Santo NG. Homocysteine and oxidative stress. Amino Acids. 2003;25(3-4):409–17.
123. Sautin YY, Johnson RJ. Uric acid: the oxidant-antioxidant paradox. Nucleosides Nucleotides Nucleic Acids. 2008;27(6):608–19.
124. Wehr H, Bednarska-Makaruk M, Graban A, Lipczyńska-Łojkowska W, Rodo M, Bochyńska A, et al. Paraoxonase activity and dementia. J Neurol Sci. 2009;283(1-2):107–8.
125. Zengi O, Karakas A, Ergun U, Senes M, Inan L, Yucel D. Urinary 8-hydroxy-2'-deoxyguanosine level and plasma paraoxonase 1 activity with Alzheimer's disease. Clin Chem Lab Med. 2012;50(3):529–34.
126. Erlich PM, Lunetta KL, Cupples LA, Abraham CR, Green RC, Baldwin CT, et al. Serum paraoxonase activity is associated with variants in the PON gene cluster and risk of Alzheimer disease. Neurobiol Aging. 2012;33(5):1015.e7–23.
127. Paragh G, Balla P, Katona E, Seres I, Egerházi A, Degrell I. Serum paraoxonase activity changes in patients with Alzheimer's disease and vascular dementia. Eur Arch Psychiatry Clin Neurosci. 2002;252(2):63–7.

128. Alam R, Tripathi M, Mansoori N, Parveen S, Luthra K, Lakshmy R, et al. Synergistic epistasis of paraoxonase 1 (rs662 and rs85460) and apolipoprotein E4 genes in pathogenesis of Alzheimer's disease and vascular dementia. Am J Alzheimers Dis Other Demen. 2014;29(8):769–76.
129. Camps J, Mackness M, Mackness B, Marsillach J, Joven J. Serum paraoxonase-1 activity and genetic polymorphisms: common errors in measurement and interpretation of results. Clin Chem Lab Med. 2010;48(6):893–4.
130. Klosinski LP, Yao J, Yin F, Fonteh AN, Harrington MG, Christensen TA, et al. White Matter Lipids as a Ketogenic Fuel Supply in Aging Female Brain: Implications for Alzheimer's Disease. EBioMedicine; 2015;2(12):1888–904.
131. Cervellati C, Bergamini C. Oxidative damage and the pathogenesis of menopause related disturbances and diseases. Clin Chem Lab.2015;[Epub ahead of print]
132. Shobab LA, Hsiung G-YR, Feldman HH. Cholesterol in Alzheimer's disease. Lancet Neurol. 2005;4(12):841–52.
133. Wang JM, Irwin RW, Brinton RD. Activation of estrogen receptor alpha increases and estrogen receptor beta decreases apolipoprotein E expression in hippocampus in vitro and in vivo. Proc Natl Acad Sci U S A. 2006;103(45):16983–8.
134. Hagberg JM, Wilund KR, Ferrell RE, Tisko R, Sopkova Z, Habalova V, et al. APO E gene and gene-environment effects on plasma lipoprotein-lipid levels. physiol genomics. 2000;4:101–8.
135. Poirier J. Apolipoprotein E and cholesterol metabolism in the pathogenesis and treatment of Alzheimer's disease. Trends Mol Med. 2003;9(3):94–100.
136. Barrow, Colin J, Small DH. Abeta Peptide and Alzheimer's Disease. In Springer; 2006. p. 142–56.
137. Karasinska JM, Hayden MR. Cholesterol metabolism in Huntington disease. Nat Rev Neurol. 2011 Sep 6;7(10):561–72.
138. Hughes TM, Rosano C, Evans RW, Kuller LH. Brain cholesterol metabolism, oxysterols, and dementia. J Alzheimer's Dis. 2013;33(4):891–911.
139. BJÖRKHEM I. Crossing the barrier: oxysterols as cholesterol transporters and metabolic modulators in the brain. J Intern Med. 2006;260(6):493–508.
140. Wildsmith KR, Holley M, Savage JC, Skerrett R, Landreth GE. Evidence for impaired amyloid β clearance in Alzheimer's disease. Alzheimers Res Ther. 2013;5(4):33.
141. Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, et al.

Decreased Clearance of CNS -Amyloid in Alzheimer's Disease. Science (80-). 2010;330(6012):1774–1774.

142. Martins IJ, Hone E, Foster JK, Sunram-Lea SI, Gnjec A, Fuller SJ, et al. Apolipoprotein E, cholesterol metabolism, diabetes, and the convergence of risk factors for Alzheimer's disease and cardiovascular disease. Mol Psychiatry. 2006;11(8):721–36.
143. Finch CE. The menopause and aging, a comparative perspective. J Steroid Biochem Mol Biol. 2014;142:132–41.
144. Harlow SD, Gass M, Hall JE, Lobo R, Maki P, Rebar RW, et al. Executive summary of the Stages of Reproductive Aging Workshop. Menopause. 2012;19(4):387–95.
145. Brinton RD. Neurosteroids as regenerative agents in the brain: therapeutic implications. Nat Rev Endocrinol. 2013;9(4):241–50.
146. Zhao L, Morgan TE, Mao Z, Lin S, Cadenas E, Finch CE, et al. Continuous versus cyclic progesterone exposure differentially regulates hippocampal gene expression and functional profiles. PLoS One. 2012;7(2):e31267.
147. Casserly I, Topol E. Convergence of atherosclerosis and Alzheimer' s disease : inflammation , cholesterol, and misflding proteins.2004;363:1139–46.
148. Cervellati C, Trentini A, Romani A, Bellini T, Bosi C, Ortolani B, et al. Serum paraoxonase and arylesterase activities of paraoxonase-1 (PON-1), mild cognitive impairment, and 2-year conversion to dementia: A pilot study. J Neurochem. 2015;135(2):395–401.