



**DOTTORATO INTERNAZIONALE DI RICERCA IN  
NEUROBIOLOGIA**

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***REDOX PROTEOMICS, THIOL HOMEOSTASIS AND  
NEUROPHYSIOLOGICAL CORRELATIONS IN AGING AND  
NEURODEGENERATION***

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***TESI DI DOTTORATO***

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## 1. INTRODUCTION

The terms "aging" and "neurodegeneration" are often used in a broad and generalized manner. Actually, they are particularly complex and multifaceted processes, involving different biochemical systems [1].

Increasing evidence supports the notion that reduction of cellular expression and activity of antioxidant proteins and the resulting increase of oxidative stress are fundamental causes in the aging processes and neurodegenerative diseases [2]. Within the frame of free radical hypothesis of aging, several lines of evidence suggest that accumulation of oxidative molecular damage is a causal factor in senescence. It is also increasingly evident that the mitochondrial genome may play a key role in aging and neurodegenerative diseases. Mitochondrial dysfunction is characteristic of several neurodegenerative disorders, and evidence for mitochondria being a site of damage in neurodegenerative disorders is partially based on decreases in respiratory chain complex activities in Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD) [3]. Such defects in respiratory complex activities, possibly associated with oxidant/antioxidant balance perturbation, are thought to underlie defects in energy metabolism and induce cellular degeneration [4]. Efficient functioning of maintenance and repair process seems to be crucial for both survival and physical quality of life. This is accomplished by a complex network of the so-called "longevity assurance processes", which are composed of several genes, termed vitagenes [5]. Among these, heat shock proteins, also known as stress proteins and molecular chaperones, are highly conserved proteins for the preservation and repair of the correct conformation of cellular macromolecules, such as proteins, RNAs and DNA. Chaperone-buffered silent mutations may be activated during the aging process and lead to the phenotypic exposure of previously hidden features and

contribute to the onset of multigenic diseases, such as age-related disorders, atherosclerosis and cancer [6,7]. Recent studies have shown that the heat-shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including ischemia and reperfusion damage, inflammation, metabolic disorders, cancer, infection, trauma, and aging [7]. The major neurodegenerative diseases, Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and Friedreich's ataxia (FA), are all associated with the presence of abnormal [3]. Given the broad cytoprotective properties of the heat-shock response, there is now strong interest in discovering and developing pharmacological agents capable of inducing the heat-shock response. These findings have opened up new perspectives in medicine and pharmacology, as molecules inducing this defense mechanism appear to be possible candidates for novel cytoprotective strategies [8]. Particularly, modulation of endogenous cellular defense mechanisms such as the heat-shock response, and the proteasomal system, through nutritional antioxidants or pharmacological compounds may represent an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. Moreover, by maintaining or recovering the activity of vitagenes, it would be possible to delay the aging process and decrease the occurrence of age-related diseases with resulting prolongation of a healthy life span.

### ***1.1 Oxidative Stress***

The brain has a large potential oxidative capacity but a limited ability to counteract oxidative stress [9,10,11]. Within the cell, reactive oxygen species (ROS) are physiologically present at minimal concentration as by-products of aerobic metabolism as well as second messengers in many signal transduction pathways and, in normal conditions, there is a steady-state balance between pro-

oxidants and antioxidants which is necessary to ensure optimal efficiency of antioxidant defenses [2,12,13,14].

However, when the rate of free radical generation exceeds the capacity of antioxidant defenses, oxidative stress ensues with consequential severe damage to DNA, proteins and lipids and plays a pivotal role in leading an irreversible cellular damage [15,16,17].

Numerous experimental data shows the involvement of oxidative stress in the mechanism of aging and neurodegeneration [18].

Oxidative stress is therefore characterized by an imbalance of the redox state of oxidants/antioxidants that may lead to altered cellular function and oxidative damage of fundamental biological macromolecules like protein (protein carbonyls, nitration of tyrosine), lipids (products of lipid peroxidation) and nucleic acids [19,20].

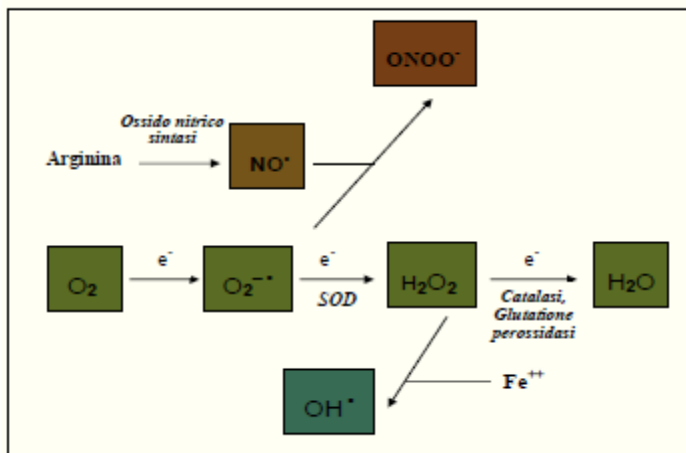
Oxidative stress is induced by both exogenous and endogenous sources. The first include drugs and toxic chemicals that change the balance of oxidants/antioxidants in favour of the oxidation; the latter includes overproduction of reactive oxygen intermediates by the mitochondrial electron transport chain. One of the main causes of oxidative stress is therefore the excessive release of reactive oxygen species (ROS) [18].

### ***1.2 Reactive oxygen species (ROS)***

ROS are defined as molecular entities that react with cellular components, causing harmful effects on their functions. ROS include both free radicals (containing highly reactive unpaired electrons) such as superoxide anion ( $O_2^{\bullet-}$ ), nitric oxide ( $NO^{\bullet}$ ) and hydroxyl radical ( $OH^{\bullet}$ ) and other molecular species, such as hydrogen peroxide ( $H_2O_2$ ) and peroxynitrite ( $ONOO^-$ ).

The majority of cellular ROS are generated during the incomplete metabolic

reduction of oxygen to water (see figure below).



The oxygen molecule is a biological paradox in which one side is an essential molecule for aerobic life, the other a biological hazard due to its high toxicity. In fact, the oxygen that is taken from the external environment through breathing, is required in mitochondrial respiration for the production of energy in the form of ATP using a complex process called "oxidative phosphorylation"; oxygen, acting as a final acceptor of electrons subtracted to molecules and combining with the protons subtracted under the same, allows the complete oxidation in water and carbon dioxide of various molecules (glycidol, fatty acids, amino acids, etc.), with release of all the energy that they contain.

At the same time, an amount equal to 2-4% of oxygen uptake by cells is converted into free radicals, highly reactive molecules with an unpaired electron, which subtract the electron they need to restore the even number of electrons in their orbital, from the molecules they are in contact with [21]. The activation of molecular oxygen can usually occur in two ways: one using electron and a means of energy [3]. The free radicals which can be formed during the sequential reduction mono electronics O<sub>2</sub> are the superoxide anion (O<sub>2</sub><sup>-•</sup>), hydrogen peroxide

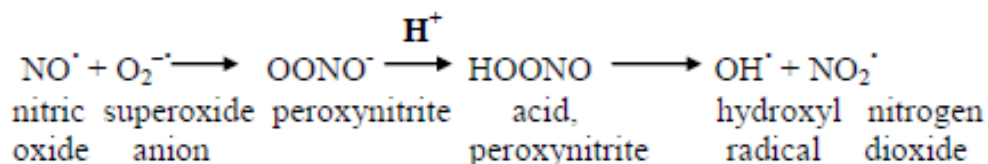
(HOOH) and hydroxyl radical (OH<sup>·</sup>).

In the energetic activation pathway, 22 kcal of energy are sufficient for transitions electronic in the orbital molecular oxygen that lead to formation of singlet oxygen (O<sub>2</sub>) that is not a radical, as it does not there is an unpaired electron, but has a strong oxidizing ability, and, through the degradation, generates superoxide anion.

Ionizing radiation, photosensitizing agents, heat, death of a cell entropic phenomena all thermodynamically favor the release of the amount of energy above this threshold and, therefore, sufficient for these electronic transitions take place [21].

Excessive formation of NO<sup>·</sup>, a physiologically important molecule for the regulation of vascular tone and immunomodulatory processes, can generate radical forms when associated with a concomitant overproduction of superoxide anion [22].

The endothelium seems to continuously produce small amounts of superoxide that can react with nitric oxide (both free radicals) to form nitrate ions, a product which is not radical. For this reason, variations in the production of nitric oxide and superoxide by the endothelium may represent a mechanism of regulation of vascular tone. The peroxynitrite anion, degrading, form the hydroxyl radical [22,23].



If two free radicals react with each other, they cancel each other out, especially when a radical reacts with a free radical molecule, it produces a new free radical

and triggers a chain reaction until it forms a stable compound. It then passes from the stage of "generation" to that of "propagation" of the free radical [21].

The formation of oxygen radical species is thus an occurrence which cannot be eliminated in the cellular environment.

### ***1.3 ROS Toxicity***

Certain clinical situations or the intensification of external factors such as environmental pollution, smoking, a high-fat diet, alcohol abuse, solar radiation, the use of certain drugs, physical and mental stress, are conditions generally associated with the overproduction of free radicals.

When the generation of free radicals exceeds the capacity of detoxifying antioxidant defenses, it establishes a condition of "oxidative stress". This represents a risk to the structural and functional integrity of important molecules such as DNA, proteins and lipids [20].

Free radicals, and in particular the  $\text{OH}^\cdot$ , can react with various molecules, dramatically altering both their chemical states and their functions. The proteins can be oxidized at the level of sulfhydryl groups through a process involving the deactivation of channel proteins, receptor or important enzyme activities [24,25]; for example, enzymes such as phosphofructokinase, complex I and complex IV of mitochondrial respiratory chain are inactivated with severe deterioration of the cell's ability to supply energy. The calcium pump is inactivated with a consequent tendency to maintain high levels of calcium citosoluble.

Nucleic acids are sensitive to free radical attack at both of the bases of the pentose resulting in rupture of the propellers with the formation of modified bases, such as 8-hydroxy-guanine, and alteration of the genetic code [21].

The best known harmful effect of ROS is lipid peroxidation, a chain reaction that



leads to the formation of lipid peroxides and hydroperoxides from the oxidation of a methylene bridge at the level of a polyunsaturated fatty acid of membrane lipids such as arachidonic acid and linolenic acid. The lipoidperoxides tend to move from the hydrophobic membrane to the surface, leading to a disorganization of the structure of the membrane itself. Consequently it causes irreversible damage to the morfofunctionality of intracellular and cellular membranes or lipoproteins [26].

The extended oxidative damage against important molecules like DNA, proteins and lipids, elicited by activated oxygen species and of NO, is considered, in light of current experimental and clinical evidence, the most important cause of fisiopathogenetic and biochemical changes observed during aging of the CNS including neurodegenerative disorders [22].

Several lines of evidence suggest that accumulation of oxidative molecular damage is a causal factor in senescence.

Among the correlative evidence supporting the involvement of oxidative stress are the following: (a) oxidative molecular damage to DNA and proteins increases exponentially with age, and concomitantly, the rates of mitochondrial  $O_2^{\cdot-}$  and  $H_2O_2$  generation as well as the susceptibility of tissues to experimentally induced oxide. Among the correlative evidence supporting the involvement of oxidative stress are the following: (b) experimental regimens that extend the lifespan, such as caloric restriction in mammals and reduction of metabolic rate in insects, decrease the accumulation rates of oxidative damage; (c) mitochondria make two rather contradictory contributions to cell survival. The classically recognized function is the synthesis of ATP for energizing endergonic reactions, the other is generation of reactive oxygen species which may compromise the long-term survival of cells and constitute a major underlying cause of the aging process. Indeed, these two rather conflicting functions are part of the same process, namely

mitochondrial respiration.

The resulting alteration in the redox and mitochondrial dysfunction is involved in the pathogenesis of various diseases including neurodegenerative disorders such as multiple sclerosis (MS), Parkinson's disease (PD), Alzheimer's disease, (AD) and aging [27].

CNS has a large potential oxidative capacity due to the high level of tissue oxygen consumption [28]. However, the ability of the brain to withstand oxidative stress is limited because of: (a) a high content of easily oxidizable substrates, such as polyunsaturated fatty acids and catecholamines; (b) relatively low levels of antioxidants such as glutathione and vitamin E and antioxidant enzymes (such as glutathione peroxidase, catalase and superoxide dismutase); (c) the endogenous generation of reactive oxygen free radicals through several specific reactions; (d) the elevated content of iron in specific areas of the human brain, such as globus pallidus and substantia nigra (SN), while cerebrospinal fluid has very little iron-binding capacity owing to its low content of transferrin; (e) CNS contains non-replicating neuronal cells which, once damaged, may be permanently dysfunctional or committed to programmed cell death (apoptosis).

Numerous experimental evidence lead to the conclusion that the dysfunction of the cellular energy metabolism is an important factor in the neurotoxicity mediated by NO and that the cellular content of thiols is crucial in determining the sensitivity of cells to oxidative and nitrosative stress [29].

#### ***1.4 Mechanisms of Antioxidant Defence***

In normal conditions, there is a steady-state balance between prooxidants and antioxidants, which is necessary to ensure optimal efficiency of antioxidant defenses during normal cellular metabolism [30].

Furthermore, recent studies show that a minimum amount of free radicals, which

until now have been considered only due to aging, is a necessary condition for optimal cell function since it leads to greater efficiency in defense systems and increased cell survival [29].

In the cell, on the front of the insult oxidative level of the cytoplasm, mitochondria and also in the extracellular fluid exist efficient enzymatic and non enzymatic mechanisms of antioxidant defense.

The enzymatic mechanisms are represented by cytoprotective enzymes (Superoxide dismutase, catalase, glutathione peroxidase) that act as "scavengers" towards free radicals removing them just formed or preventing their formation. The non-enzymatic antioxidants mechanisms work through substrates capable of reacting with free radicals, abducting and neutralizing them, blocking the reactions of lipid peroxidation, thus preventing their detrimental action and the propagation of free radicals. These include: molecules able to bind the singlet O<sub>2</sub> ( $\beta$ -carotenes, retinoids), inhibitors of xanthine oxidase (allopurinol), low molecular weight molecules, both water-soluble (ascorbic acid and glutathione, operating in blood plasma and in the cytosol) both fat-soluble, such as  $\alpha$ -tocopherol (vitamin E), the bilirubin, uric acid, estrogen, which instead exert their action in the hydrophobic core of cell membrane or plasma lipoproteins [21].

In recent years, since oxidative stress has been considered the basis of some aspects of neurodegeneration, numerous experimental investigations have been conducted in order to reduce the effects of oxidative stress through the use of scavengers of free radicals.

There are two general classes of antioxidants, endogenous and exogenous. Among the former there are the tripeptide glutathione (GSH), various vitamins, and products of reactions catalyzed by enzymes that are upregulated in response to oxidative stress, e.g., bilirubin from heme oxygenase and products of antioxidant response elements (ARE) [8]. Among the exogenous ones, nutritionally derived

antioxidants, there are different classes of molecules: some that increase endogenous GSH levels and others that have reactive SH functionalities, vitamins, and phenolic and polyphenolic compounds [31,32]. The major regulator of intracellular redox state is glutathione ( $\gamma$ -glutamyl-cysteinyl-glycine), a cysteine-containing tripeptide with reducing and nucleophilic properties. Glutathione (GSH) is required for maintaining the thiol redox status of cell, particularly in the brain, protecting against oxidative damage, detoxifying of endogenous and exogenous reactive metals and electrophiles, storage and transport of cysteine, as well as for protein and DNA synthesis, cell cycle regulation and cell differentiation [33].

Glutathione and glutathione-related enzymes play a key role in protecting the cell against the effects of reactive oxygen species.

The key functional element of glutathione is the cysteine moiety, which provides the reactive thiol group. Glutathione is the predominant defense against reactive oxygen species (ROS), which are reduced by GSH in the presence of GSH peroxidase. As a result, GSH is oxidized to GSSG, which, in turn, is rapidly reduced back to GSH by GSSG reductase at the expense of NADPH. The thiol-disulfide redox cycle also aids in maintaining reduced protein and enzyme thiols. Lacking a process to reduce protein disulfides, vulnerable cysteinyl residues of essential enzymes might remain oxidized, leading to changes in catalytic activity. Glutathione also aids in the storage and transfer of cysteine as well. Cysteine self-oxidizes rapidly into cystine, producing toxic oxygen radicals. To avoid the toxicity of cystine, most of the nonprotein cysteine is stored in glutathione. In addition to protection against ROS, glutathione is an excellent scavenger of lipid peroxidation products such as HNE and acrolein, both of which have been found to bind proteins inhibiting their activities. Glutathione also reacts with saturated carbon atoms (epoxides), unsaturated carbon atoms (quinones, esters), and

aromatic carbon atoms (arylnitro compounds). This detoxification involves nucleophilic attack by GSH on an electrophilic carbon. This reaction can occur spontaneously, but most often is catalyzed by glutathione S-transferase. Glutathione also forms metal complexes via nonenzymatic reactions.

GSH functions in the storage, mobilization and delivery of metal ions between ligands, in the transport of metal across cell membranes, as a source of cysteine for metal binding, and as a reductant in redox reactions involving metals [33]. The sulfhydryl group of the cysteine moiety of GSH has a high affinity for metal ions such as mercury, silver, cadmium, arsenic, lead, gold, zinc, and copper, forming a thermodynamically stable complex that can be eliminated from the body.

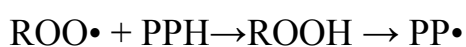
Recent data demonstrate that, besides intracellular functions, GSH has also important extracellular functions in brain. In this respect astrocytes appear to play a key role in the GSH metabolism of the brain, since astroglial GSH export is essential for providing GSH precursors to neurons. Of the different brain cell types studied *in vitro* only astrocytes release substantial amounts of GSH. In addition, during oxidative stress astrocytes efficiently export glutathione disulfide (GSSG) [34].

*Vitamin E*, a phenolic compound, acts as an antioxidant by scavenging free radicals via the phenolic H-atom. The reactions of vitamin E, vitamin C, and glutathione may be linked by various recycling pathways, thereby increasing efficiency of these moieties against oxidative stress.

*Polyphenols* are natural substances ubiquitously present in fruits and vegetables, as well as, beverages obtained from plants such as tea, red wine and olive oil. Flavonoids compose the largest group of polyphenols. Their skeletal structure consists of an aromatic ring condensed to a heterocyclic ring, attached to a second aromatic ring. Flavonoids are mainly divided into: anthocyanins, glycosylated

derivative of anthocyanidin, present in colorful flowers and fruits, and anthoxantins, colorless compounds further divided in several categories including flavones, flavans, flavonols, flavanols, and isoflavones. The remarkable antioxidant activity of these compounds is conferred by the numerous phenolic hydroxyl groups on the aromatic ring.

The rapid donation of a hydrogen atom to lipid peroxy radical results in the formation of the polyphenol phenoxyl radical (PP•) according to the reaction



that can be stabilized by further donation of another hydrogen or by reacting with another radical. In addition, flavonoids present efficient iron chelating activity, for which the 3-OH is important [35]. The physiological effects of flavonoids are particularly significant in those pathologies where the oxidative stress hypothesis is accepted and supported by experimental data, such as AD. *In vitro*, flavonoids are capable of scavenging superoxide anions and hydroxyl radicals [36,37]. Once ingested, these compounds are capable of elevating the redox and antioxidant level [38]. In red blood cells, polyphenols enhance cell resistance to oxidative insult, as well as inhibit LDL oxidation in plasma [39, 40]. The importance of these molecules in protecting cells from oxidative stress goes beyond the simple radical oxygen species (ROS) scavenging properties. In a recent study on neuronal cells, three different mechanisms of protection have been identified: flavonoids can prevent cell death after glutamate injury by scavenging radicals, maintaining the correct glutathione levels and inhibiting Ca<sup>2+</sup> influx, which represents the last step in the cell death cascade [41]. These properties, together with anti-inflammatory properties attributed to some polyphenols renders this class of compounds suitable for application where oxidative stress, together with

inflammation and antioxidant defense depletion take place, such as AD [42].

### ***1.5 Defense mechanism "Heat shock response"***

Increasing evidence demonstrates that oxidative stress alters the expression of antioxidant enzymes and enhances expression and/or DNA binding of numerous transcription factors, including AP-1, fos, jun, myc, erg-1, SAPK and NfκB(61) [43].

Cellular stress response is the ability of a cell to counteract stressful conditions (Figure 1a, 1b). This phenomenon, which includes heat shock response (HSR), represents an ancient and highly conserved cytoprotective mechanism [44-52]. Production of heat shock proteins, including protein chaperones, is essential for the folding and repair of damaged proteins, serving thus to promote cell survival conditions that would otherwise result in apoptosis [53-57]. The term 'molecular chaperone' denotes a large family of ubiquitous proteins that function as part of an ancient defense system in our cells. Chaperones promote cell survival by sequestering damaged proteins and preventing their aggregation. During stressful conditions, such as elevated temperature they prevent protein aggregation by facilitating the refolding or elimination of misfolded proteins. The stress-induced response to damaged proteins is helped by a sophisticated regulatory system, which shuts down most cellular functions and, in parallel, induces the synthesis of several chaperones and other survival-promoting proteins. Therefore, many of the chaperones are also called stress or 'heat shock' proteins in reference to the archetype of cellular stress, heat shock.

Besides their role during stress, chaperones have multiple roles under normal conditions, as such they promote the transport of macromolecules (e.g. proteins or RNA) and participate in remodeling events involving larger protein complexes, including signaling, transcription, cell division, migration and differentiation. Cellular

stress response requires the activation of pro-survival pathways which, under control of protective genes called vitagenes [58] produce molecules (heat shock proteins, glutathione, bilirubin) endowed with anti-oxidant and anti-apoptotic activities.

Generally, molecular chaperones help a multitude of signaling molecules to keep their activation-competent state, and regulate various signaling processes ranging from signaling at the plasma membrane to transcription. In addition to these specific regulatory roles, recent studies have revealed that chaperones act as genetic buffers stabilizing the phenotypes of various cells and organisms. Among the cellular pathways conferring protection against oxidative stress, a key role is played by the products of vitagenes [59-61]. These include members of the heat shock protein (Hsp) family, such as heme oxygenase-1 and Hsp72, sirtuins and the thioredoxin/thioredoxin reductase system [62]. Recent studies have shown that the heat shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging and neurodegenerative disorders [63]. Given the broad cytoprotective properties of the heat shock response there is now strong interest in discovering and developing pharmacological agents capable of inducing the heat shock response [64]. Molecular chaperones are known to disrupt aggregates but also to promote active aggregation when the concentration of the aggregating protein is high. Consistent with this notion, although protein aggregation is hazardous under certain circumstances, the creation of apparently less-toxic large aggregates is protective. This hypothesis is the basis of the therapeutic potential of heat shock proteins (HSPs), which prevent protein misfolding and aggregation [65].

Cellular stress response is regulated at the transcriptional, translational and post-translational levels by a family of heat shock transcription factors (HSFs) that are expressed and maintained in an inactive state under nonstress conditions [66].



Hsps consist of both stress inducible and constitutive family members. The constitutive form performs basic physiological functions. However, some of them are up-regulated by stress. The inducible form prevents the denaturation of proteins and assembly of abnormal polypeptides during exposure to stressful conditions. Denaturated proteins induced stress protein. The 70 kDa family of stress proteins is one of the most extensively studied. Included in this family are HSC70 (heat shock cognate, the constitutive form), HSP70 (the inducible form, also referred to as HSP72).

Another important family is HSP 32, or heme oxygenase (HO). There are three isoforms of heme oxygenase: HO-1, or inducible isoform; HO-2, or constitutive isoform; and the recently discovered HO-3.

Heme oxygenase-1 exerts protective role, by degrading the intracellular levels of prooxidant heme and by producing biliverdin, the precursor of bilirubin, this latter being an endogenous molecule with potent antioxidant and antinitrosative features and also produces carbon monoxide, a molecule involved in regulating vessel active pathway of NO [67].

Increasing evidence suggests that the HO-1 gene is redox regulated and contains in its promoter region the antioxidant responsive element (ARE), similarly to other antioxidants that bind specific transcription factors Nrf2 as sensitive to the alteration or NFkB redox balance; in fact, nitrosative stress and depletion of GSH up-regulate the protein. It was seen that the cells overexpressing the Hsps are resistant to several types of oxidizing agents and to the heat shock; the Hsps play a protective role against oxidative damage to DNA [68]. The experimental evidence that: a) inhibition of antioxidant defenses increases the susceptibility to the heat shock, b) the Hsps confers resistance to oxidative stress [69]; c) the induction of Hsp-70, suggests, inhibited by antioxidant compounds, a correlation between oxidative stress mechanisms and the induction of Hsps. Furthermore, NO plays a

role in induction of Hsp70 [70].

The *thioredoxin* (Trx) system (Trx and Trx reductase), has received a considerable attention in the last years, as a stress responsive gene, redox in light of new experimental evidence, is a leading member of the ubiquitous multifunctional redox regulation of cell redox [71].

Trx is a ubiquitous thiol oxidoreductase system that regulates cellular redox balance and constitute a family of proteins all of which have a conserved catalytic site (Cys-Gly-Pro-Cys) which undergoes reversible oxidation of the cysteine pair while reducing disulfide bridges of various proteins [72]. The thioredoxin has evolved similar to a protein chaperone ensuring the maintenance of the structure dithiol/ disulphydryl biological function of proteins. Indeed, scientific evidence shows that Trx binds to specific proteins, modulating the structural conformation.

The thioredoxin system, originally identified in *Escherichia coli*, in 1964, as a hydrogen donor for ribonucleotide reductase required for DNA synthesis, plays a key role in cell function by limiting oxidative stress directly via antioxidant effects and indirectly by protein–protein interactions [73].

It is well established that, in mammals, cellular redox regulation of many processes is provided by the cooperation between the Trx and glutathione systems [71].

Indeed, Trx and GSH systems are involved in a variety of redoxdependent pathways such as supplying reducing equivalents for ribonucleotide reductase, and peptide methionine sulfoxide reductase, the latter being involved in antioxidant defence and regulation of the cellular redox state [71].

Therefore, Trx and GSH form a powerful system controlling redox regulation of gene expression, signal transduction, cell proliferation, protection against oxidative stress, anti-apoptotic functions, growth factor and co-cytokine effects, as well as regulation of the redox state of the extracellular environment [74]. The

promoter of the Trx gene contains a series of stress responsive elements, various transcription factor binding sites, such as SP1, AP-1, NF- $\kappa$ B, and the antioxidant response element (ARE) [75].

GSH is thought to be largely responsible for maintaining a low redox potential and free thiol levels inside cells and organelles due to its high intracellular concentration (1–10 mM). The Trx system, rather may play a critical role in the redox regulation of protein thiols involved in signal transduction and gene regulation [76].

In addition, the thioredoxin (Trx), which essentially acts as a soluble protein after breakup cells, exists in an isoform cytoplasmic (Trx-1) and in a mitochondrial (Trx-2) [77].

Molecular studies show that the cytoplasmic isoform of the mitochondrial Trx as well the mitochondrial one protect against oxidative stress and both are essential for the survival of mammalian cells [78]. Given the large amount of functions performed by Trx redox seems reasonable to say that it is a critical molecule essential for cell survival. Overexpression of Trx system/TrxR is generally associated with activation of cellular mechanisms of tolerance to stress and in general, a resistance to oxidative damage and/or nitrosative mediated a wide variety of stressors, including compounds such as doxorubicin and etoposide [79-81].

The Trx plays a cytoprotective role against different forms of stress in a variety of biological systems. It is considered basically as a stress inducible protein with a typical intracellular cytosolic localization [77].

Many physicochemical stimuli, such as UV irradiation and hydrogen peroxide, have been shown to induce Trx expression and secretion, as a redox-sensitive molecule with cytokine-like and chemokine-like activities in the prevention of cellular damage from oxidative stress. In addition to UV irradiation, treatment of cells in culture with phorbol esters, hydrogen peroxide, hypoxia, the cancer drug

cisplatin and hemin has been reported to cause the translocation of Trx from the cytoplasm to the nucleus, where it regulates the redox-activation and DNA binding activity of critical transcription factors (Jun, Fos, p53, CREB, PEBP2/CBF, Myb), all involved in fundamental processes, such as gene expression, cell growth and apoptosis.

The Trx-1, the most extensively studied isoform, is primarily a cytosolic soluble protein without a specific localization signal. Several studies indicate that the Trx is expressed constitutively associated with protein sulfhydryl on the surface of the plasma membrane of different cell types [77].

Thioredoxin plasma levels in normal individuals vary between 20 and 30 ng/ml (80, 81) and increase in certain human diseases including HIV infection and cancer [75]. Elevated Trx levels may contribute to increased cancer cell proliferation and resistance to chemotherapy by several mechanisms as the stimulation of DNA synthesis and the activation of redox-modulated transcription factors.

Recent work suggests that Trx-1 is involved in nerve growth factor (NGF) signaling pathways. NGF, a neurotrophic factor regulating development, maintenance and function of the CNS, has been shown to activate Trx-1 expression via cyclic AMP (cAMP)-response elements (AREs) present in the Trx-1 gene promoter, and also to induce nuclear translocation of Trx1 [75]. Several data suggest that, beyond its ability to regulate the function of proteins through thiol-disulfide exchange reactions, Trx and its substrates may also have beneficial effects during oxidative stress by upregulating HO-1, with important cytoprotective pleiotropic effects deriving from heme degradation and bilirubin formation [82, 83]. Besides the role as a source of reducing equivalents, Trx *per se* acts as antioxidant or ROS scavenger. In fact, Trx eliminates singlet oxygen, hydroxyl radical and hydrogen peroxide. Finally, the NO-dependent expression of

Trx has been shown to be involved in the neuroprotection against oxidative stress mediated by estrogens [75]. It has also been reported that some of the neuroprotective effects of GSNO on beta-amyloid- or ferrous citrate-induced toxicity in rat cortical neurons or in rat substantia nigra can be due to the activation of multiple signalling pathways including thioredoxin [84,85;].

The sirtuins are a group of proteins linked to aging, metabolism and stress tolerance in several organisms. In mammalian cells seven sirtuins have been identified. SIRT1, 2, 3, 6 and possibly 5 are NAD-dependent deacetylases, SIRT4 and 6 are ADP-ribosyltransferases, and the activity of SIRT7 has not been defined [86]. The sirtuin family of histone deacetylases (HDACs) was named after their homology to the *Saccharomyces cerevisiae* gene silent information regulator 2 (Sir2). In the yeast, Sir2 has been shown to mediate the effects of caloric restriction on the extension of life span, with high levels of Sir2 activity promoting longevity [87]. Like their yeast homologs, the mammalian sirtuins (SIRT1-7) are class III HDACs and require NAD<sup>+</sup> as a cofactor to deacetylate substrates ranging from histones to transcriptional regulators. Through this activity, sirtuins are shown to regulate important biological processes, such as apoptosis, cell differentiation, energy transduction or glucose homeostasis [88]. In particular, the NAD<sup>+</sup>/NADH ratio can be considered as a “biochemical sensor” to evaluate the energetic status of the cell; in fact, among the several mechanisms through which dietary antioxidants may be useful for tissues, it is noteworthy to mention the improvement of metabolic conditions secondary to proinflammatory damage [88]. In this light, the interaction between NAD<sup>+</sup>/NADH and the members of the sirtuins family, puts in a single frame the cytoprotective activity of dietary antioxidants through the regulation of both cellular redox and metabolic state [88]. Since the Sir2 family of proteins exert their enzymatic activity not only on histones but also on numerous other proteins, including transcriptional factors,

they are involved in many cellular processes, e.g., gene silencing, DNA repair, progression of the cell cycle, whereby controlling the mechanism of cellular ageing [88].

Several experimental evidences have shown the role of SIRT1 protein in human cell survival. SIRT1 specifically associates with the p53 tumor suppressor protein and deacetylates it, resulting in negative regulation of p53-mediated transcriptional activation. Importantly, p53 deacetylation by SIRT1 also prevents cellular senescence and apoptosis induced by DNA damage and stress. SIRT1 regulates important aspects of mitochondrial biology, e.g. it deacetylates the essential cofactor PGC-1 $\alpha$  (PPAR-c coactivator-1 $\alpha$ ) in mitochondrial biogenesis. An up regulation of the mitochondrial activity might be of therapeutic benefit for various diseases related to aging such as metabolic disorders (e.g. diabetes type 2) or mitochondrial disorders.

These studies provide important information on the activity of SIRT1 and offer a promising approach for the treatment of metabolic disorders. In addition, SIRT1 activation significantly decreases neuronal cell death induced by amyloid-beta (A $\beta$ ) peptides through inhibition of NF- $\kappa$ B signaling [88].

## ***1.6 Aging***

Aging is a complex biological process characterized by a gradual decline in biochemical and physiological functions of most organs and is considered one of the most significant risk factors for age-related neurodegenerative diseases, such as Alzheimer's, Parkinson's disease, ALS, Huntington disease, Friedreich ataxia and multiple sclerosis [89, 90, 47, 52]. In general, aging is associated with changes in physiological characteristics, including muscle weakness and hair decolorization, and many physiological functions. The causes, however, are

multifactorial and several studies have suggested that oxidative stress plays an important role. Age-related changes in the brain include reduction of trophic supports, decreased proteosomal enzyme activities, mitochondrial dysfunction, change in the redox status which promotes a more pro-inflammatory environment associated with increased formation of reactive oxygen species (ROS) [91, 92]. The free radical theory of aging postulates that ROS may produce oxidative damage directly to critical biological molecules including proteins, DNA and lipids [92, 93, 94]. To counteract increasing levels of ROS, the cell has developed a number of antioxidant defense systems such as antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) as well as non-enzymatic antioxidant molecules (carotenoids, vitamin E, GSH). The imbalance between the activity of free radicals generation and scavenging systems is known as oxidative stress and is considered one of the most important mediators in the progressive decline of cellular function during aging [90, 95].

In particular, the aging process is accompanied by a general decline of physiological functions in the CNS. The CNS is particularly vulnerable to oxidative injury for its high oxygen consumption per unit weight, consistent with the generation of high levels of ROS, and the little amount of ROS defence systems (lower in the nervous system than other tissues) that allow ROS to remain elevated once formed. Since the brain contains high levels of polyunsaturated fatty acids, which upon oxidation form neurotoxic lipid peroxidation products (MDA, HNE), neuronal tissue is extremely vulnerable to oxidative modification of its cellular components. The oxidative damage to cellular macromolecules such as DNA, proteins, and lipids accumulates with age and has been postulated to be the main, but not the only, type of endogenous damage strongly involved in the aging process [96]. Indeed, when post mitotic neurons are injured by oxidative stress they cannot be replaced, carrying the detrimental effects of oxidative injury

over the lifetime of the neuron population. The chemical reactions resulting from attack of ROS/RNS on proteins are complex and lead to a variety of products, many as yet uncharacterized. The oxidative damage to proteins is reflected by increasing levels of protein carbonyls and decreasing levels of protein thiols [97, 98] Protein carbonylation, among different types of post-translational modifications, serve as useful biomarker for the accumulation of oxidatively modified proteins [97]. It appears that such modifications target very specific proteins and can affect the integrity and functioning of the proteome.

A number of studies indicated that the levels of oxidized proteins, exhibiting carbonyl groups, increase progressively with age in brain extracts of rats of different ages [99]. Furthermore, due to its central role in producing energy (ATP), mitochondria were brought to attention in aging biology, in order to understand the decline of basal metabolic rate and, consequently, physiological performances observed in aged mammals. Mitochondria produce the majority of free radicals (49%) and as a powerful source of these toxic oxidants are also their potential victim. In fact, the mitochondrial components (e.g. mtDNA or mt enzymes) are surely more susceptible to oxidative damage than all the other components [13, 100, 101]. This damage increase might have important consequences on mitochondrial structure, on the activity of the respiratory chain complex and on the global functionality of these organelles. Increasing body of evidence also suggests that the decay of mitochondria accompanied by an impairment of cell energy metabolism are important factors in the pathogenesis of most important neurodegenerative disorders [102]. Dysfunctional mitochondria are observed in aging, and also in pathological situations as ischemia-reperfusion and inflammation, moreover studies on senescence-accelerated mice (SAMP8) showed the mitochondria decay as one of the main contributor to the acceleration of aging [103, 104]



### ***1.7 Multiple Sclerosis***

Multiple sclerosis (MS) is an autoimmune-mediated neurodegenerative disease with characteristic foci of inflammatory demyelination in the brain, spinal cord, and optic nerves. Recent studies have demonstrated not only that axonal damage and neuronal loss are significant pathologic components of MS and experimental autoimmune encephalomyelitis (EAE), but that this neuronal damage is thought to cause the permanent neurologic disability often seen in MS patients. Current treatments for MS involve immunomodulation, which can reduce the incidence of inflammatory relapses. However, existing therapies are often not fully effective, and limited evidence suggests that these therapies prevent the long-term neuronal damage and physical disability of MS patients [105, 106]. New therapies that prevent neurodegeneration through nonimmunomodulatory mechanisms have a tremendous potential to work synergistically with current MS therapies [107]. MS pathology is characterized by perivenous infiltration of lymphocytes and macrophage leading to damage of myelin and axons in the brain and spinal cord, which underlie the clinical disease course usually occurring with recurrent and reversible episodes of neurological dysfunction affecting one or several sites, during late adolescence and early adulthood; this form is called relapsing-remitting form (RR) and it is the most prevalent. Usually, approximately 20 years later, this clinical pattern transforms into a secondary progressive phase with continuous and progressive neurological decline [108]. Although evidence indicates that MS is a multifactorial disease caused by a complex interplay between genetic and environmental factors, it is still unclear which are the causes or the factors that contribute to its unpredictable course. It is generally accepted that, virus infections of the CNS, vascular factors and/or disturbed immune mechanisms are implicated in the pathogenesis of MS [109, 110]. Once triggered,

the immune system attacks and destroys myelin and the myelin-forming cell, leading to the pathological hallmarks of MS: the classical actively demyelinating lesions, the cortical demyelination and the diffuse white matter injury, the latter particularly evident in the later stage of the disease [110, 111]. Existing evidence indicates that CNS responds to the attack by immune cells and their secreted products through modulation of its metabolism and gene expression [112]. In addition, cytokines, immunoglobulins, and complement complexes may elicit a survival response in the oligodendrocytes, involving the induction of endogenous heat shock proteins and other protective molecules, which indicates that redox systems and therefore the oxidant/antioxidant balance in these cells are of great importance in MS [113-116]. The adaptation and survival of cells and organisms requires the ability to sense proteotoxic insults and to coordinate protective cellular stress response pathways and chaperone networks related to protein quality control [47]. Despite the abundance and apparent capacity of chaperones and other components of homeostasis to restore folding equilibrium, brain cells appears poorly adapted for chronic proteotoxic stress which increases in neurodegenerative diseases such as MS [50].

### ***1.8 Alzheimer's disease***

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and represents the most common cause of dementia in the elderly, accounting for 50-60% of all cases in Western world [117, 118]. The prevalence rates for AD rise exponentially with age, increasing markedly after 65 years. AD is characterized by cognitive decline beginning usually with impairment of episodic memory, involving progressively all cognitive functions in the late stage [119]. Although some cases are familial, sporadic AD is more common, affecting more than 15 million people worldwide [120].

The pathological hallmarks of AD are amyloid plaques, containing amyloid- $\beta$  peptide, derived from the transmembrane amyloid precursor protein, and neurofibrillary tangles, composed of hyperphosphorylated tau protein, in the medial temporal lobe structures and cortical areas of the brain together with neuronal death and synapses loss [121, 122].

Many approaches have been undertaken to understand AD, including A $\beta$  aggregation, but the heterogeneity of the etiologic factors makes it difficult to define the clinically most important factor determining the onset and progression of the disease [123]. However, increasing evidence indicates that factors such as oxidative stress and disturbed protein metabolism and their interaction in a vicious cycle are central to AD pathogenesis and progression [124-127]. Amyloid- $\beta$  peptide (1–42) has been shown to induce protein oxidation in both *in vitro* and *in vivo* studies [128-131]. As a result, amyloid- $\beta$  peptide (1–42) has been proposed to play a central role in the pathogenesis of AD [132]. A previous study has shown that increased protein oxidation and lipid peroxidation are present in the brain from patients with mild cognitive impairment (MCI), as compared to aged-matched control brain [133, 134]. Because many researchers consider MCI to be the transition zone between normal cognition and the dementia of early AD, these findings suggest that oxidative stress is fundamental to the progression of AD and not simply a consequence of AD [44, 135].

## **2. AIM OF THE RESEARCH**

The cell antioxidant defence systems operate very efficiently and there is a balance between pro-oxidant and antioxidant factors, that, during normal cellular metabolism, is able to eliminate all free radicals that are produced [30].

Numerous experimental data show evidence of the involvement of oxidative stress in aging and neurodegenerative disorders [18]. In recent years, since oxidative stress has been considered the basis for some aspects of neurodegeneration, a number of experimental studies have been carried out in order to identify a way to counter the effects of oxidative stress through scavengers of free radicals [8].

From a molecular point of view, the central nervous system (CNS) cells are able to fight oxidative stress with many resources including bioactive molecules (glutathione, thioredoxin, flavonoids), lipoic acid, enzymes (heat shock proteins, superoxide dismutase, catalase, glutathione peroxidase, thioredoxin reductase, etc.) and redox sensitive transcription factor protein [136].

The heat shock proteins (HSP) is one of the most studied active defence systems against oxidative damage. The heat shock response is able to produce a cytoprotective state in a wide variety of human diseases such as inflammation, cancer, aging and neurodegenerative disorders, opening new perspective in medicine and pharmacology about molecules capable of activating these defence mechanisms as potential target for novel cytoprotective strategies [7, 137-140].

## ***2.1 Aging***

In this study, we examine the free radical hypothesis of aging employing a redox proteomics technique. More in details, the aims of the study were:

- to dose levels of expression of stress proteins by Western blot analysis, in brain areas of aged rats, using antibodies specific: Hsp-90, Hsp-70, CN1, 4-HNE, DPNH.
- to study the role of free radicals in the aging process, through a redox proteomics approach.
- to investigate the oxidation of specific proteins by measuring the protein carbonyl levels in four different brain regions of rats "(Hippocampus, cerebellum, cortex and striatum) of 28 months (senescent) and 12 months (adults).
- to identify proteins that are specifically oxidized during the process of aging in different brain regions because many of these proteins are related to the functionality of mitochondria, the energy metabolism and activity of chaperones.

## ***2.2 Multiple sclerosis***

The present study was undertaken in order to investigate systemic stress response and the associated oxidative stress measured through the determination of markers of protein and lipid oxidation in plasma, lymphocytes and CSF of patients with active MS, as compared to age-matched controls, in order to gain a better insight into the molecular mechanisms regulating the cellular stress response during the progression of the disease and, as such, provide a potential target for novel cytoprotective strategies impacting the clinical settings of this degenerative disease.

The research included the following objectives:

- determining the level of Hsp-72 and HO-1 and SIRT in plasma and in lymphocytes from control subjects and patients with MS by Western blot analysis.

- study, using a redox proteomics approach, the role played by free radicals in multiple sclerosis.
- investigate the oxidation of specific proteins by measuring the protein carbonyl levels in the serum of patients with MS and control subjects.

### ***2.3 Alzheimer's disease***

In the present study we evaluate stress response mechanisms in plasma and lymphocytes of control patients compared to AD patients, in order to provide evidence of an imbalance of oxidant/antioxidant mechanisms and oxidative damage in AD patients and the possible protective role of vitagenes.

The research has been focused on determining the levels of Sirt-1, Sirt-2 and Trx in plasma and in lymphocytes from control subjects and patients with AD by Western blot analysis.

### 3. MATERIALS AND METHODS

#### *3.1 Aging*

##### *3.1.1 Chemicals*

5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), 1,1,3,3 tetraethoxypropane, purified bovine blood SOD, NADH, reduced glutathione (GSH), oxidized glutathione (GSSG),  $\beta$ - NADPH (type 1, tetrasodium salt), glutathione reductase (GR; Type II from Bakers Yeast), SIN-1 (3-Morpholinosydnonimine hydrochloridte) were from Sigma Chemicals Co, St. Louis (USA). All other chemicals were from Merck (Germany) and of the highest grade available.

##### *3.1.2 Animals and samples preparation*

All animal protocols were approved by the University of Catania laboratory Animal Care Advisory Committee. Male Wistar rats purchased from Harlan (Udine, Italy) were maintained in a temperature and humidity-controlled room with a 12 h light: dark cycle. Rats ( $n = 8$ , per group) of 12 (aged) and 28 (senescent) months, were fed ad libitum a certified diet prepared according to the recommendations of the AIN.

After sacrifice, brains were quickly removed and dissected into the cerebral cortex, cerebellum, and striatum according to a standardized procedure, in a cold anatomical chamber and following a protocol that allows a maximum of 50 s time-variability for each sample across animals. Brain samples from hippocampus, cerebellum, striatum and cerebral cortex were minced and suspended in 10 mM HEPES buffer (pH 7.4) containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.1 mM EDTA, and 0.6 mM MgSO<sub>4</sub> as well as proteinase inhibitors: leupeptin (0.5 mg/ml), pepstatin (0.7  $\mu$ g/ml), type II S soybean trypsin inhibitor (0.5  $\mu$ g/ml), and PMSF (40  $\mu$ g/ml). Homogenates were

centrifuged at  $14,000 \times g$  for 10 min to remove debris. Protein concentration in the supernatant was determined by the “Coomassie Plus Protein Assay” (Pierce, Rockford, IL, USA).

### ***3.1.3. Two-dimensional gel electrophoresis***

Samples (200  $\mu$ g) were incubated at room temperature for 30 min in four volumes of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in either 2 M HCl for protein carbonyl derivatization/oxyblots or 2 M HCl for gel maps and mass spectrometry analysis. This was followed by precipitation of proteins by addition of ice-cold 100% trichloroacetic acid (TCA) to a final concentration of 15% and samples were placed on ice for 10 min.

Precipitates were centrifuged at 15,800 g for 2 min. The pellets were washed with 0.5 ml of 1:1 (v/v) ethanol/ethyl acetate solution. After centrifugation and washing with ethanol/ethyl acetate solution three times, the samples were then dissolved with 185  $\mu$ l of rehydration buffer (8 M urea, 20 mM dithiothreitol, 2.0% (w/v) CHAPS, 0.2% Biolytes, 2 M thiourea and bromophenol blue). For the first-dimension electrophoresis, 200  $\mu$ l of sample solution were applied to a ReadyStrip™ IPG strip (Bio-Rad). The strips were soaked in the sample solution for 1 hour to allow uptake of the proteins. The strip was then actively rehydrated in protean IEF cell (Bio-Rad) for 16 hours at 50V. The isoelectric focusing was performed at 300V for 2 hours linearly; 500V for 2 hr linearly; 1000V for 2 hr linearly, 8000V for 8 hr linearly and 8000V for 10 hr rapidly. All the processes above were carried out at 22°C. The focused IEF strip was stored at  $-80^{\circ}\text{C}$  until second dimension electrophoresis was performed. For second dimension electrophoresis, thawed IPG® Strips pH 3-10 were equilibrated for 10 min in 50 mM Tris-HCl (pH 6.8) containing 6M urea, 1% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 0.5% dithiothreitol, and then re-equilibrated for 15



min in the same buffer containing 4.5% iodacetamide in place of dithiothreitol. Linear Gradient (8-16%) Precast criterion Tris-HCl gels (Bio-Rad) were used to perform second dimension electrophoresis. Precision Protein™ Standards (Bio-Rad) were run along with the sample at 200V for 65 min. After electrophoresis, the gels were incubated in fixing solution (7% acetic acid, 10% methanol) for 20 min. Approximately 40 ml of Coomassie Safe Gel Stain (Bio-Rad) were used to stain the gels for 1 hour, on a gently continuous rocker. The gels were placed in deionized water overnight for destaining.

#### ***3.1.4. Western Blotting***

The same amount of protein samples (200 µg) was used for detecting specific protein carbonyl levels and the electrophoresis was carried out in the same way as described above. Proteins (200 µg) were incubated with 4 volumes of 20 mM 2,4-dinitrophenylhydrazine (DNPH) at room temperature (25°C) for 20 min. The gels were prepared in the same manner as 2D-electrophoresis. The proteins from the second dimension electrophoresis gels were transferred to nitrocellulose (Bio-Rad) using a Criterion Blotter Apparatus (Bio-Rad) at 15V for 2 h. The 2,4-dinitrophenyl hydrazone (DNP) adducts of the carbonyls of the proteins were detected on the nitrocellulose paper using a primary rabbit antibody (Chemicon) specific for DNP-protein adduct (1:100), followed by a secondary goat anti-rabbit IgG (Sigma, St Louis, MO, USA) antibody. The resultant stain was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution (SigmaFast tablets; Sigma).

### ***3.1.5. Image Analysis***

The gels (n=8 aged and n=8 senescent) and nitrocellulose blots were scanned and saved in TIF format using a HP Deskjet H2180 (Hewlett Packard). PDQuest 2-D Analysis Software (Bio-Rad, Inc.) was used for matching and analysis of visualized protein spots among differential gels and membranes to compare protein and DNP immunoreactivity content between senescent and aged rats brain samples. This sophisticated software offers powerful comparative analysis and is specifically designed to analyze many gels or blots at once. Powerful automatching algorithms quickly and accurately match gels or blots and sophisticated statistical analysis tools identify experimentally significant spots. The principles of measuring intensity values by 2-D analysis software were similar to those of densitometric measurement. The average mode of background subtraction was used to normalize intensity values, which represents the amount of protein (total protein on gel and DNP-bound protein on the membrane) per spot. After completion of spot matching, the normalized intensity of each protein spot from individual gels (or membranes) was compared between groups using statistical analysis. Statistical significance was assessed by a two-tailed Student's t-test. P values <0.05 were considered significant for comparison between aged and senescent rats. This is the method of statistical analysis most appropriate for proteomic analysis of small number of protein spots in contrast to statistical approach used for large analysis common in gene microarray studies (Maurer et al., 2005).

### ***3.1.6. Trypsin digestion***

The selected protein spots were excised with a clean blade and transferred into clean microcentrifuge tubes. The protein spots were then washed with 0.1 M

ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ) at room temperature for 15 min. Acetonitrile was added to the gel pieces and incubated at room temperature for 15 min. The solvent was removed, and the gel pieces were dried in a flow hood. The protein spots were incubated with 20  $\mu\text{l}$  of 20 mM DTT in 0.1M  $\text{NH}_4\text{HCO}_3$  at 56°C for 45 min. The DTT solution was then removed and replaced with 20  $\mu\text{l}$  of 55 mM iodacetamide in 0.1 M  $\text{NH}_4\text{HCO}_3$ . The solution was incubated at room temperature in the dark for 30 min. The iodacetamide was removed and replaced with 0.2 ml of 50 mM  $\text{NH}_4\text{HCO}_3$  and incubated at room temperature for 15 min. 200  $\mu\text{l}$  of acetonitrile was added. After 15 min incubation, the solvent was removed, and the gel spots were dried in a flow hood for 30 min. The gel pieces were rehydrated with 20 ng/ $\mu\text{l}$  methylated trypsin (Promega, Madison, WI) in 50 mM  $\text{NH}_4\text{HCO}_3$  with the minimal volume to cover the gel pieces. The gel pieces were chopped into smaller pieces and incubated at 37°C overnight in shaking incubator.

### ***3.1.7. Protein identification by mass spectrometry***

Selected spots were manually excised from gels and submitted to trypsin proteolysis. Briefly, after three destaining steps using a solution of 50 mM ammonium bicarbonate (5 min), 50% acetonitrile in 50 mM ammonium bicarbonate (15 min) and 100% acetonitrile (15 min), about 100 ng of trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega, Madison, WI, USA), solubilised in 10  $\mu\text{l}$  of a 25 mM ammonium bicarbonate digestion buffer, were added to each vacuum-dried gel spot. Digestion was performed at 37°C overnight. An aliquot (1  $\mu\text{l}$ ) of each peptide mixture was mixed with the same volume of  $\alpha$ -cyano-4-hydroxy-trans-cinnamic acid matrix solution (10mg/ml) in 70% acetonitrile containing 0.2% TFA (v/v) and spotted onto a MALDI target plate.

MALDI-ToF MS analyses were performed in a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA) equipped with a 337 nm nitrogen laser and operating in reflector mode. Mass data were obtained by accumulating several spectra from laser shots with an accelerating voltage of 20 kV. Two tryptic autolytic peptides were used for the internal calibration ( $m/z$  842.5100 and 2807.3145). Identification by peptide mass fingerprint (PMF), with the monoisotopic mass list obtained from each spot, was performed after exclusion of expected contaminant mass values by Peak Erazor program (<http://www.protein.sdu.dk/gpmaw/Help/PeakErazor/peakerazor.html>), using the Mascot search engine (v. 2.2) against SwissProt database (v. 55.3, 366226 sequences). Up to one missed cleavage, 50 ppm measurement tolerance, oxidation at methionine (variable modification) and carbamidomethyl cysteine (fixed modification) were considered. Posttranslational modifications were not considered. Identifications were validated when the probability-based Mowse protein score was significant according to Mascot.

### ***3.1.8. Determination of reduced glutathione and oxidized glutathione in the cytosol.***

Brain regions were homogenized on ice for 10 s in 100 mM potassium phosphate, pH 7.5, which contained 12 mM disodium EDTA. The homogenate was divided in two aliquot, for total glutathione (GSH+GSSG) assay 0.25 ml of homogenate was added to equal volume of 100mM potassium phosphate buffer pH7.5, containing 17.5mM EDTA and 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sample SS1). For oxidized glutathione (GSSG) assay 0.25 ml of homogenate was added to 100 mM potassium phosphate buffer pH 6.5, containing 17.5 mM EDTA and 10 mM N-ethylmaleimide NEM (Sample SS2). The samples were centrifuged at 800g for 20 min, and the supernatant fractions were then

centrifuged at 10,000 g for 30 min. The supernatant of SS1 and SS2 represented the cytosolic fractions and were used for the spectrophotometric assay of total or oxidized glutathione. Before spectrophotometric determination, 0.25 ml aliquot of SS2 sample was passed through a C18 Sep-Pak cartridge (Waters, Watford, U.K.) to remove the excess of NEM and washed with 0.5 ml of buffer 100 mM potassium phosphate buffer pH 7.5, containing 5 mM EDTA. Spectrophotometric assay of glutathione was performed adding the samples to a cuvette containing 0.5 unit of glutathione reductase, 0.2mM DTNB in a final volume of 1ml of 100 mM potassium phosphate buffer pH 7.5, 5 mM EDTA and the reaction initiated by adding NADPH (220 nmoles). The change in absorbance at 412 nm was recorded over a period of 5 min for SS1 sample or 10 min for SS2 sample using a reference cuvette containing equal concentrations of NADPH, DTNB and enzyme. The GSH and GSSG content, expressed as nmol/mg protein, was determined by comparison with a standard curve obtained with GSH and GSSG solution.

### ***3.1.9. Determination of total glutathione (GSH+GSSG) and oxidized glutathione (GSSG) in mitochondria.***

The pellet of SS1 and SS2 samples obtained after centrifugation at 10,000 g was utilized for determination of total and oxidized glutathione in mitochondria. The SS1 pellet was resuspended in 0.32 M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, 5 mM DTNB. The SS2 pellet was resuspended in 0.32 M sucrose, 1 mM EDTA, 100 mM potassium phosphate buffer pH6.5, containing 5mM N-ethylmaleimide (NEM). Low and high speed differential centrifugations for mitochondrial isolation were, respectively, 1,300 g for 3 min and 21,200 g for 10 min for all brain regions examined; mitochondrial preparations were washed once with a 100mM potassium phosphate buffer pH 7.5, containing 5mM EDTA. This washing step was found to be critical in order to remove excess GSH and GSSG

from the cytosolic fraction. The mitochondria were then mixed, sonicated and centrifuged at 10,000 g per 11 min., the supernatant was used for spectrophotometric determination assay. Before of spectrophotometric determination 0.25 ml aliquots of SS2 sample were passed through a C18 Sep-Pak cartridge (Waters, Watford, U.K.) to remove the excess of NEM and washing with 0.5 ml of buffer 100 mM potassium phosphate buffer pH 7.5, containing 5 mM EDTA.

Spectrophotometric assay of glutathione was performed adding the samples to a cuvette containing 0.5 unit of glutathione reductase, 0.2 mM DTNB in a final volume of 1 ml of 100 mM potassium phosphate buffer pH 7.5, 5 mM EDTA and the reaction initiated by adding NADPH (220  $\mu$ M). The change in absorbance at 412 nm was recorded over a period of 5 min for SS1 sample or 10 min for SS2 sample using a reference cuvette containing equal concentrations of NADPH, DTNB and enzyme. Protein concentration was determined in the samples with NEM, according to Bradford method, using BSA as standard. The GSH and GSSG content, expressed as nmol/mg prot, was determined by comparison with a standard curve obtained with known concentrations of GSH and GSSG solution.

### ***3.1.10 Enzyme assays***

**Pyruvate kinase:** The enzyme activity of pyruvate kinase (PK) was determined at 37 °C by lactate dehydrogenase-coupled spectrophotometric assay (Hayashi et al., 1979). The standard reaction mixture contained 100 mM Tris pH 8.0, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.2 mM NADH, 10  $\mu$ g LDH, 10 mM Phosphoenolpyruvate, and 1.5 mM ADP in a final volume of 1 ml. The reaction was started by adding enzyme solution (0.5–1  $\mu$ g).

One unit of activity is the amount of enzyme catalyzing the oxidation of 1  $\mu\text{mol}$  NADH/min under the above conditions. The assay was carried out in a microplate reader (Labsystem Multiscan MS).

**Glyceraldehyde-3-phosphate dehydrogenase:** To determine the activity of GAPDH, 20  $\mu\text{g}$  of protein homogenate was added to an assay mixture (100 mM 3-phosphoglyceric acid, 200 U/ml 3-phosphoglyceric phosphokinase, 200 mM cysteine, 100 mM  $\text{MgSO}_4$ , 34 mM ATP, 7.0 mM  $\beta$ -NADH) in a UV-transparent microtiter plate (Corning). The change in absorbance at 340 nm was monitored during a 5-min period with a microplate reader (Labsystem Multiscan MS).

#### ***3.1.11. Statistical analysis***

Results were expressed as means  $\pm$  SEM of at least eight separate experiments. Statistical analyses were performed using the software package SYSTAT (Systat Inc., Evanston IL, USA). The significance of the differences, evaluated by two-way ANOVA, followed by Duncan's new multiple-range test, was considered significant at  $P < 0.05$ .

## **3.2 Multiple sclerosis**

### ***3.2.1. Ethical permission***

The study was conducted according to guidelines of local Ethics Committee, and informed consent was obtained from all patients.

### ***3.2.2. Patients***

Twenty-six patients with an age range of 20–60 years were used as the “test” group for this study. The mean disease duration was  $3.9 \pm 2.0$  years. All these patients had a confirmed clinical diagnosis of MS according to the diagnostic criteria of McDonald et al. [141]. Furthermore, all the subjects were clinically classified having the relapsing remitting form of MS according to the criteria described in Lublin and Reingold [142]. The patients had not been undergoing corticosteroid or immunosuppressive treatment for at least 2 months before the CSF samples were collected. Twenty control patients, with an age range of 30–60 years, underwent lumbar puncture because of suspected subarachnoid hemorrhage, pseudotumor cerebri, oculomotor palsies, or other indications in the usual neurological survey. Laboratory and neuroimaging tests were normal. Therefore the final diagnosis was mainly tension headache or conversion disorder. Clinical and demographical data of patients are shown in Table 1.

### ***3.2.3. Sampling***

Blood was collected from controls and patients by venipuncture from an antecubital vein into tubes containing EDTA as an anticoagulant. Immediately after sampling, 1 ml the blood was centrifuged at  $10,000 \times g$  for 10 min at  $4 \pm 8^\circ C$  to separate serum from red blood cells and 4 mL were utilized for lymphocytes purification. Lymphocytes from peripheral blood were purified using the Ficoll Paque System following the procedure provided by the manufacturer (GE Healthcare, Piscataway, NJ, USA). CSF was obtained (on ice) from all subjects by lumbar spinal tap. The CSF samples were immediately centrifuged at  $10,000 \times g$  for 3 min at  $4^\circ C$  to remove any contaminating cells and kept on ice until the biochemical assays were performed. Chemical analysis of CSF showed no heme present in the CSF in any of the control or MS CSF samples. CSF lymphocytes



count was  $1.05 \pm 1.17$  in the first MS CSF sample and  $1.04 \pm 1.15$  in second ones. The control group lymphocytes count was  $1 \pm 0.12$ .

#### ***3.2.4. Western blot analysis***

Carbonyls (DPNH), HNE, Hsp60, Hsc70, Hsp-72, HO-1, HO-2, Trx, TrxR-1, Sirt-1 were evaluated by Western blot analyses. In brief, proteins (40 mg) for each sample were separated by a 12.5% SDS-PAGE and electroblotted (1 h at 100 V) to nitrocellulose membranes (Bio-Rad) using 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. Equal protein loading was confirmed by staining with 0.2% (v/v) Ponceau S in 7% acetic acid. Blotted membranes were blocked with 3% non fat dry milk in phosphate-buffered saline and challenged with appropriate primary antibodies, namely anti-Hsc70, monoclonal antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), that recognizes the constitutive form of Hsp 70, or with a monoclonal anti-Hsp72 antibody (RPN 1197, Amersham) that recognizes only the inducible form, anti-DPNH (V0401 DAKO, Glostrup, Denmark), anti-HNE (HNE11-S Alpha Diagnostic International, San Antonio, TX, USA), anti-HO-1, anti- HO-2 (Stressgen, Victoria, BC, Canada) anti-Trx, anti TrxR1 and anti Sirt-1 rabbit polyclonal antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), for 1 h at room temperature. Immunodetection of Hsp60 was performed using a polyclonal goat antibody sc-1052 (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA). Unbound antibodies were removed by washing TBS-T for 5 min. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody diluted 1:10,000. Protein bands were visualized with ECL Plus™ (Amersham, NJ, USA) according to the manufacturer's protocol. Immunoreactive

bands were quantified by scanning Western blot-imaged films with a laser densitometer (LKB-Ultrascan, XL model, Pharmacia, American Instruments, Haverhill, MA, USA). Molecular weights of the proteins detected were determined using a standard curve obtained with proteins of known molecular weight.

### ***3.2.5. Protein measurements***

Samples protein concentrations were determined by the bicinchoninic acid protein assay (Cod 23227 Pierce Protein Research Products, Rockford, IL 61101 U.S.A.) according to the method described in Smith et al. 1985 and using bovine serum albumin as standard.

### ***3.2.6. Statistical analysis***

All results are expressed as means  $\pm$  standard error of mean. Each experiment was performed, unless otherwise specified, in triplicate. Data were analyzed by one-way analysis of variance, followed by inspection of all differences by Duncan's new multiple-range test. Differences were considered significant at  $P < 0.05$ .

## **3.3 Alzheimer's disease**

### ***3.3.1. Patients***

The study was conducted according to guidelines of local Ethics Committee, and informed consent was obtained from all patients. Thirty patients (13 men and 17 women), with an age range of 69-81 years were enrolled in the study. All patients had progressive cognitive and memory impairment for at least 12 months and were diagnosed as suffering of probable AD, according to the criteria of the National Institute of Neurological and communicative Disorder and Stroke

Alzheimer Disease and Related Disorder Association (NINCDS-ADRADA) [124]. The evaluation of the stage of dementia was assessed by the Mini Mental State Examination (MMSE) [143]. Status of basic and instrumental activities of daily living (Activity of Daily Living, ADL, Instrumental Activity of Daily Living, IADL) was also assessed. None of our patients had a history of major psychiatric illness or other neurological disorders (i.e. Parkinson's disease, stroke, dementia, multiple sclerosis, etc), history of head trauma or epilepsy, acute or chronic medical illness, endocrinopathies or vitamin B deficiency affecting cognitive functions, alcohol or drug abuse, and conditions precluding MRI or CT execution. Thirteen patients were classified as mild and seventeen as moderate. All patients were under acetylcholinesterase inhibitor (AChE-I) medication. Computed tomography (CT) or magnetic resonance imaging (MRI) scan showed widespread cortical atrophy in most patients (Figure 28). In addition ten subjects (5 men and 5 women) with an age range of 60-79 years were studied as a control group. Controls showed no impairment in neuropsychological evaluation. Laboratory and neuroimaging tests were normal. Clinical and demographic data of patients and control subjects are shown in Table 2.

### ***3.3.2. Sampling***

Blood was collected from controls and patients by venipuncture from an antecubital vein into tubes containing EDTA as an anticoagulant. Immediately after sampling, 1 ml the blood was centrifuged at 3000 x g for 10 min at 4 °C to separate serum from red blood cells and 4 mL were utilized for lymphocytes purification. Lymphocytes from peripheral blood were purified using the Ficoll Paque System following the procedure provided by the manufacturer (GE Healthcare, Piscataway, NJ, USA). The control group lymphocytes count was  $1 \pm 0.12$ .

### ***3.3.3. Western blot analysis***

Trx, Sirt-1, Sirt-2 were evaluated by Western blot analyses. Plasma samples were ready to use, while the lymphocyte pellet was homogenized and centrifuged at 10,000 x g for 10 min and the supernatant was used for analysis after dosage of proteins.

Equal concentrations of protein extracted for each sample (40 mg) were separated on a polyacrylamide mini gels precasting 4-20% (cod NB10420 NuSept Ltd Australia). Before being loaded on the gel, samples were boiled for 3 minutes in sample buffer (containing 40 mM Tris-HCl pH 7.4, 2.5% SDS, 5% 2-mercaptoethanol, 5% glycerol, 0.025 mg/ml of bromophenol blue). The proteins were transferred onto nitrocellulose membrane (0.45mM) (BIO-RAD Hercules, CA, USA) in transfer buffer containing (0.05% di SDS, 25mM di Tris, 192mM glycine and 20% v/v methanol).

The transfer of the proteins on the nitrocellulose membrane was confirmed by staining with Ponceau Red which was then removed by 3 washes in PBS (phosphate buffered saline) for 5 min. each. The membranes were then incubated for 1 hour at room temperature in 20 mM Tris pH 7.4, 150 mM NaCl and Tween 20 (TBS-T) containing 2% milk powder and incubated with appropriate primary antibodies, namely anti-Trx, anti Sirt-1, anti Sirt-2, rabbit polyclonal antibody (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), overnight at 4°C in TBS-T.

Excess unbound antibodies were removed by 3 washes are with TBS-T for 5 minutes. After incubation with primary antibody, the membranes were washed 3 times for 5 min. in TBS-T and then incubated for 1 h at room temperature with the secondary polyclonal antibody conjugated with horseradish peroxidase (dilution 1:500).

The membranes were then washed 3 times with TBS-T for 5 minutes. The same

membrane was incubated with a polyclonal antibody anti-beta-actin (SC 1615 Santa Cruz Biotech. Inc., CA, USA, dilution 1:1000) to verify that the concentration of protein loaded in the gel was the same in each sample. Finally, the membranes were incubated for 3 minutes with SuperSignal chemiluminescence detection system kit (Cod 34080 Pierce Chemical Co, Rockford, USA) to display the specific protein bands for each antibody. The immunoreactive bands were quantified by capturing the luminescence signal emitted from the membranes with the Gel Logic 2200 PRO (Bioscience) and analyzed with Molecular Imaging software for the complete analysis of regions of interest for measuring expression ratios. The molecular weight of proteins analyzed was determined using a standard curve prepared with protein molecular weight.

#### ***3.3.4. Determination of protein***

Samples protein concentrations were determined by the bicinchoninic acid protein assay.

#### ***3.3.5. Statistical Analysis***

All results are expressed as means  $\pm$  S.E.M. Each experiment was performed, unless otherwise specified, in triplicate. Data were analyzed by one-way ANOVA, followed by inspection of all differences by Duncan's new multiple-range test. Differences were considered significant at  $P < 0.05$ .

## 4. RESULTS

### 4.1 Aging

#### 4.1.1. *Protein carbonylation in senescent vs aged rats:*

**Cortex.** Two-dimensional (2D) electrophoresis offers an efficient tool for screening for abundant protein changes in different disease states as well as differences in metabolic pathways [144]. Western blot and subsequent immunochemical detection of DNP-adducts allowed identification of carbonylated proteins in the cerebral tissue samples of senescent in comparison with aged rats. We used a parallel approach to quantify the protein levels by Coomassie staining and the carbonyl levels by immunohistochemistry. Coomassie stain achieves a linear and sensitive staining of gel slabs, and immunoblotting with DNP antibody allows specific detection of DNP-adducts in hippocampus samples. The specific carbonyl levels were obtained by dividing the carbonyl level of a protein spot on the nitrocellulose membrane by the protein level of its corresponding protein spot on the gel. Such numbers give the carbonyl level per unit of protein. In senescent rat cortex 14 proteins result significantly more oxidized than aged rat cortex (Figure 2). These proteins showed on Figure 3 and listed on Table 3 are: Creatine kinase B-type, Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoforms, Aspartate aminotransferase, Fructose-bisphosphate aldolase A, Peroxiredoxin- 2, Guanine nucleotide-binding protein subunit beta-1, Vacuolar ATP synthase subunit B, Stress-70 protein, Aldehyde dehydrogenase, Pyruvate kinase isozymes M1/M2, ATP synthase subunit alpha, Triosephosphate isomerase. Glyceraldehyde-3-phosphate dehydrogenase occurs in double spot

suggesting that a post-translational modification alter the heterogeneity of this particular protein, affecting protein isoelectric point (pI).

All the others proteins occur in a single spot with a significant p. value (<0.05).

**Striatum.** In senescent rat striatum 9 proteins result significantly more oxidized than in aged rats (Figure 2). These proteins showed on Figure 4 and listed on Table 4 are: 78 kDa glucose-regulated protein, Stress-70 protein, Adenylate kinase, Elongation factor Tu, Pyruvate kinase isozymes M1/M2, Aconitate hydratase, Triosephosphate isomerase, Glyceraldehyde-3-phosphate dehydrogenase. All the proteins but Pyruvate kinase isozymes M1/M2 occur in a single spot. All these proteins were identified as a single spot on the gel and present a significant p. value (<0.05). A representative two-dimensional carbonyl immunoblots from aged (A) and senescent rat striatum is reported in Figure 5.

**Hippocampus.** Figure 6 shows representative 2D-electrophoresis gels of hippocampus from senescent (B) and aged rats (A) after Coomassie staining. Figure 7 shows representative 2D Western blots of senescent (B) and aged rats (A). In comparison with aged rats, senescent animals have eleven proteins significantly more oxidized. These proteins are: heat shock protein 90 (Hsp90), Cytochrome b-c1 complex subunit 1, Creatine kinase, malate dehydrogenase,  $\alpha$ -enolase, glutamate dehydrogenase, pyruvate kinase, fructose-bishosphate aldolase C, ATP synthase subnit alpha and glutamine synthase (Table 5).

Table 4 shows the proteins that were successfully identified by mass spectrometry along with the peptides matched, percentage coverage, pI, Mw values and the increase of specific carbonyl levels, indexed as percentage of control.

**Cerebellum.** In senescent rats cerebellum 11 proteins result significantly more oxidized than aged rat cerebellum (Figure 8). In Figure 9 two-dimensional carbonyl immunoblots of senescent (B) and aged rats (A) cerebellum proteins are showed. In Table 6 are showed the proteins successfully identified by mass

spectrometry along with the p value, percentage of oxidation based on CTR value, Mowse score, *pI* and theoretical mass.

These proteins are: Heat shock cognate (Hsc)72 protein, Malate dehydrogenase, Isocitrate dehydrogenase, Vesicle-fusing ATPase, Pyruvate kinase isozymes M1/M2, Aspartate aminotransferase, Phosphoglycerate kinase 1, Voltage-dependent anion-selective channel protein, Adenylate kinase isoenzyme 4. All these proteins were identified as a single spot on the gel and present a significant p value (<0.05).

#### ***4.1.2. Validation of proteomic results***

To verify the results obtained through redox proteomics analysis we performed a validation study on the Hsp70 carbonylation using traditional immunochemistry. Consistent with the proteomics results, the carbonyl levels of HSP 70 were significantly increased by about 40 % in cerebral cortex of senescent rats compared to aged rats (Figure 10). The increased carbonyl levels of Hsp70 were stronger when detected by proteomics method. The differences in the magnitude of fold changes of carbonyl levels between the two techniques are likely because proteomics measures the carbonyl level per unit of protein, whereas Western blotting measures the carbonyl level of total protein.

Clearly, both techniques showed that Hsp70 is oxidatively modified in cerebral cortex of senescent rats, thus validating our proteomics results.

#### ***4.1.3. Glutathione redox state analysis***

The content of reduced glutathione (GSH) and oxidized glutathione (GSSG) was measured in both cytosol and mitochondria of different brain regions of aged and senescent rats. In senescent rats, GSH significantly ( $p < 0.05$ ) diminished in all brain regions examined except for the cerebellum, where this decrease was not



significant, compared to aged control animals (Figure 11 A). Consistent with this finding, mitochondrial GSH showed a significant decrease in all regions, particularly in the brain areas of hippocampus, followed by cortex, striatum and cerebellum (Figure 11 A).

Analysis of GSSG in the same brain regions showed to increase significantly in senescent rat brain areas, both in the cytosol and mitochondrial compartments, particularly in the hippocampus, followed by striatum, cortex and cerebellum (Figure 11 B). We also measured GSH/GSSG ratios in the different brain regions examined. In the cytosol, GSH/GSSG ratios varied in the brain from  $87.2 \pm 11$  of aged to  $33.5 \pm 8$  of senescent animals, whereas mitochondrial ratios of GSH/GSSG varied from  $56.3 \pm 9$  to  $19.9 \pm 7$ , that is 62% and 64%, respectively, indicating that no significant shift in the mitochondrial GSH redox state occur, compared to that in the cytosol, among the two experimental group of animals.

#### ***4.1.4. Glyceraldehyde-3-phosphate dehydrogenase and Pyruvate Kinase enzymatic activity***

Several studies from our laboratory and others demonstrated that protein oxidation likely lead to protein dysfunction [145, 146, 147]. Most of the oxidative post-translational modifications are irreversible and they are responsible of the impairment of multiple cellular functions.

Therefore, we measure the enzyme activity of some of the enzymes we found oxidized with the redox proteomics approach. Specifically, pyruvate kinase was found to be decreased in all the brain regions of senescent vs aged rats (Figure 12). In addition, the enzyme activity of GAPDH was found to be significantly decreased in both cortex and striatum of senescent rats compared with aged rats (Figure 13). These results are in line with our previous findings of decreased

enzyme activity of PK and GAPDH upon oxidation in AD brain in comparison with control brain [148].

#### ***4.2 Multiple sclerosis***

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) of unclear etiology, characterized by perivenous infiltration of lymphocytes and macrophage leading damage of myelin and axons in the brain and spinal cord. Clinical disease usually occurs with recurrent and reversible episodes of neurological dysfunction affecting one or several sites, during late adolescence and early adulthood; this form being the most prevalent is called relapsing-remitting form (RR) and was chosen in this study. Table 1 summarizes clinical data of patients and control a disease duration of  $3.92 \pm 2.08$  years, a relatively short duration typical of the relapsing remitting form not yet changed into the secondary progressive form. The mean value of Expanded Disability Status Scale (EDSS), which the most used method of quantifying disability in multiple sclerosis, was  $1.5 \pm 1.18$  and this indicates that patients have a low degree of disability linked to the short period of disease. Brain MRI from MS patients presenting two hypointense areas distributed bilaterally on the white matter of the semioval center (A), enhanced by gadolinium injection (B) is shown in Fig. 14 A and B.

The heat shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging and neurodegenerative disorders. The vitagene family is composed of the heat shock proteins HO-1 (also called Hsp32), Hsp70, by the thioredoxin system and by sirtuin proteins [5, 19, 149]. We therefore evaluated the expression levels of cellular stress response proteins in the CSF, plasma and lymphocytes in control

and in MS patients. Western blot analysis of CSF probed for heme oxygenase-1 is reported in Figure 15. HO-1 expression is significantly ( $P < 0.05$ ) increased in the CSF of MS patients, compared to controls. This finding was associated with increased levels of protein oxidation (Figure 16A) as well as lipid oxidation (Figure 16C). Protein oxidation was evaluated by measuring the amount of protein carbonyls (DNPH). Protein carbonyls most often are detected by two methods, i.e., derivatization with 2,4-dinitrophenylhydrazine (DNPH), followed by immunochemical detection with an antibody against the resulting protein hydrazone, or formation of the Schiff base by biotin hydrazide followed by detection of the protein-bound imine with enzyme- or fluorophore-linked avidin or streptavidin. Here we describe the DNPH-based detection method that is routinely used for detection of carbonylated proteins. In the DNPH method, samples are derivatized with DNPH. DNPH reacts with carbonyl groups to form protein-resident 2,4-dinitrophenylhydrazone (DNP), which is then detected using commercially available anti-DNP antibodies (Figure 16B). As shown, protein carbonyls were found at a significantly ( $P < 0.05$ ) higher level in the CSF of MS patients than in control subjects.

Furthermore, one measure of oxidative marker is lipid oxidation, indexed by HNE [150, 151], which can also occur in brain under oxidative stress [152]. HNE, formed from arachidonic acid or other unsaturated fatty acids following free radical attack, binds by Michael addition to proteins, particularly at cysteine, histidine, or lysine residues [152]. Examination of HNE levels in the CSF of MS patients showed a significant ( $P < 0.05$ ) elevation of proteinbound HNE as compared to control group (Fig. 21D). The 70 kDa family of stress proteins is one of the most extensively studied cytoprotective system. Included in this family are the constitutive isoform, Hsc70 (heat shock cognate), and the inducible isoform, Hsp70 (also referred to as Hsp72). Figures. 17 and 18 show a significant ( $P <$

0.05) increase in the expression level of Hsc70 and Hsp70, respectively, in lymphocytes of MS patients with respect to controls (Figs. 17A and 18A). Representative immunoblots are also shown (Figs. 17B and 18B). Heme oxygenase-1 (HO-1), also referred to as Hsp32, belongs to the Hsp family and protects brain cells from oxidative stress by degrading toxic heme into free iron, carbon monoxide and biliverdin [149, 153]. This latter is then reduced by biliverdin reductase (BVR) into bilirubin (BR), a linear tetrapyrrole with antioxidant properties; very recently,

BR has been shown to effectively counteract also nitrosative stress, due to its ability to bind and inactivate NO and RNS [50, 153]. The constitutive isoform of heme oxygenase is HO-2. We evaluated the expression of both isoforms in lymphocytes of MS patients and in controls. As shown in Figure 19, HO-1 protein expression was higher in MS samples compared to control.

Notably, increase in HO-1 was associated with a significant ( $P < 0.05$ ) decrease in HO-2 expression in the same experimental group (Figure 20).

Analysis of lymphocytes in MS patients, compared to control group, revealed also a significant ( $P < 0.05$ ) increase in thioredoxin expression, whereas expression of the related enzyme thioredoxin reductase-1 was decreased (Figures 21A, B and 22A, B). These changes were associated with significantly ( $P < 0.05$ ) increased formation of protein carbonyls (Fig. 23A and B) and HNE (Fig. 23C and D). Consistently to the observed changes in MS lymphocytes, analysis of plasma in MS patients showed higher and significant ( $P < 0.05$ ) expression levels of HO-1, Hsp60 than in control subjects (Figures 24 and 25), while no difference was found to a significant extent between the two experimental groups in Hsp70 protein levels (Figure 26). Interestingly, we found in the plasma significantly ( $P < 0.05$ ) higher levels of sirtuin-1 protein in MS patients than in control group (Figure 27).

We cannot exclude that this might not be a specific alteration of this progressive inflammatory neurodegenerative disease.

### ***4.3 Alzheimer's disease***

Alzheimer's disease (AD) is the most common form of dementia and is characterized pathologically by senile plaques, neurofibrillary tangles and cerebral amyloid angiopathy [154]. Both senile plaques and cerebral amyloid angiopathy consist primarily of aggregated protein deposits of the amyloid-beta protein [155, 156]. Accumulation of A $\beta$  characterizes AD as a protein misfolding disease, suggesting a pathogenic role for defective protein clearance by the ubiquitin-proteasome system [157, 158]. Furthermore, misfolded A $\beta$  is considered to be the key mediator of cellular oxidative stress in AD and different evidences exist that oxidative stress is central to neurodegeneration in AD [159-161] In a previous work, it has been shown in brain, peripheral lymphocytes, and plasma that oxidative and nitrosative stress is evident in AD compared with control sample [135].

The heat shock response contributes to establishing a cytoprotective state in a wide variety of human diseases, including inflammation, cancer, aging and neurodegenerative disorders. The vitagene family is composed of the heat shock proteins, by the thioredoxin system and by sirtuin proteins [5, 19, 149]. Therefore, we evaluated the expression levels of Trx and Sirtuin in the plasma and lymphocytes in control and in AD patients.

Sirtuins are a family of histone deacetylases that, in humans, includes at least seven members that exhibit different cellular and subcellular localizations and substrate specificities. Western blot analysis of lymphocytes probed for Sirt-1 is

reported in Figure 29. Sirt-1 expression is significantly increased in AD patients, compared to controls. In accord with the results described in Calabrese et al. 2006 this finding was associated with a significant increased levels of protein oxidation as well as lipid oxidation: the protein oxidation was evaluated by measuring the amount of protein carbonyls (DNPH); protein carbonyls were found at a significantly higher level in the AD patients than in control subjects [135]. Furthermore, our study group previously demonstrated that HNE, considered a marker of lipid peroxidation, is elevated in AD plasma and lymphocytes compared with to control group [135].

Figure 30 shows a significant increase in the expression level of Sirt 2 in lymphocytes of AD patients compared to controls.

As shown in Figure 31, analysis of lymphocytes in AD patients, compared to control group, revealed also a increases in thioredoxin expression. Consistently to the observed changes in AD lymphocytes, analysis of plasma in AD patients showed higher expression levels of Sirt-1 and Sirt-2 (Figure 32 and 33) Interestingly, we investigated in our experimental conditions the expression of Trx and we found in the plasma higher levels of Trx protein in AD patients compared with control group (Figure 34). The increased expression of these proteins seemed to be consequent to a strong oxidant environment.

## 5. DISCUSSION

### *5.1 Aging*

Aging is characterized by a general decline in physiological functions that affects especially the brain which is particularly susceptible to the effects oxidant injury. In this context, increasing evidence supports the notion that reduction of cellular expression and activity of antioxidant proteins and the resulting increase of oxidative stress are fundamental causes in the aging processes and neurodegenerative diseases [47, 48, 52].

Reduced glutathione (GSH) is the most prevalent non-protein thiol in animal cells. De novo and salvage synthesis of GSH maintains a reduced cellular environment where the tripeptide is a co-factor for cytoplasmic enzymes and can act as important post-translational modifier in a number of cellular proteins [162-164]. Owing to the cysteine thiol, it reacts as a nucleophile with exogenous and endogenous electrophilic species. As a consequence, reactive oxygen and nitrogen species (ROS, RNS) are frequently targeted by GSH in both spontaneous and catalytic reactions [153, 165]. Since free radicals have defined roles in cell signalling events as well as in human disease pathologies [52], an imbalance in expression of GSH and associated enzymes has been implicated in a variety of pathological conditions [50]. Thus, impaired function of the central nervous system (CNS) in aged animals is associated with increased susceptibility to the development of many neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [75, 166]. In the present study we show that oxidative stress increases during aging in brain, as revealed by decreased GSH content and increases in GSSG, particularly in the mitochondria and this was associated with specific pattern of protein

oxidation at the inner mitochondrial membrane, where protein of mitochondrial bioenergetics reside [165, 167].

Oxidatively damaged proteins are known to increase markedly with age [145, 168, 169]. In addition the loss of protein sulfhydryl groups and the reduced activity of important metabolic enzymes have been documented to occur in brain as a function of aging [92, 170, 171]. Studies on the induction of HSPs response, a cytoprotective mechanism to counteract oxidative damage, showed a regional specificity indicating that different brain areas might undergo oxidation differently and react to protect themselves based on the strength of the insult [13, 49, 172].

Previous studies performed on SAMP8 mice, an animal model of aging, showed increased levels of oxidized proteins in old animal versus young, a number of which were also identified to be involved in energy metabolism process with impaired activity [103].

In this study we used a redox proteomics approach to identify the oxidatively modified proteins in four different brain regions of 28 months old rats compared with 12 months old rats. We analyzed the hippocampus, cerebellum, cortex and striatum, all known to be involved in cognitive function such as working memory, spatial cognition, thought and consciousness. All these brain regions are recognized to be involved in the aging process and also in neurodegeneration and have been demonstrated that their dysfunction is associated with many protein conformational disease like AD, PD, amyotrophic lateral sclerosis, Huntington's disease and Friedreich's ataxia, all associated with the presence of abnormal proteins [103, 173, 174].

Our results showed the oxidation of a large number of proteins involved in energy metabolism, including ATP production, glycolysis and Krebs cycle. In addition, protein oxidation also affected other component of the cell involved in cell structure and signal transduction, all fundamental processes for the correct



functionality of neuronal cells. Here we describe the specific function of every carbonylated protein identified and we posit about all the possible implication of the proteins in cellular dysfunction during aging.

### ***5.1.1. Energy metabolism proteins***

We found a significant increase in the oxidation of several *Glycolytic* enzymes, these are:

Fructose-bisphosphate aldolase (FBP) catalyzes the reversible aldol cleavage or condensation of fructose-1,6-bisphosphate into dihydroxyacetone-phosphate and glyceraldehydes-3-phosphate. In vertebrates, three forms of this enzyme are found and aldolase C expression is the prevalent form in brain, heart and ovary. The different isozymes have different catalytic functions: aldolases C is mainly involved in glycolysis [175].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the oxidation of glyceraldehyde-3-phosphate to 1,3-phosphoglycerate and NADH, the first oxidation/reduction reaction in the glycolytic pathway. Other than its role in glycolysis, recent studies now support the idea of diverse activities for GAPDH. Emerging roles include membrane fusion and transport, accumulation of glutamate into pre-synaptic vesicles, and acting as a cellular sensor of oxidative stress, raising questions on its cellular localization [176]. GAPDH undergoes significant nitration, another form of oxidative modification, in the hippocampus of AD patients and also in rats after intracerebral injection with A $\beta$  (1-42) [177, 178].

Recent studies have demonstrated that oxidative inactivation of GAPDH may be involved in neurotoxicity [178].

Phosphoglycerate kinase 1 (PGK1) catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate in glycolysis. This reaction converts

ADP to ATP to ensure maximum glutamate accumulation into presynaptic vesicles. Several studies show that oxidative modification of this enzyme possibly due to the decrease of activity during aging [173, 179].

Triosephosphate isomerase (TPI) catalyzes the reversible interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Previous studies on SAMP8 mice showed an increase of oxidation of TPI but no change in enzyme activity was observed [173].

Pyruvate kinase (PK) catalyzes the final step in glycolysis, with the concomitant transfer of the high-energy phosphate group from phosphoenolpyruvate to ADP, thereby generating ATP. Under aerobic conditions, pyruvate can be transported to the mitochondria, where it enters the TCA cycle and is further broken down to produce considerably more ATP through oxidative phosphorylation. This enzyme controls the flow of glucose into synthetic pathways or into metabolism, so it is crucial to the energy regulation of the cell [179]. A decrease in ATP production would accordingly lead to dysfunction in electrochemical gradients, ion pumps, and voltage-gated ion channels, altering cell potential [178]. Other studies report an impairment of PK, caused by oxidative damage, in mild cognitive impairment [180] or Down syndrome [181].

Taken together, these results suggest the idea about the involvement of glycolysis failure as one of the first event associated with aging progression. In fact impairment of glycolysis, as indicated by the reduced activity of the enzymes listed above, could lead to reduction in glucose metabolism and therefore decreased ATP production, all described in aging brain. In our model of aging also *the Krebs's cycle* turned out as a target of oxidation. Among the identified enzymes those which showed increased carbonyl levels were isocitrate dehydrogenase, malate dehydrogenase, aconitase.

Isocitrate dehydrogenase (ISD) that catalyzes the first oxidative conversion in the TCA cycle. Isocitrate and NAD<sup>+</sup> are converted to  $\alpha$ -ketoglutarate, NADH and CO<sub>2</sub>. Isocitrate dehydrogenase is a highly regulated enzyme. The enzyme is stimulated by NAD<sup>+</sup> and ADP and inhibited by NADH, making it very sensitive to the NADH-to-NAD<sup>+</sup> ratio [182].

Malate dehydrogenase (MDH) is also essential to energy production and regulation. This enzyme is involved in the malate–aspartate shuttle catalyzing the conversion from malate to oxaloacetate in glycolysis, also producing ATP from ADP. MDH is located within the mitochondrial matrix in order to connect glycolysis to mitochondrial respiration. This protein transfers NADH across the mitochondrial membrane to respiratory complex I [182].

Aconitase (ACO) an iron-sulfur protein of the mitochondrial matrix catalyzes the stereospecific isomerization of citrate to isocitrate via *cis*-aconitate, a reaction essential to normal metabolic function. The particular susceptibility of mitochondrial aconitase to oxidative damage may be related to the iron-sulfur cluster [4Fe-4S] in its active site [183]. Age-related oxidative inhibition of this enzyme has already been observed and may block normal electron flow to oxygen, leading to an accumulation of reduced metabolites such as NADH [183, 184].

Collectively, the oxidation of TCA enzymes and the consequent impairment of their activity may affect ATP levels. We also found the oxidation of others proteins directly involved in ATP production such as ATP synthases or taking part in other processes of the total energy metabolism pathway.

ATP synthase is localized in the inner membrane of mitochondria and is a part of the complex V that plays a key role in energy production. It is responsible for proton transport and oxidative phosphorylation as part of the mitochondrial electron transport chain. Impairment in ATP synthase activity has been reported in

cerebral cortex of aging rats [185]. We found increased carbonyl levels of ATP synthase in the cortex of senescent rats that could lead to the inactivation of the mitochondrial complex. Dysfunction of ATP synthase could contribute to a decrease in the activity of the entire electron transport chain and impaired ATP production, resulting in possible electron leakage from their carrier molecules to generate ROS, with consequent dysfunction in electrochemical gradients, ion pumps, and voltage-gated ion channels, as well as lower efficiency on such processes as Ca<sup>2+</sup> homeostasis, cell potential, and signal transduction [179]. The proteomic identification of ATP synthase impairment in senescent rats brain provides further evidence of the role of mitochondria in the aging process.

Voltage-dependent anion channel (VDAC) is a component of the mitochondrial permeability transition pore, a structure that plays an essential role in movement of metabolites like ATP in and out of mitochondria by passive diffusion and in calcium homeostasis. ATP production and mitochondrial calcium buffering are essential for normal synaptic transmission [186]. In addition, VDAC also plays an important role in apoptotic process involving release of several apoptogenic factors such as cytochrome C, apoptosis inducing factor, smac and caspases from mitochondria [178]. Interestingly, VDAC was recently identified as a selective oxidized target in AD brain [178]. In one way, this oxidation could possibly lead to a conformational change of the porin, inhibiting the exchange of ATP-ADP, thus compromising cellular energetic. We found an increase of oxidation of VDAC in cerebellum of senescent rats in comparison with young rats.

Adenylate kinases (AKs) are ubiquitous enzymes which are involved in maintaining the homeostasis of adenine and guanine nucleotide composition in various organisms [187]. In vertebrates, three isozymes, AK1 and AK2 and AK3, were originally classified. Recently, two additional AK isozymes, AK4 and AK5, were identified [188]. Each AK has been demonstrated to catalyze a similar

reaction. In the central nervous system, ATP and GTP are thought to be not only the energy source but also the principal neurotransmitter or neuromodulator at purinergic synapses, and the evidence for the presence of a synaptic plasma membrane associated AK has been reported. Furthermore, it has been found that there is substantial AK activity in developing mouse brain during the period of rapid neural growth and it has been concluded that AK is involved in neural functioning. We found an increased oxidation of AK4 in cerebellum and striatum of senescent rats compared to aged. This increased oxidation might lead to the impairment of its activity and subsequently to the loss of adenine and guanine nucleotide homeostasis in the cell. Furthermore the decreased activity of AK4, may result in a decline of synaptic transmission.

Mitochondrial Aldehyde dehydrogenase (ALDH2), is a polymorphic enzyme known to metabolize acetaldehyde produced from ethanol into acetate. Several studies suggested a main role of the brain ALDH2 in acetaldehyde conversion and its dysfunction has been associated with age-associated neurodegenerative diseases [189]. ALDH belongs to a large family consisting of at least 16 different genes in humans, and are involved in the metabolic systems of various alcohols and aldehydes according to their distribution and substrate specificity [190]. This enzyme is a known target for oxidation under conditions of oxidative stress and is thought to be protective against oxidative stress [191]. In humans, ALDH2 deficiency was found to contribute to the risk of complication with diabetes mellitus, hypertension, myocardial infarction, osteoporosis, and cancer presumably because of increased oxidative stress [146, 192, 193].

We here report an increased oxidation of ALDH2 in the cortex of senescent rats compared to young rats, and its deficiency could be associated with an increased risk of neurodegeneration and elevated oxidative stress as described by other groups [194]. Because highly toxic aldehydes are generated spontaneously by

lipid peroxidation, there are a variety of mechanisms for their detoxification including binding with glutathione and reduction by aldose reductase, ADH and multiple. It is quite interesting that mitochondrial ALDH2 plays an important role in the detoxification of HNE in the CNS [195]. The accumulation of aldehydes, including HNE, not only induces neuronal death but also causes synaptic dysfunction because of mechanisms such as reducing Na-K-ATPase activity and markedly inhibits microtubule formation and neurite outgrowth [191]. Furthermore, there have been a number of reports on the relationship between neurofibrillary tangle (NFT) and HNE-induced oxidative stress [196]. These findings suggest that earlier accumulation of HNE in the ALDH2-deficient brain is responsible for a trigger of cognitive decline [197]. Although, the molecular mechanisms that trigger dementia symptoms have been highly elusive, marked increases in oxidative stress, typically represented by HNE, as reported, either in the hippocampus and superior and middle temporal gyrus of patients with mild cognitive impairment and in those with early AD compared with age-matched healthy individuals, or in CSF, are strongly suggestive that oxidative stress, typically represented by HNE, is involved in the causes of the onset of dementia [197, 198].

The creatine kinase (CK) system is the most important immediate energy buffering and transport system especially in muscle and neuronal tissue [175]. Creatine is phosphorylated to phosphocreatine in the intermembrane space of mitochondria where mitochondrial CK is located and is then transported into the cytosol. There the energy pool can be regenerated by transphosphorylation of phosphocreatine to ATP, which is catalyzed by cytosolic CK located in close vicinity of cellular ATPases [175]. CKs are prime targets of oxidative damage, and it is also well established that oxidative modification of CK-BB decreases its activity in aging, such as in the brain of old brown Norway rats, in AD and other

neurodegenerative diseases [183, 199, 200]. Consistent with these studies we show increased oxidative modifications of CK B monomer that could lead to decreased activity during aging of rats cortex brain region.

Aspartate aminotransferase (AAT) is a cytoplasmic enzyme that catalyzes the conversion of aspartate to its corresponding oxo acid  $\alpha$ -ketoglutarate, which is produced downstream to the aconitase reaction. Thus, the inactivation of aconitase blocks the subsequent production of  $\alpha$ -ketoglutarate, which may be partially restored through the transamination of aspartate operated by aspartate aminotransferase [183].

We show an increase in the oxidation of AAT in cortex and cerebellum of senescent rats that coupled with the impairment of Aconitase, as above reported, could be connected to the impairment of the energy metabolism pathway related to glycolysis failure.

### ***5.1.2. Chaperon proteins***

The 70 Da family of stress proteins is one of the most extensively studied. Included in this family are Hsc70 (heat shock cognate, the constitutive form), HSP70 (the inducible form, also referred to as Hsp72), and GRP78 (a constitutively expressed glucose-regulated protein found in the endoplasmic reticulum) [201].

Hsp70 is a chaperone molecule that may contribute to cellular protection against a variety of stresses by preventing protein aggregation, assisting in the refolding of damaged proteins, and chaperoning nascent polypeptides along ribosomes. Hsp70 shows very low expression levels in brain under physiological conditions, but it is induced after certain oxidative stresses. Hsp70 is present in cytosol, nucleus and endoplasmic reticulum [5]. In the nervous system Hsps, may respond to the protein denaturation with aging and may have a role to suppress protein

denaturation [19]. Inducible Hsp70 (Hsp72) has been found to have the highest levels in the substantia nigra, followed by septum, cerebellum, striatum, hippocampus and cortex [162, 172].

Hsc 72 is a constitutive isoform, recruited by the cell as a primary defense against unfavorable conditions. It is involved in the degradation of proteins with abnormal conformation by binding to a particular peptide region and labeling it for proteolysis. HSC-72 might be also involved in the structural maintenance of the proteasome and conformational recognition of mis-folded proteins by proteases, raising the possibility that HSC-72 has a functional role in the cell [202]. Previous studies performed on aged rats found that the basal level of Hsc70, was significantly higher in the substantia nigra, followed by septum and hippocampus, striatum and cerebellum, with lowest levels in the cortex [162].

Glucose-regulated protein 78 (GRP78) act as molecular chaperones by binding transiently to proteins crossing through the ER and helping their folding, assembly, and transport GRP78 is the main constituent involved in the unfolded protein response (UPR). GRP78 recognizes unfolded polypeptides, inhibits intra- and intermolecular aggregation, and promotes oligomerization and proper folding [203]. Decreased functionality of GRP 78 can lead to a decreased activity of the proteasome with consequent enhance of misfolded protein inside the cell [204].

Our finding about the oxidative modification of these chaperone molecules in striatum, cerebellum and cortex could lead to an increased accumulation of misfolded proteins, one of the main common traits of aging and neurodegeneration.



## ***5.2 MULTIPLE SCLEROSIS***

In this study, significant increases in oxidative stress markers, such as carbonyls and HNE were found in the CSF, peripheral lymphocytes and plasma of MS patients, as compared to control subjects. These changes were associated with induction of stress responsive proteins, such as HO-1, Hsp72, Hsp60 and thioredoxin (TRX), measured in CSF, lymphocytes and in plasma. Heme oxygenase is the rate-limiting enzyme in the production of bilirubin. In the past decade the heme oxygenase (HO) system has been focused for its potential significance in maintaining cellular homeostasis. It is located in the endoplasmic reticulum in a complex with NADPH cytochrome c P450 reductase. The reaction catalyzed by HO is the oxidative cleavage of the heme molecule to form biliverdin and carbon monoxide (CO). Iron is reduced to its ferrous state through the action of NADPH cytochrome c P450 reductase. Further degradation of biliverdin to bilirubin occurs through the action of a cytosolic enzyme, biliverdin reductase [205]. HO is present in various tissues with the highest activity in the brain, liver, spleen, and testes. There are three isoforms of heme oxygenase, HO-1 or inducible isoform [19, 47, 206], HO-2 or constitutive isoform [207], and the recently discovered HO-3, cloned only in rat to date [201]. Elevation of HO-1 expression and activity in MS is probably due to elevated oxidative stress. This finding is consistent with evidence suggesting that the HO-1 gene is redox regulated and, similar to other antioxidant enzymes [201], this occurs because it contains in its promoter region the antioxidant responsive element (ARE). Therefore, the HO-1 gene undergoes a redox sensitive modulation by transcription factors recognizing specific binding sites within the promoter and distal enhancer regions of the HO-1 gene [50, 208]. In addition, heme oxygenase-1 is rapidly upregulated by oxidative and nitrosative stresses, as well as by glutathione depletion [67]. It has been also

suggested that inappropriate stress response within the CNS could influence both the permeability of the blood–brain barrier and the expression of Hsps, thereby initiating the MS lesion [10, 44, 209]. In addition, cytokines, immunoglobulins, and complement complexes may elicit a survival response involving the induction of endogenous Hsps and other protective molecules, which indicates that redox dependent mechanisms in these cells are of great importance in MS [210-212]. The heat shock response contributes to establish a cytoprotective state in a variety of metabolic disturbances and injuries, including stroke, epilepsy, cell and tissue trauma, neurodegenerative disease, and aging [149, 208]. In mammalian cells, the induction of the heat shock response requires the activation and translocation to the nucleus of one or more heat shock transcription factors (HSFs), which control the expression of a specific set of genes encoding cytoprotective Hsps. Production of Hsps is essential for the folding and repair of damaged proteins, serving thus to promote cell survival [44, 209]. Hsps serve as chaperones that bind to other proteins and regulate their conformation, regulate protein movement across membranes or through organelles, or regulate the availability of a receptor or activity of an enzyme. The 70-kDa family of stress proteins is one of the most extensively studied. This family comprises Hsc70 (heatshock cognate, the constitutive form), Hsp70 (the inducible form or Hsp72), and GRP75 (a constitutively expressed glucose-regulated protein found in the endoplasmic reticulum). After a CNS injury, Hsp70 is synthesized at high levels and is present in the cytosol, nucleus, and endoplasmic reticulum [44]. Hsp60 chaperone proteins within mitochondria and, also, protect brain cells against ischemia and seizures in vivo, after viral-induced overexpression [75]. Hsp60 is encoded in the nucleus and resides mainly in the mitochondria [10]. Hsp60 forms the chaperonin complex, which is implicated in protein folding and assembly within the mitochondria under normal conditions [10]. Hsp60 together with its

cochaperonin Hsp10 produce a large, efficient protein-editing machinery that facilitates proper folding and assembly of mitochondrial-imported proteins and corrects misfolded polypeptides generated under mitochondrial oxidative stress [213]. Mutations in the HSPD1 gene encoding Hsp60 have recently been found to underlie spastic paraplegia 13 (SPG13 [MIM 605280]), an autosomal-dominant spinal-cord neurodegenerative disorder of late onset, characterized by progressive weakness and spasticity of the lower limbs, and, more recently, a homozygous missense mutation, D29G, in HSPD1, encoding the mitochondrial Hsp60 chaperonin, causing a mitochondrial Hsp60 chaperonopathy linked to brain hypomyelination and leukodystrophy. This finding provides evidence for the pivotal role of the mitochondrial Hsp60 chaperonin in the process of normal brain myelination and in the pathogenesis of hypomyelinating neurodegenerative disease [214].

Notably, we found increased expression of thioredoxin in MS as compared to control subjects. Thioredoxin (TRX) represents an intracellular redox regulator that has been shown to be important for the regulation of redox-sensitive transcription factors [75]. In its reduced state TRX can oxidatively reactivate inactive transcription factors such as Jun, Fos, AP-1, redox factor-1 (ref-1), and Nrf-2 [48]. Thioredoxin reductase (TRXr) is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized thioredoxin protein. It is usually located in the cytosol, but it translocates into the nucleus in response to various stimuli associated with oxidative stress. TRXr plays a pivotal role in protecting against oxidative stress and in regulating cell growth and cell death. Constitutive TRXr expression has been observed in several cell types of the mammals, including neuronal cells after nitrosative stress. Both in vivo and in vitro studies demonstrated that TRX and TRXr have protective roles against cytotoxicity mediated by the generation of ROS [48]. In our study we demonstrate that

thioredoxin reductase is decreased in MS lymphocytes. This may be relevant to the pathogenesis of MS, as we have previously demonstrated in CSF and plasma of MS patients a significant decrease of GSH/GSSG ratio [109, 113]. Consistent with this notion, it is well established that cellular redox regulation of many processes is provided by the cooperation between the Trx and glutathione systems [215]. In fact, Trx and GSH systems are involved in a variety of redox-dependent reactions, such as supplying reducing equivalents for ribonucleotide reductase, and peptide methionine sulfoxide reductase, the latter being involved in antioxidant defense and regulation of the cellular redox state [48]. Therefore, Trx and GSH form a powerful system controlling redox regulation of gene expression, signal transduction, cell proliferation, protection against oxidative stress, anti-apoptotic functions, growth factor and co-cytokine effects, as well as regulation of the redox state of the extracellular environment [216, 217]. In addition, increased Trx-1 expression has been described in many human primary cancers and tumor cell lines [218]. Changes in MS redox status observed in this study were reflected also by the interesting finding that Sirtuin-1 is present in the plasma of MS patients at significantly higher levels compared to control subjects. Sirtuins are a family of histone deacetylase, that, in humans, includes at least seven members, with different cellular and subcellular localization and substrate binding activity [88, 208, 216]. The most studied is the SIRT1, a NAD<sup>+</sup> dependent enzyme, involved in deacetylation of different proteins and in the regulation of energy metabolism and redox state. Sirtuins are involved in the regulation of important biological processes, such as apoptosis, cell differentiation, energy transduction, glucose homeostasis [75, 219], as well as in mediating enhancement of synaptic plasticity and neurogenesis in response to exercise, dietary energy restriction, and other hormetic environmental factors [208, 220]. Because of their roles in cellular stress responses, sirtuins would be expected to play particularly important roles in

adaptive responses of neural cells to stress, such as that associated with the MS pathogenesis. In support of this conceivable possibility, SIRT1 has been shown to interact either directly or indirectly with several pathways known to be involved in adaptive neural plasticity, including the Ca<sup>2+</sup>-calmodulin-cyclic adenosine monophosphate (AMP) response element binding protein and NF- $\kappa$ B systems [208]. Thus, our finding may be relevant to the MS pathogenesis, in view of the fact that SIRT1 are emerging as a promising candidate target for therapeutic interventions in metabolic and neurodegenerative disorders [221].

### **5.3 ALZHEIMER'S DISEASE**

Alzheimer's disease is a progressive disorder characterized usually by early memory loss, but affecting all intellectual functions in the late stage and leading to complete dependence for basic functions of life. The pathological features of AD are a variable degree of cortical atrophy, in the frontal, parietal, and temporal lobes. The pathological lesions in AD include neurofibrillary tangles, neurite, plaques, the central core of which is amyloid- $\beta$  peptide, derived from the transmembrane amyloid precursor protein (APP), amyloid angiopathy [123, 124]. AD brain has been reported to be under oxidative stress that may play an important role in the pathogenesis and progression of AD [125, 126, 127]. Several lines of evidence support a fundamental role for free radical mediated event in the pathogenesis of the disease. Amyloid- $\beta$  peptide (1-42) has been shown to induce protein oxidation in both *in vitro* and *in vivo* studies [128, 129, 130, 131]. As a result, amyloid- $\beta$  peptide (1-42) has been proposed to play a central role in the pathogenesis of AD [126, 132]. We demonstrated that brain from patients with

mild cognitive impairment (MCI) showed increased protein oxidation and lipid peroxidation relative to aged-matched control brain [133, 134]. Because many researchers consider MCI to be the transition zone between normal cognition and the dementia of early AD, these findings suggest that oxidative stress is fundamental to the progression of AD and not simply a consequence of AD [201]. (Cells have evolved different adaptive responses to manage oxidative stress which includes the recognition of cellular redox potential, reactive oxygen species and protein oxidation and responding with changes in gene expression. To deal with oxidative stress cells activate the NRF-2 (nuclear factor-erythroid 2-related factor 2) transcription factor which leads to the upregulation of antioxidant gene expression and other protective proteins, NRF2 binds to the antioxidant response elements (ARE) within promoters of antioxidant enzymes and detoxifying enzymes, [223, 224]. Antioxidants counteract oxidative stress, employing a variety of mechanisms to decrease ROS levels in the intracellular environment [225]. Sirt-1 and Sirt-2 are stress induced proteins that have been implicated in defense mechanisms against agents that may induce oxidative injury, and its induction represents a common feature in a number of neurodegenerative diseases [226]. In addition, another protein, thioredoxin reductase (TrxR), is emerging as critical vitagene involved in brain stress tolerance. As such, it has been demonstrated that Trx plays an important role in protecting against oxidative stress and in regulating cell growth and cell death [227, 228]. In particular, Sirt-1, has received considerable attention, as it has been recently demonstrated that Sirt-1 induction could represent a protective system potentially active against brain oxidative injury [88, 220, 222]. Several studies suggest that the Sirt-1 gene is redox-regulated and its expression appears closely related to conditions of oxidative stress[222, 230]. Different experimental evidences indicate that increased rate of free radical generation and decreased efficiency of the reparative-degradative

mechanisms, such as proteolysis, are factors that primarily contribute to age-related elevation in the level of oxidative stress and brain damage [44]. Another protein, in addition, thioredoxin reductase (Trx), is emerging as critical vitagene involved in brain stress tolerance. As such, it has been demonstrated that Trx plays an important role in protecting against oxidative stress and in regulating cell growth and cell death [77, 228, 231].

In the present study, the role of the vitagenes Sirt-1, Sirt-2 and Trx, along with thiol homeostasis in the brain and peripheral blood of AD patients, was investigated to gain further insight into the heat shock signal pathways, and the oxidant/antioxidant balance as critical factors operating in the pathogenesis of AD. The role of oxidative stress in the pathogenesis of AD and the importance of therapeutic strategies focusing on antioxidants and/or upregulation of stress-responsive genes.

Particularly, manipulation of endogenous cellular defense mechanisms such as the heat shock response, through nutritional antioxidants or pharmacological compounds, represents an innovative approach to therapeutic intervention in diseases causing tissue damage, such as neurodegeneration. Our data support a role for oxidative stress in the pathogenesis of AD and indicate that the stress responsive genes may represent important targets for novel cytoprotective strategies.

These findings have led to new perspectives in medicine and pharmacology, as molecules inducing this defense mechanism appear to be possible candidates for novel, cytoprotective strategies.

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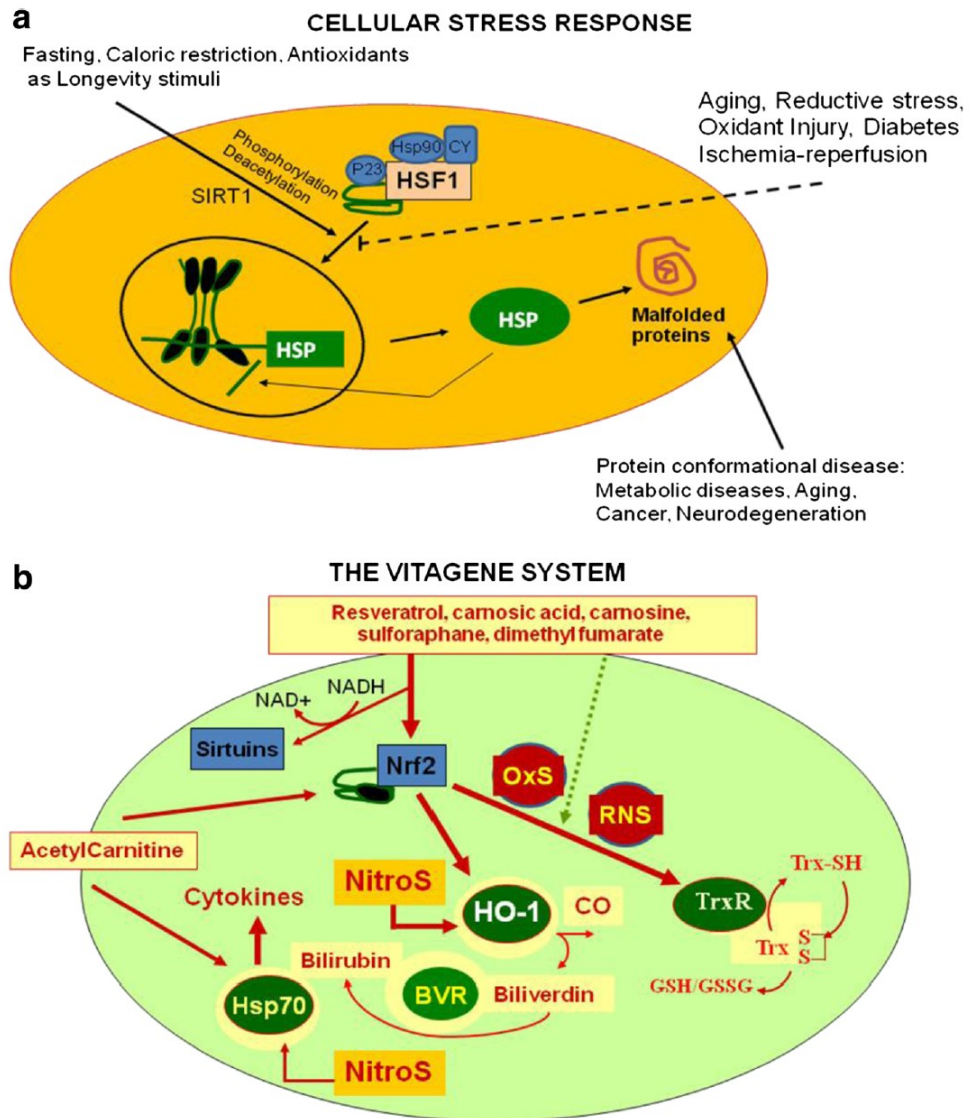
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**Figure 1 a, b:** Vitagenes and the pathway of cellular stress response. Cumulating misfolded proteins in response to proteotoxic environmental stress conditions triggers the cellular stress response (Figure 1a). HSPs that are normally bound to HSF1, maintaining it in a repressed state before stress, are titrate away by damaged or misfolded proteins with resulting HSF-1 activation. Multi-step activation of HSF1 involves post-translational modifications, such as hyperphosphorylation and deacetylation, which allow HSF1 to trimerize, translocate into the nucleus, and bind to heat-shock elements (HSEs) in the promoter regions of its target hsp genes. Nutritional antioxidants, including carnosic acid, resveratrol, sulforaphane, dimethyl fumarate, acetyl-L-carnitine or carnosine are able to activate vitagenes, such as heme oxygenase, Hsp70, thioredoxin reductase and sirtuins which represent an integrated system for cellular stress tolerance. Phytochemicals and Acetyl-L-carnitine act through the activation of the vitagene system, with up-regulation of HO-1, Thioredoxin, the GSH and Sirtuin system, results in counteraction of pro-oxidant conditions (Figure 1b). During aging, a gradual decline in potency of the heat shock response occur and this may prevent repair of protein damage, leading to degeneration and cell death.

**Table 1:** Clinical and demographic data of MS patients and control subjects.

	<b>Number of subjects</b>	<b>Age (mean±SD)</b>	<b>Gender (F/M)</b>	<b>Age at disease onset (mean±SD)</b>	<b>Disease duration (mean±SD)</b>	<b>EDSS (mean±SD)</b>
<b>Patients</b>	26	35.3±9.9	16/10	31.4±9.2	3.92±2.08	1.5±1.18
<b>Controls</b>	20	36.9±10.9	12/8			

EDSS: Expanded Disability Status Scale.

The table 1 summarizes clinical data of patients and control subjects. As shown in the table the mean age at disease onset is 31.4±9.2 years and the disease duration is 3.92±2.08 years, a relatively short duration and is typical of the RR form, which has not yet changed the SP form.

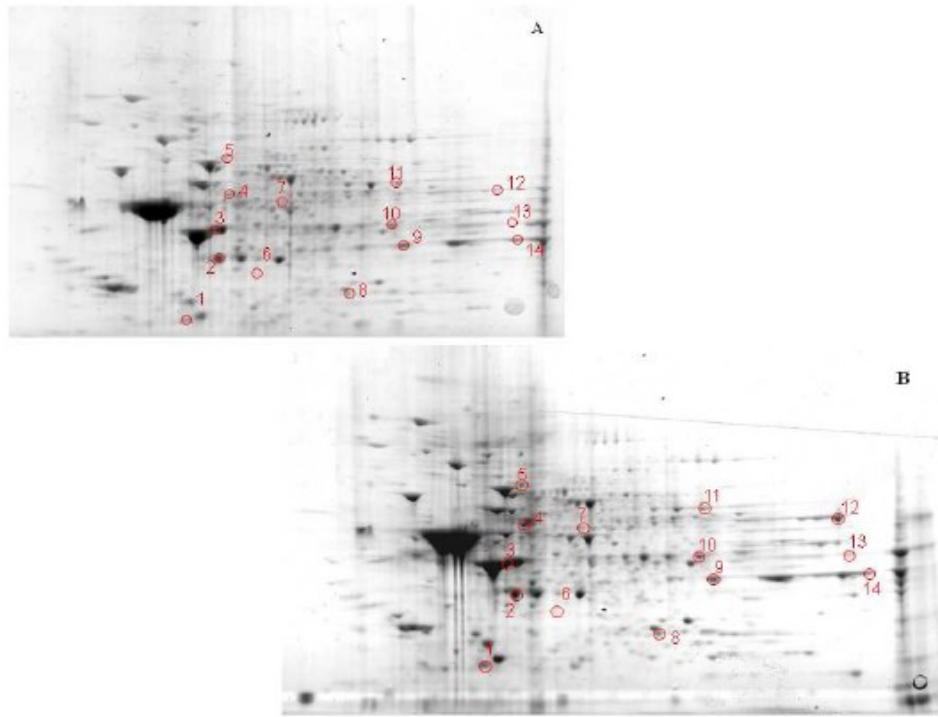
**Table 2:** Clinical and demographic data of AD patients and control subjects.

	<b>Number of Subjects</b>	<b>Age (Mean ± SD)</b>	<b>Gender (F/M)</b>	<b>Disease Duration (mean±SD)</b>	<b>MMSE (mean±SD)</b>	<b>ADL (mean±SD)</b>	<b>IADL (mean±SD)</b>
Patients	30	74.6±4.28	17/13	2.7±1.7	17.5±3.8	4.9±1.2	3.7±2.9
Controls	10	69.3±5.77	5/5		27.9±2	5.6±0.5	7.9±0.3

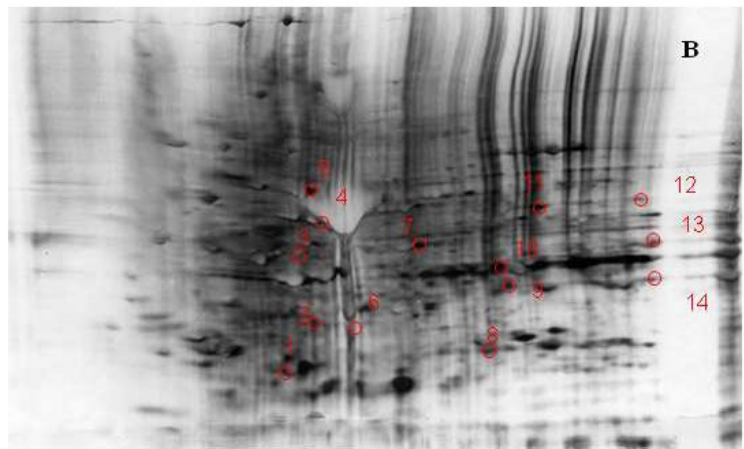
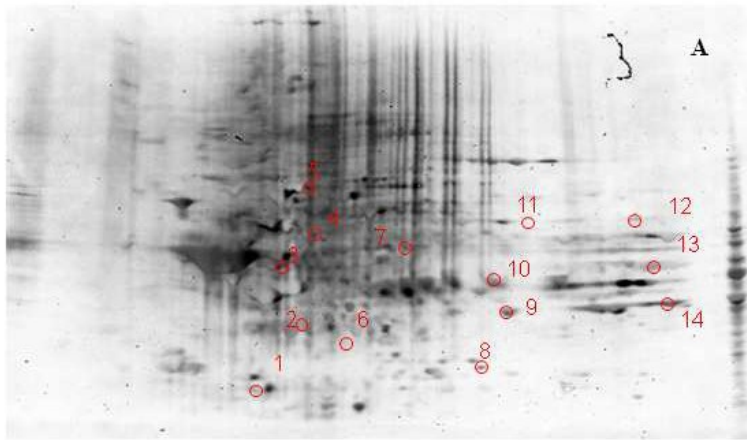
MMSE: Mini Mental State Examination (normal values: >24/30)

ADL: Activity Daily Living (normal values: 6/6)

IADL: Instrumental Activity of Daily Living (normal values: 8/8)



**Figure 2:** Representative 2-D gels of aged (A) and senescent rat cortex (B). Proteins (150  $\mu$ g) were separated on immobilized pH 3–10 IPG strips followed by separation on 8–16% gradient SDS-PAGE gels and stained with Biosafe Coomassie.

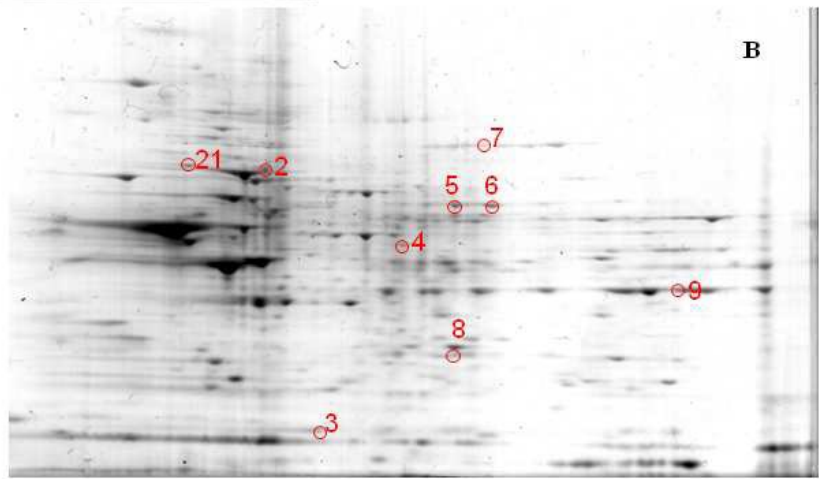
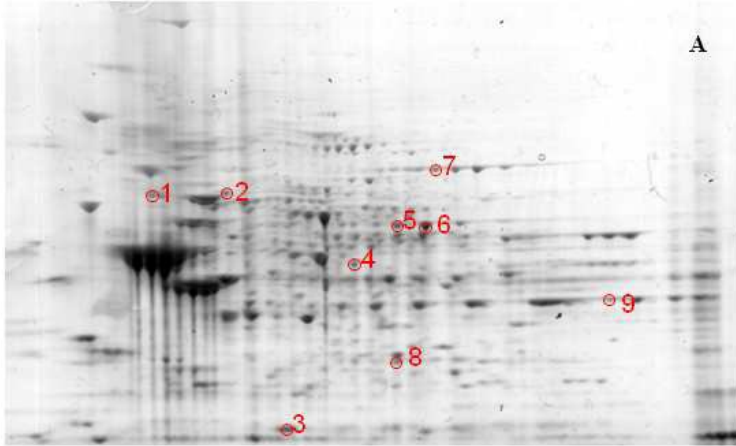


**Figure 3:** Two-dimensional carbonyl immunoblots from aged (A) and senescent rat cortex (B). Relative change in carbonyl immunoreactivity, after normalization of the immunostaining intensities to the protein content, was significant for six proteins.

Table 2

Spot n°	Protein name	Fold oxidation	P value	MOWSE Score*	Mr/pI
1	Peroxiredoxin-2	10.1	0.05	91	21941/ 5.34
2	Guanine nucleotide-binding protein subunit beta-1	2.2	0.01	161	38151/ 5.60
3	Creatine kinase B-type	10.0	0.05	72	42052/ 5.39
4	Vacuolar ATP synthase subunit B, brain isoform	5.9	0.005	214	56857/ 5.95
5	Stress-70 protein, mitochondrial precursor	3.5	0.04	208	74094/5.37
6	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	4.1	0.04	86	59291/ 5.58
7	Aldehyde dehydrogenase, mitochondrial precursor	29.0	0.01	192	56966/ 6.63
8	Alpha-centractin	2.66	0.01	117	42701/ 6.19
9	Triosephosphate isomerase	4.8	0.01	161	27345/ 6.89
10	Glyceraldehyde-3-phosphate dehydrogenase	7.04	0.02	82	36090/8.97
11	Aspartate aminotransferase, cytoplasmic	3.4	0.01	239	46628/ 6.73
12	Pyruvate kinase isozymes M1/M2	2.8	0.04	175	58294/ 6.63
13	ATP synthase subunit alpha, mitochondrial precursor	5.1	0.04	205	59831/ 9.22
14	Fructose-bisphosphate aldolase A	4.7	0.04	188	39783/ 8.31
15	Glyceraldehyde-3-phosphate dehydrogenase	34.8	0.01	146	36090/8.14

**Table 3:** Summary of the proteins identified by redox proteomics that are increasingly carbonylated in the cortex from senescent rats versus aged rats. For each protein, the carbonyl immunoreactivity/protein expression values were averaged ( $n = 8$ ) and expressed as fold oxidation compared to control. The  $p$ -value listed is the significance of increased carbonyl levels relative to control aged samples (see text). pI = isoelectric point; Mr = relative mobility.

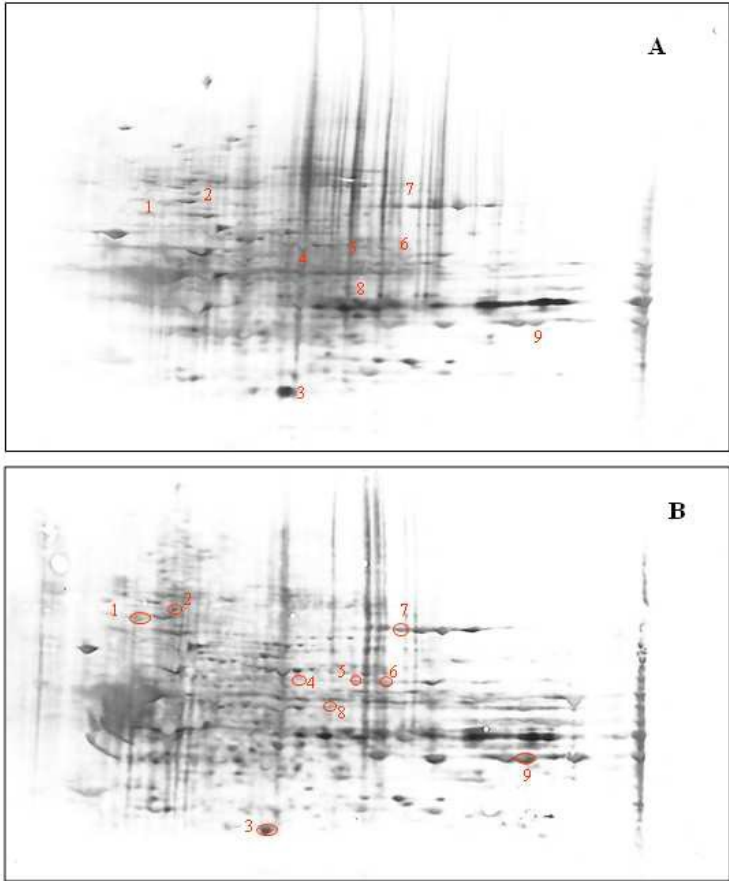


**Figure 4:** Representative 2-D gels of aged (A) and senescent (B) rat striatum.

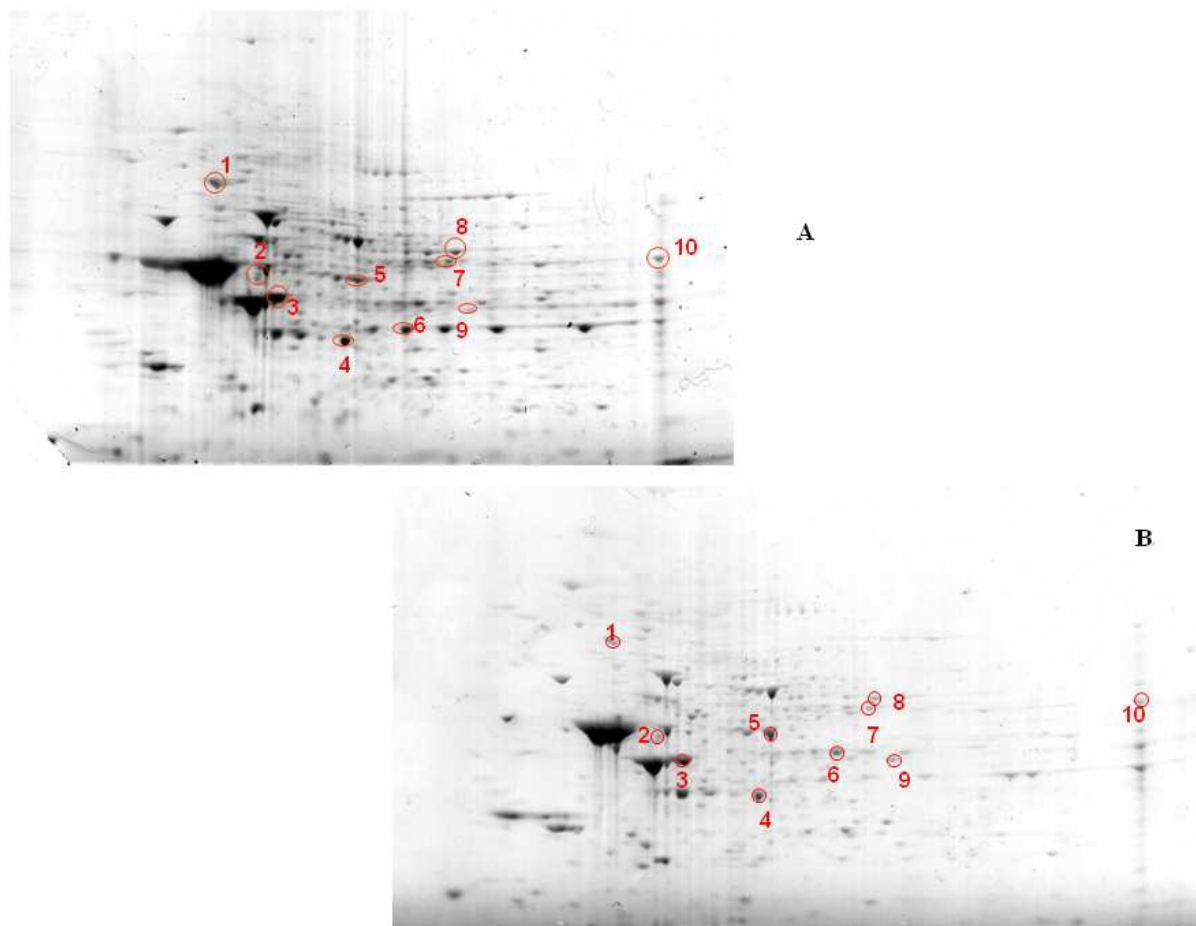


Spot n°	Protein name	P value	Fold oxidation	MOWSE Score*	Mr/pI
1	Heat shock protein HSP 90-beta	<0.05	5.3	169	83571/4.97
2	Cytochrome b-c1 complex subunit 1, mitochondrial precursor	<0.01	10.8	139	53500/ 5.57
3	Creatine kinase B-type	<0.05	5	181	42983/ 5.39
4	Malate dehydrogenase, cytoplasmic	<0.04	9.4	144	36631/ 6.16
5	Alpha-enolase	<0.01	3.9	109	47440/ 6.16
6	Dihydropyrimidinase-related protein 2	<0.05	6	125	62638/ 5.95
7	Glutamine synthetase	<0.05	6.4	91	42982/ 6.64
8	Glutamate dehydrogenase 1, mitochondrial precursor	<0.05	16.6	133	61640/ 8.05
9	Pyruvate kinase isozymes M1/M2	<0.05	5.9	227	58294/ 6.63
10	Fructose-bisphosphate aldolase C	<0.01	10.3	109	39658/ 6.67
11	ATP synthase subunit alpha, mitochondrial precursor	<0.04	9	232	59831/ 9.22

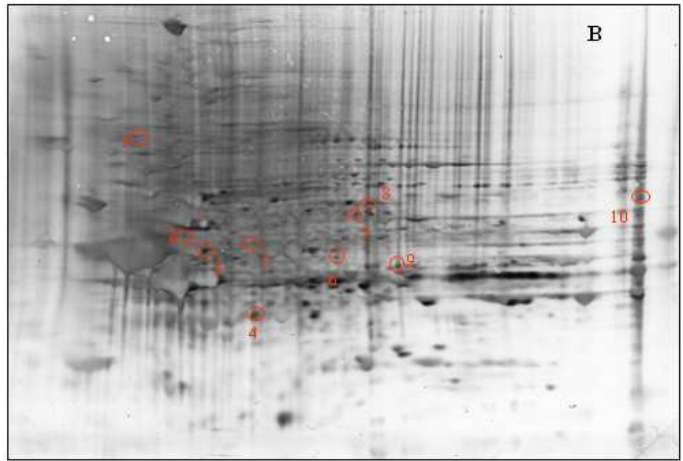
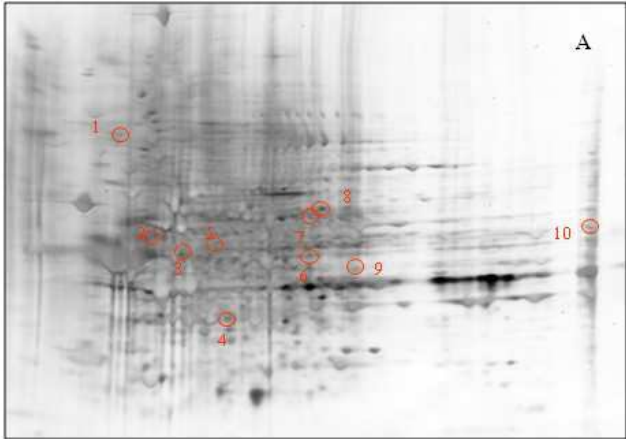
**Table 4.** Summary of the proteins identified by redox proteomics that are increasingly carbonylated in the striatum from senescent versus aged rats.



**Figure 5:** Two-dimensional carbonyl immunoblots from aged (A) and senescent rat striatum (B).



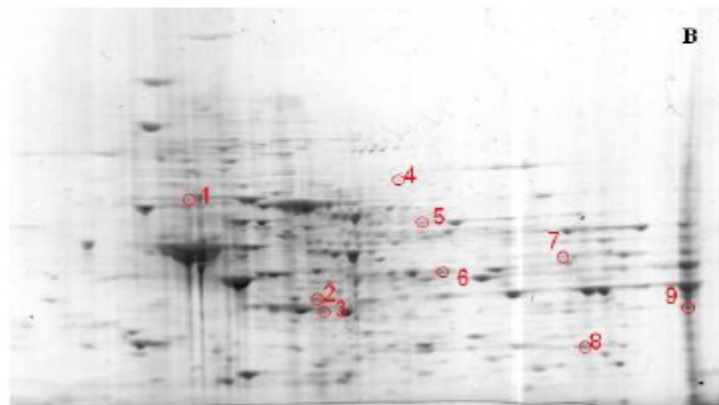
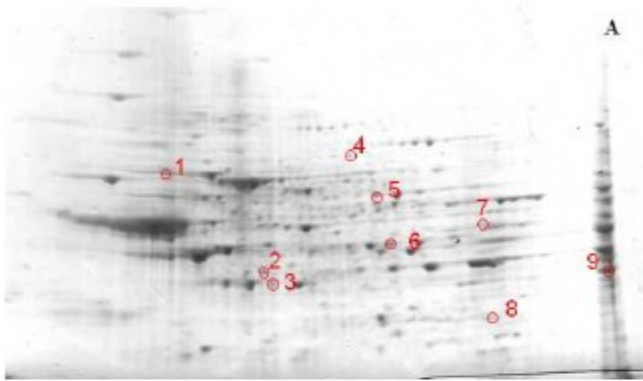
**Figure 6:** Representative 2-D gels of aged (A) and senescent rat hippocampus (B).



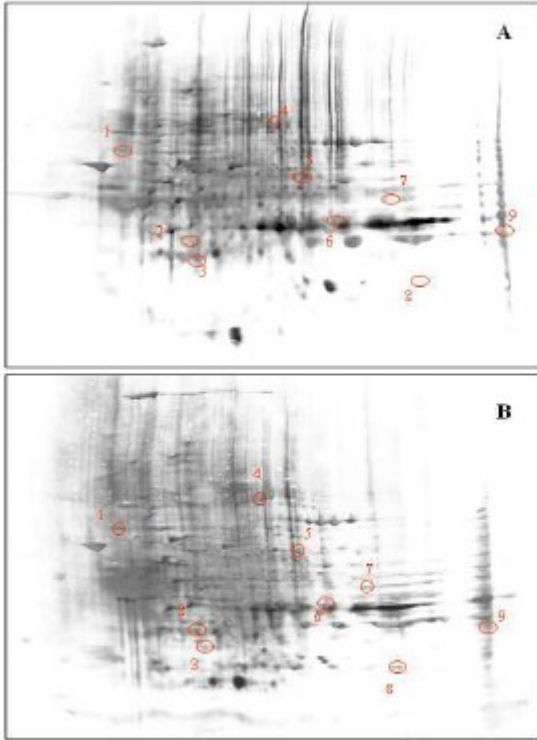
**Figure 7:** Two-dimensional carbonyl immunoblots from aged (A) and senescent rat hippocampus (B).

Spot n°	Protein name	P value	Fold oxidation	MOWSE Score*	Mr/pI
1	Tubulin alpha-1A chain	<0.01	2.9	260	50788/4.94
2	78 kDa glucose-regulated protein	<0.05	3.8	146	72473/5.07
3	Stress-70 protein, mitochondria	<0.01	3.1	179	74097/5.37
4	Adenylate kinase isoenzyme	<0.05	3.0	94	21684/6.32
5	Elongation factor Tu, mitochondrial	<0.01	4.5	184	49890/7.23
6	Pyruvate kinase isozymes M1/M2	<0.05	10.6	71	49890/7.23
7	Pyruvate kinase isozymes M1/M2	<0.05	4.3	194	58294/6.63
8	Aconitate hydratase, mitochondrial	<0.05	3.3	240	86121/7.87
9	Triosephosphate isomerase	<0.05	4.0	97	27346/6.67
10	Glyceraldehyde-3-phosphate dehydrogenase	<0.05	9.9	151	36090/8.14

**Table 5.** Summary of the proteins identified by redox proteomics that are increasingly carbonylated in the hippocampus from senescent versus aged rats.



**Figure 8:** Representative 2-D gels of aged (A) and senescent rat cerebellum (B).

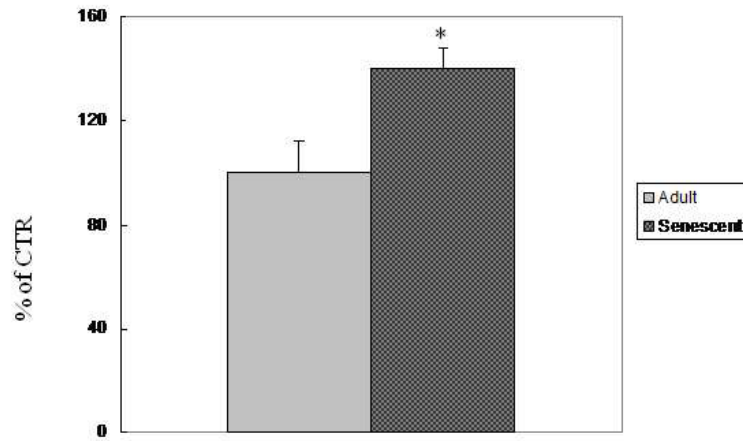


**Figure 9:** Two-dimensional carbonyl immunoblots from aged (A) and senescent rat cerebellum (B).

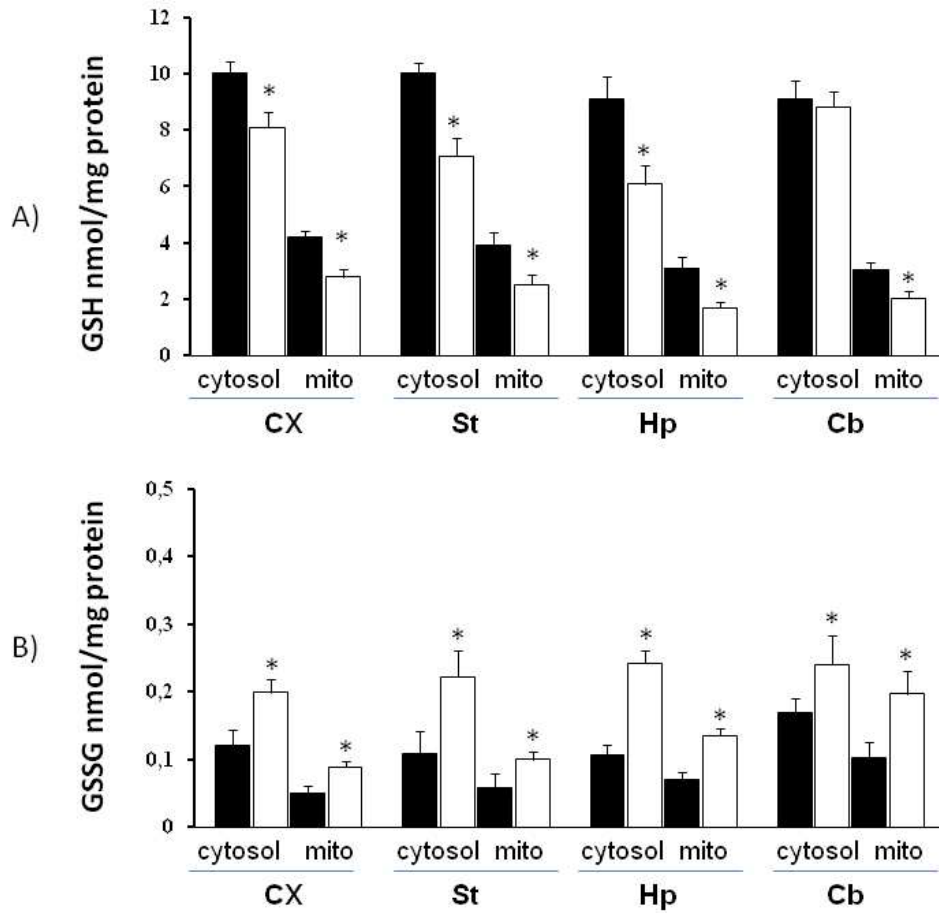
Spot n°	Protein name	P value	Fold oxidation	MOWSE Score*	Mr/pI
1	Heat shock cognate 71 kDa protein	<0.05	7.1	83	71244/5.37
2	Tubulin beta chain	<0.05	3.2	99	46994/4.78
3	Malate dehydrogenase, cytoplasmic	<0.05	2.8	75	36659/6.16
4	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondria	<0.05	4.1	86	40044/5.94
5	Vesicle-fusing ATPase	<0.01	3.4	132	83170/6.55
6	Pyruvate kinase isozymes M1/M2	<0.05	4.4	114	58294/6.63
7	Synapsin-2	<0.05	2.8	75	63702/8.73
8	Aspartate aminotransferase, cytoplasmic	<0.01	5.2	131	46628/6.73
9	Phosphoglycerate kinase 1	<0.05	3.8	95	44909/8.02
10	Voltage-dependent anion-selective channel protein	<0.01	2.7	168	30851/8.62
11	Adenylate kinase isoenzyme 4, mitochondrial	<0.05	4.2	137	25301/7.79

**Table 6:** Summary of the proteins identified by redox proteomics that are increasingly carbonylated in the cerebellum from senescent versus aged rats.

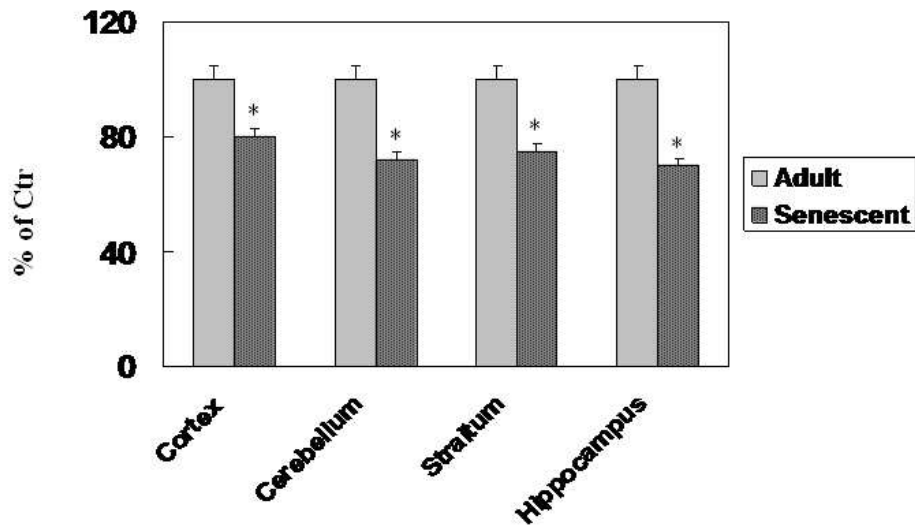




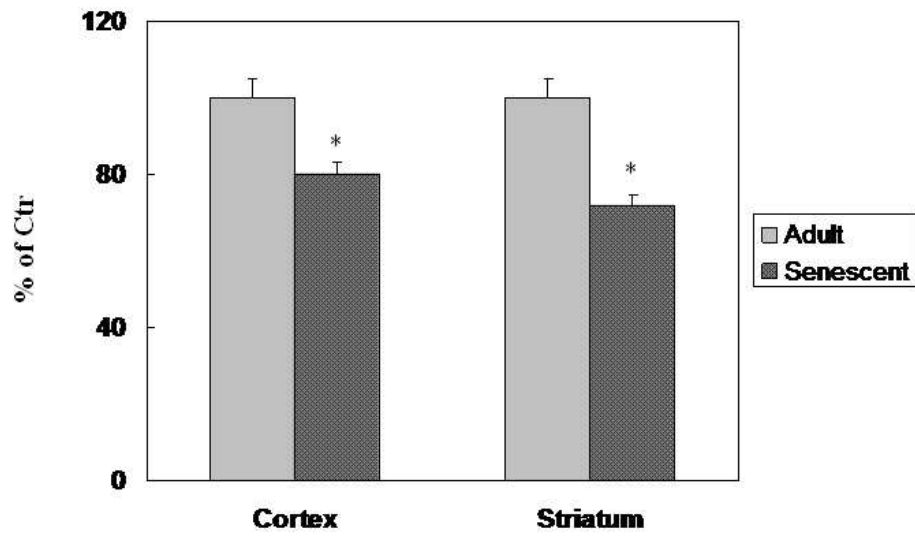
**Figure 10:** Western blot analysis confirmed proteomic results of increased carbonyl levels of Hsp70 in senescent rat hippocampus compared with aged rat using traditional immunochemical detection. Error bars indicate S.E.M for 3 samples in each group.  
\* $P < 0.05$



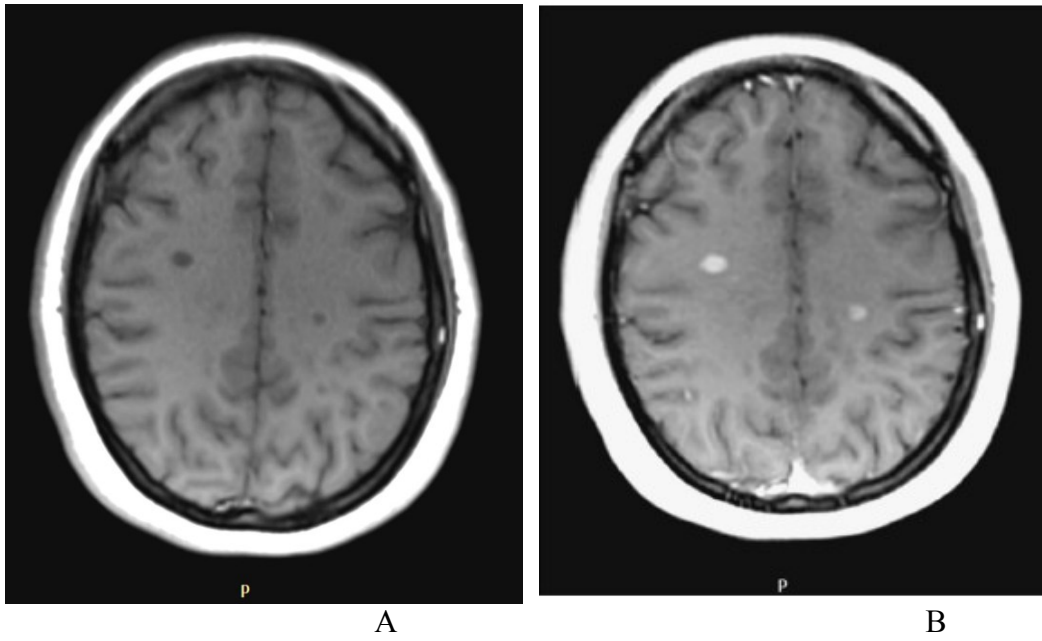
**Figure 11.** Regional distribution of reduced glutathione (GSH) and oxidized glutathione (GSSG) in different brain regions in adult and senescent rats. Cytosolic (cytosol) and mitochondrial (mito) GSH (A) and GSSG (B) in cortex (CX), striatum (St), hippocampus (Hp), and cerebellum (Cb) were measured as described in Materials and Methods. Results are expressed in nmol/mg protein. Data are means  $\pm$  SEM of eight animals. (\*)  $p < 0.05$  vs. control adult, 12-month-old, rats.



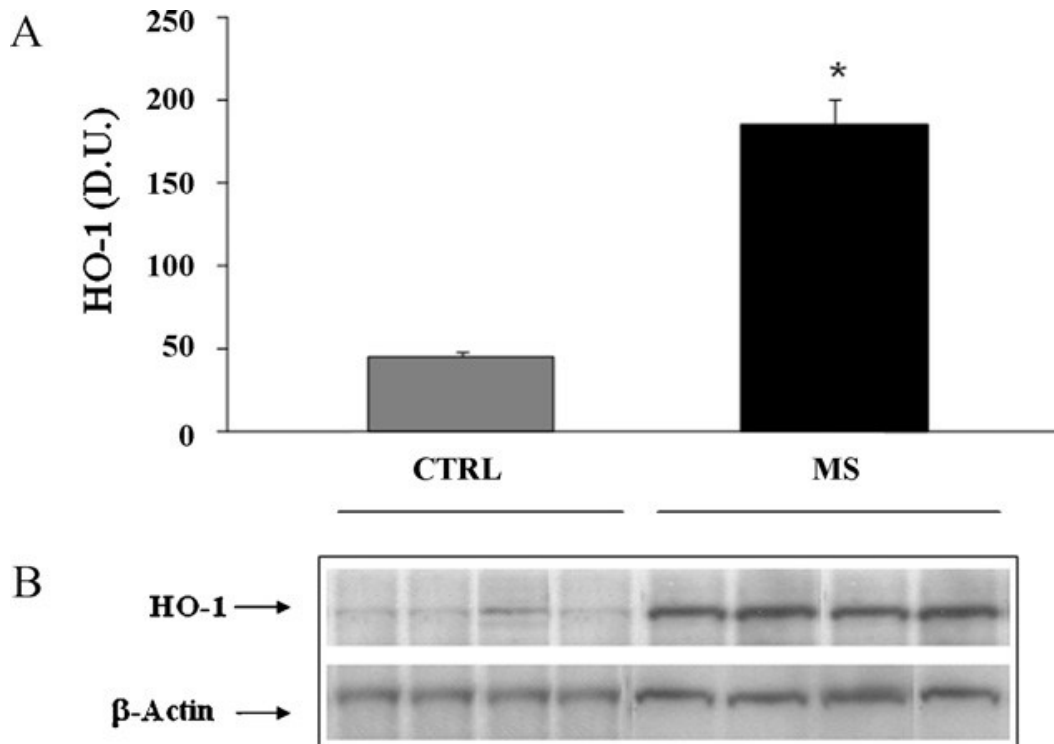
**Figure 12:** Activity of PK in all the brain regions of senescent rats compared with aged controls. The activity of PK is significantly decreased in all the brain regions. Bars represent mean  $\pm$  S.E. ,  $p < 0.05$ ;  $n = 8$  for each group.



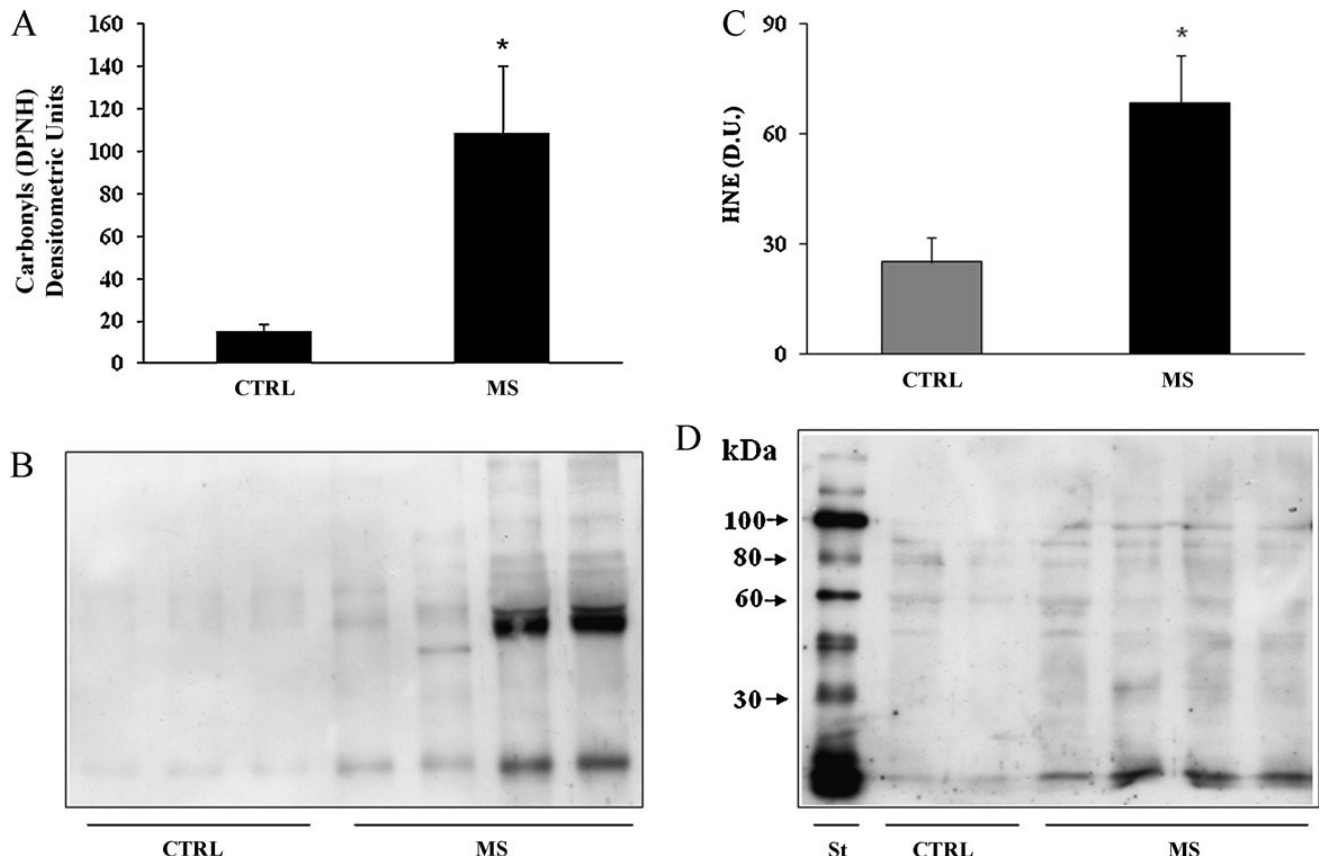
**Figure 13:** Activity of GAPDH in cortex and striatum of senescent rats compared with aged control animals.



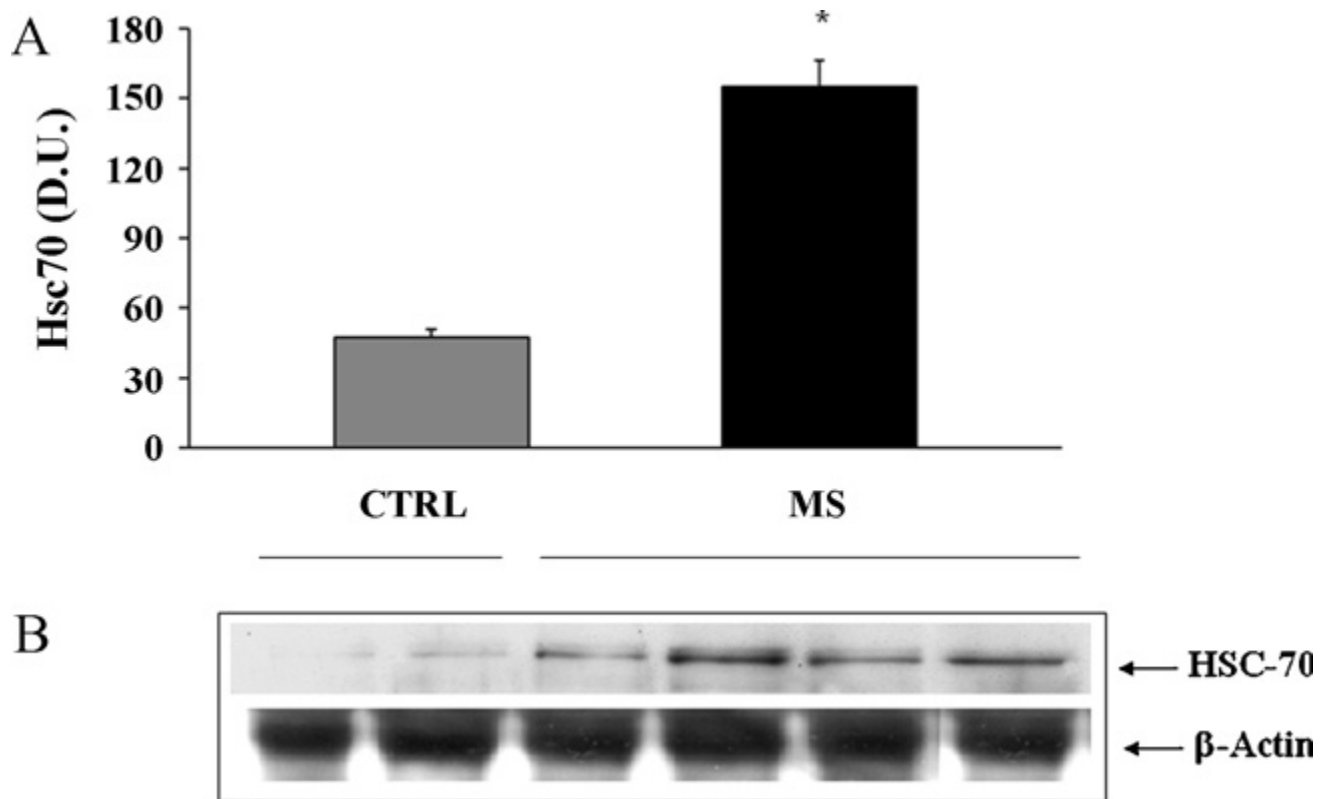
**Figure 14:** Brain MRI from MS patients: axial T1WI shows two hypointense areas on the white matter of the semioval center bilaterally (A) that present enhancement after gadolinium injection (B).



**Figure 15:** (A) Heme oxygenase-1 (HO-1) levels in the CSF of MS patients. CSF samples were assayed for HO-1 by Western blot. (B) A representative immunoblot is shown.  $\beta$ -Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control; St, standard.

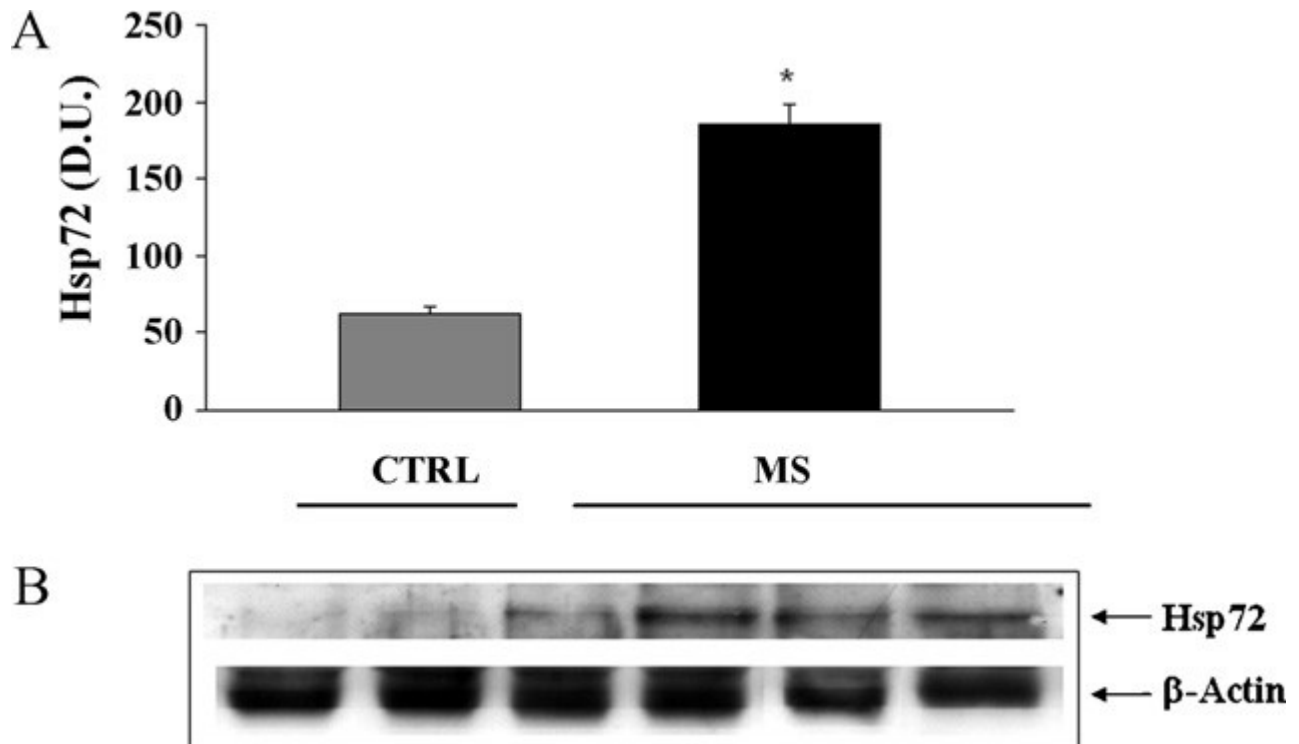


**Figure 16:** Protein carbonyls and HNE (4-hydroxy-2-nonenals) levels in the CSF of MS patients. CSF samples were assayed for protein carbonyls (A, B), and HNE (C, D) by Western blot. Representative immunoblots are shown (B, D). The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.

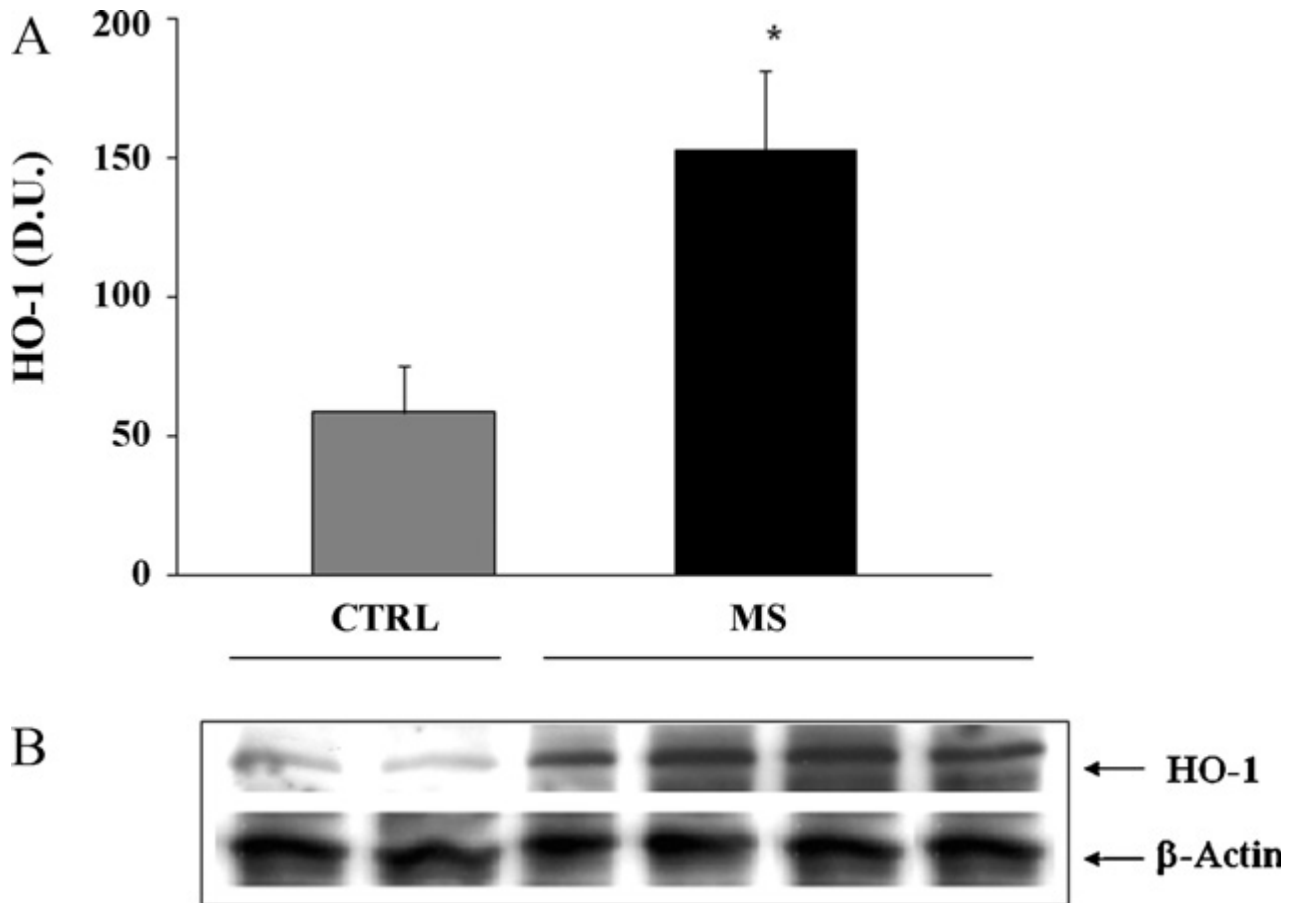


**Figure 17:** (A) Heat shock protein 70 (Hsc70) levels in MS lymphocytes. Lymphocytes were assayed for Hsc-70 by Western blot. (B) A representative immunoblot is shown. b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.

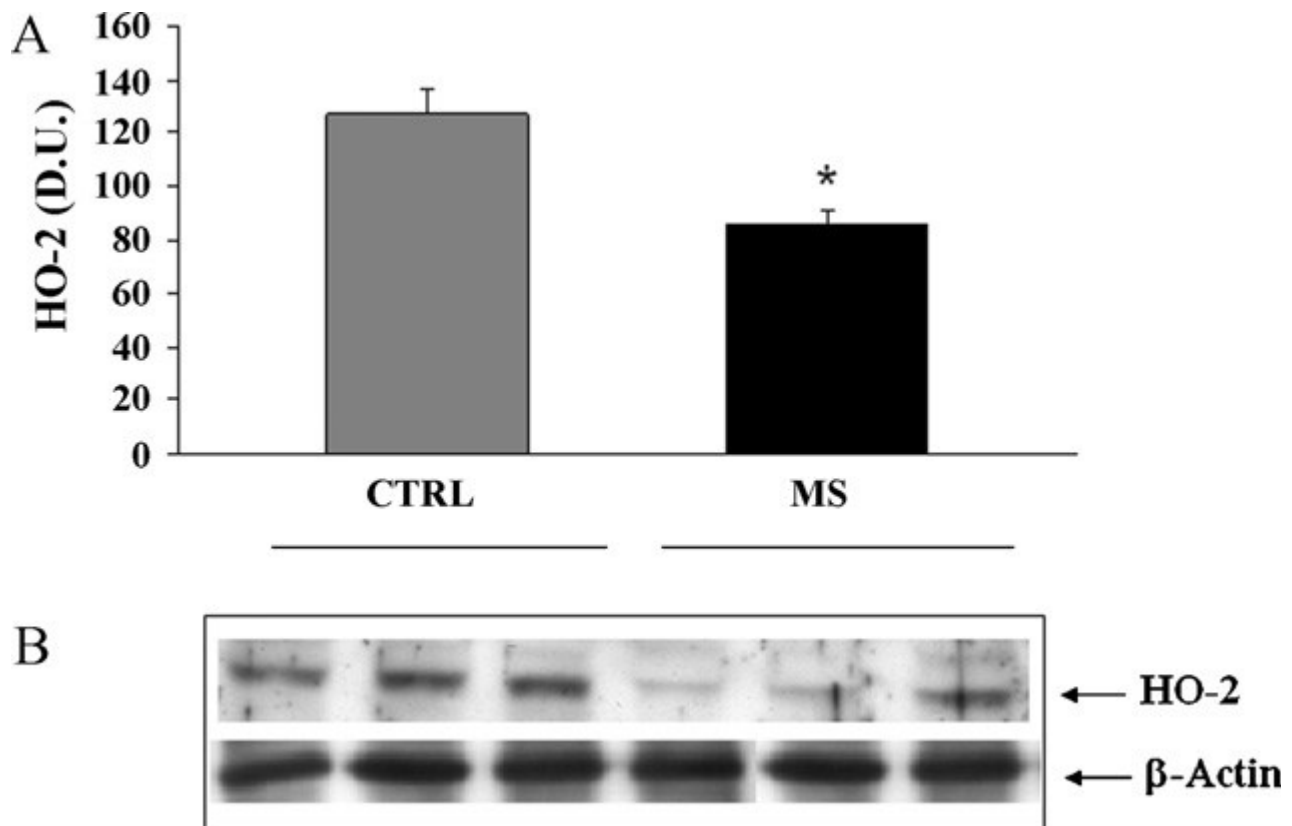




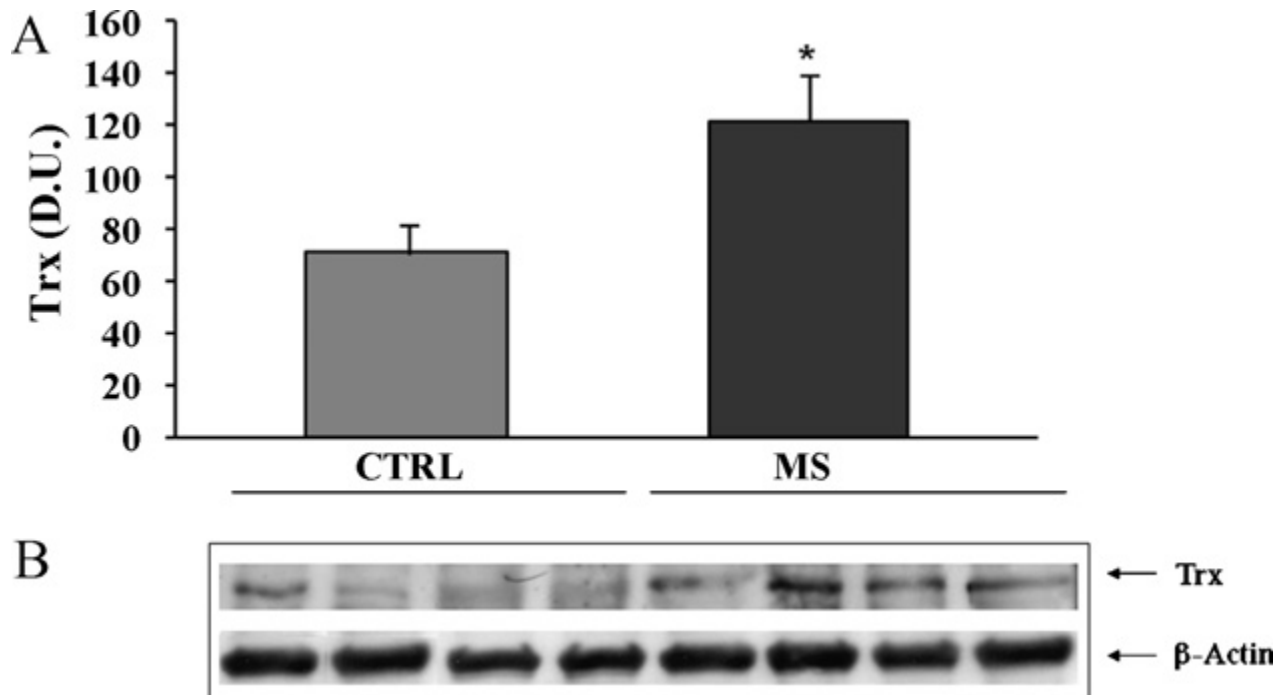
**Figure 18:** (A) Heat shock protein 72 (Hsp72) levels in multiple sclerosis patients. Lymphocytes samples were assayed for Hsc-72 by Western blot. (B) A representative immunoblot is shown. b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.



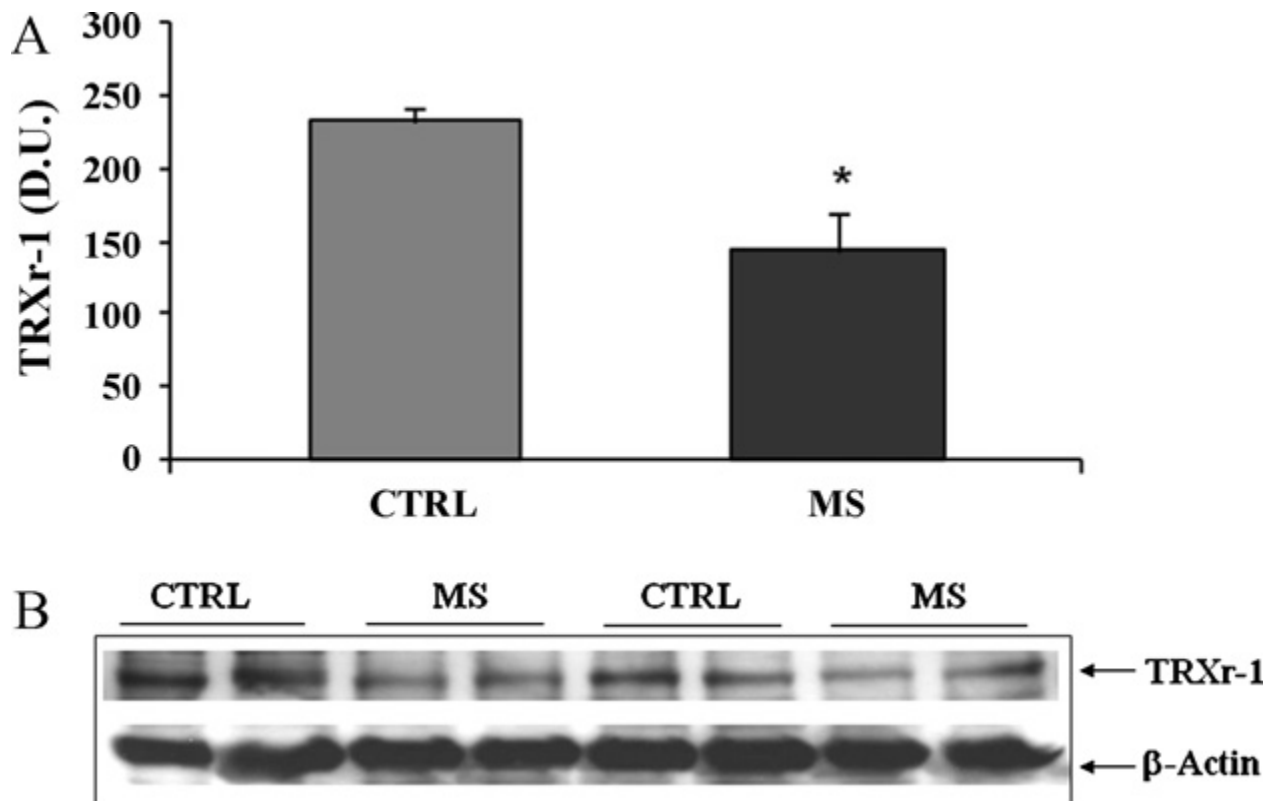
**Figure 19:** (A) Heme oxygenase-1 (HO-1) levels in lymphocytes of MS patients. Lymphocytes samples were assayed for HO-1 by Western blot. (B) A representative immunoblot is shown. b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.



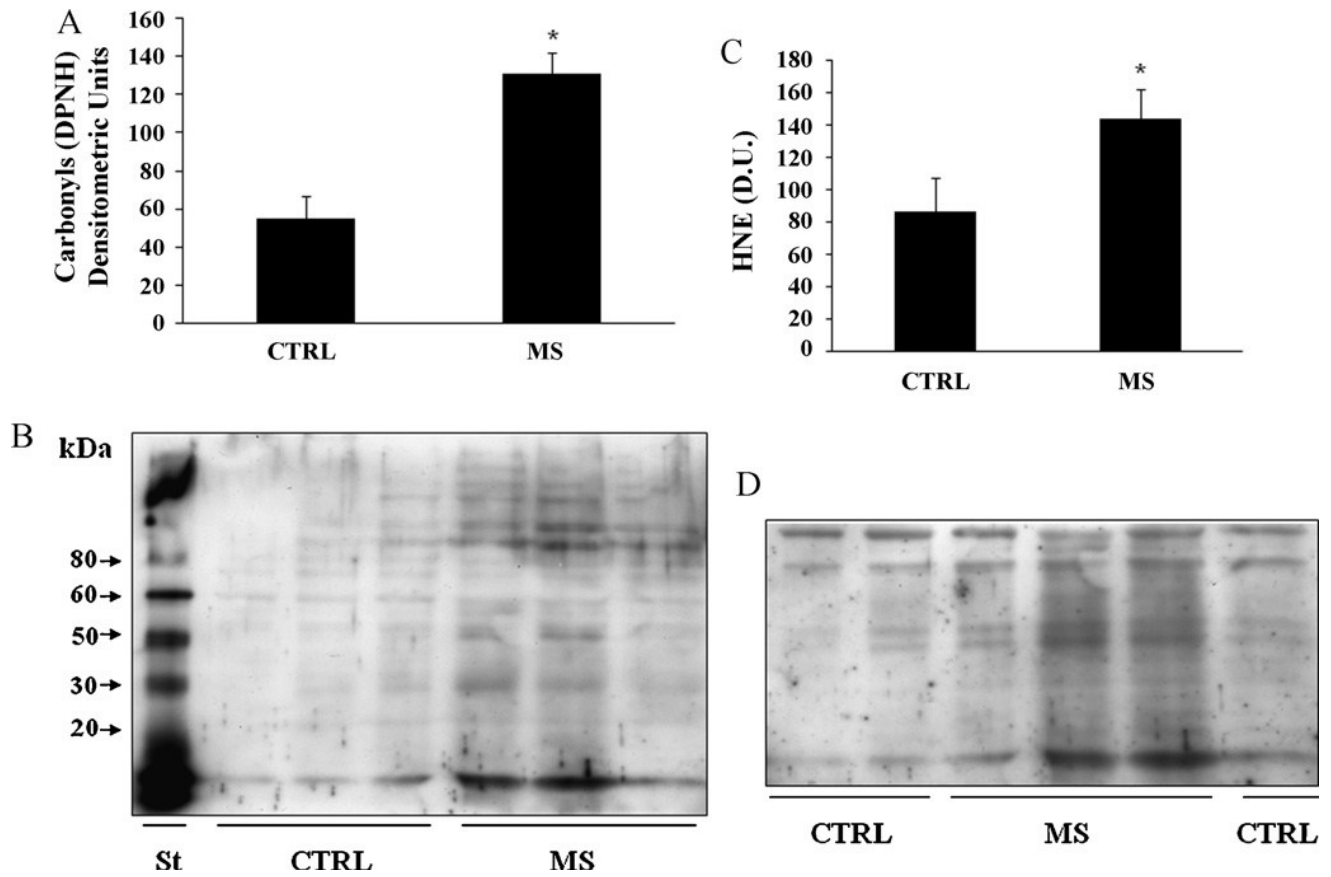
**Figure 20:** (A) Heme oxygenase-2 (HO-2) levels in lymphocytes of MS patients. Lymphocytes samples were assayed for HO-2 by Western blot. (B) A representative immunoblot is shown. b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.



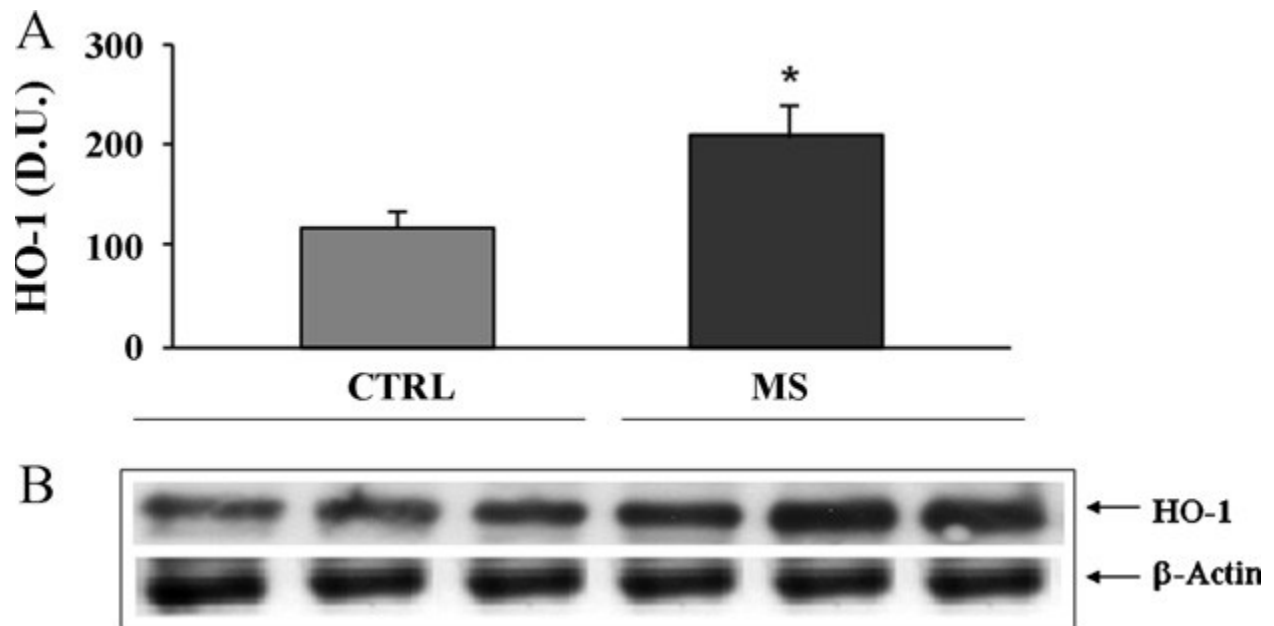
**Figure 21:** (A) Thioredoxin (Trx) levels in lymphocytes from multiple sclerosis patients. Lymphocytes samples were assayed for Trx by Western blot. (B) A representative immunoblot is shown. b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.



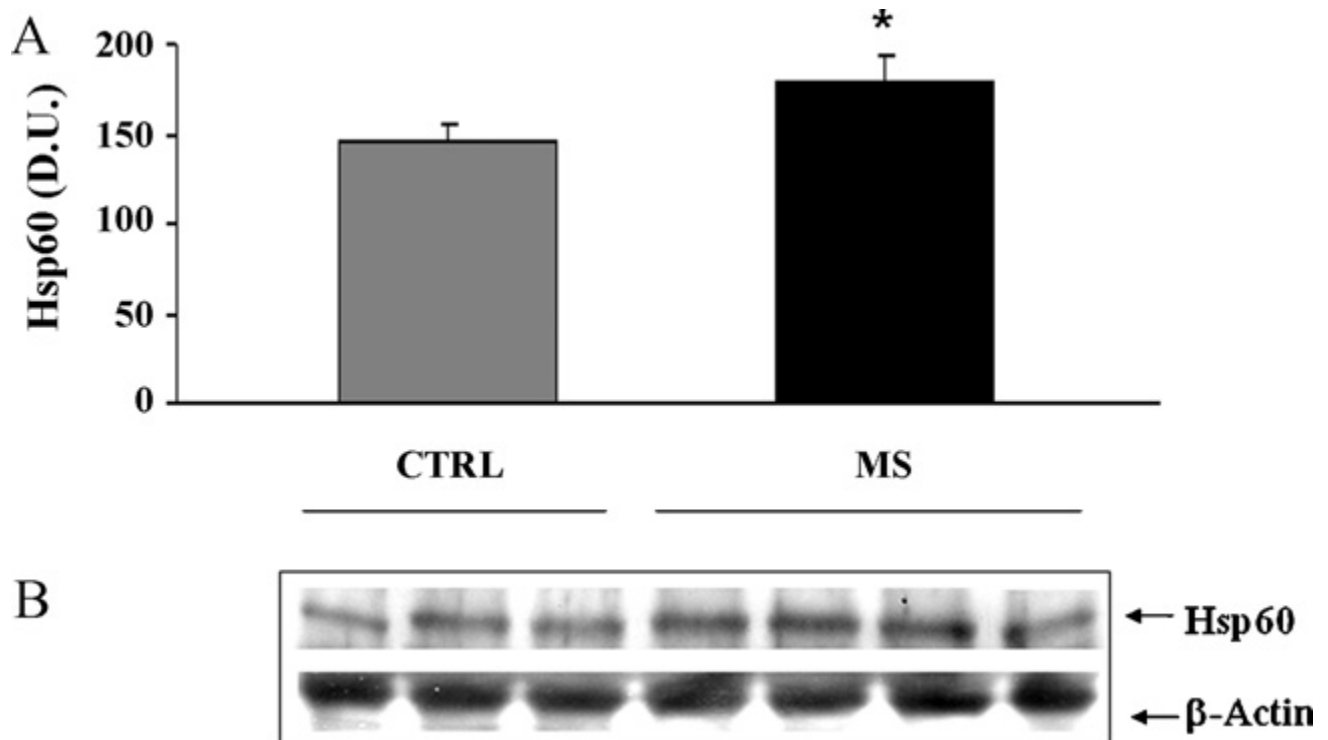
**Figure 22:** (A) Thioredoxin reductase-1 (TrxR-1) levels in lymphocytes from patients with multiple sclerosis. Lymphocytes samples were assayed for TrxR-1 by Western blot. (B) A representative immunoblot is shown. b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.



**Figures 23:** (A) Protein carbonyls (DPNH) and (C) HNE levels in lymphocytes from multiple sclerosis and control patients. Lymphocytes samples were assayed for carbonyls and HNE by Western blot as described in Section 2. Representative immunoblots are shown (B, D). The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units, MS, multiple sclerosis; CTRL, control; St, standard.

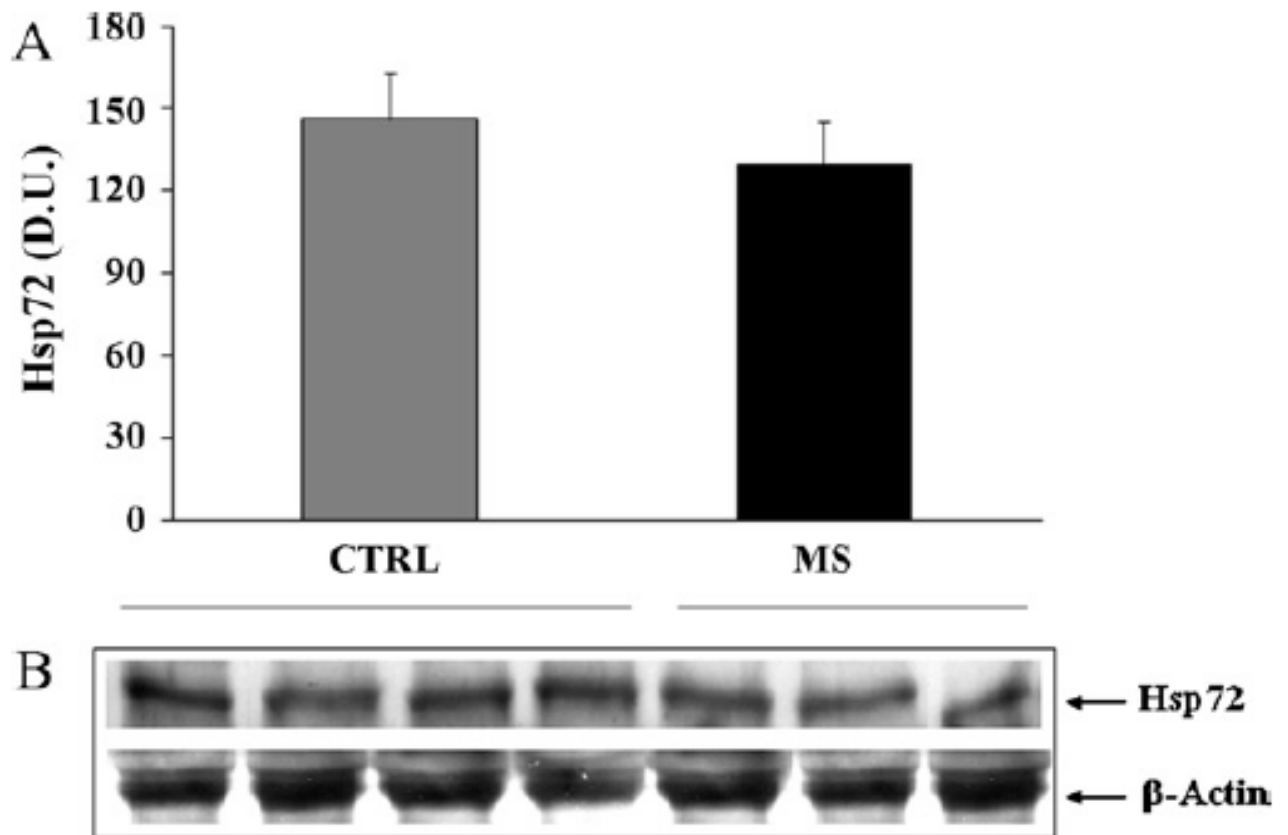


**Figure 24:** (A) Heme oxygenase-1 (HO-1) levels in plasma from multiple sclerosis patients. Plasma samples were assayed for HO-1 by Western blot. (B) A representative immunoblot is shown. b-Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.

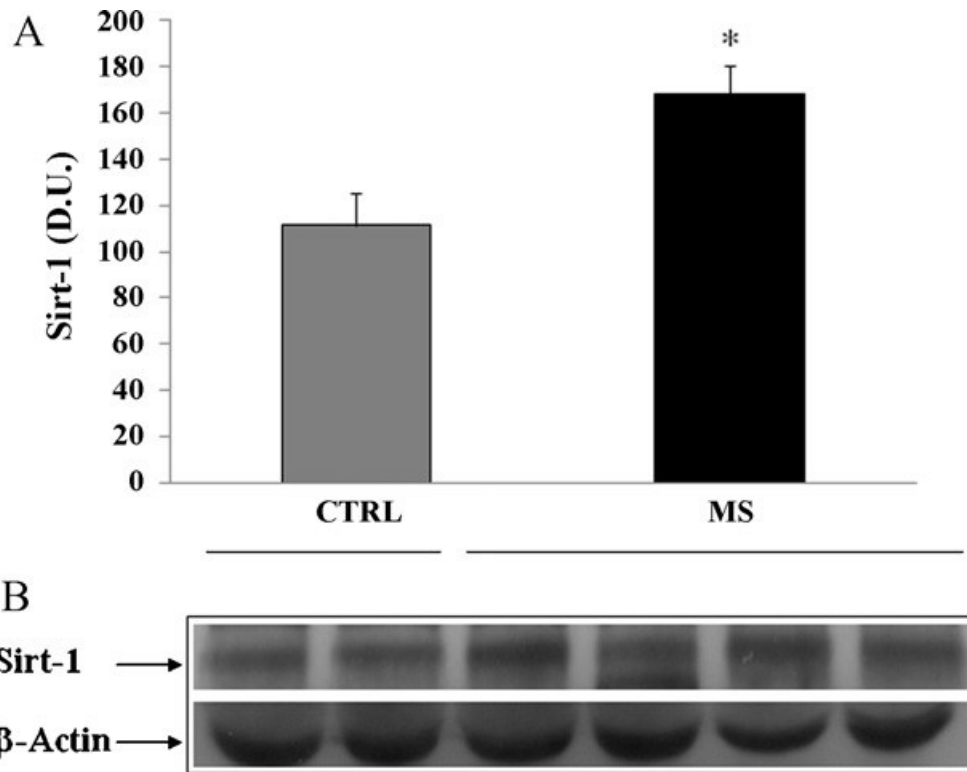


**Figure 25:** (A) Heat shock protein 60 (Hsp60) levels in plasma from multiple sclerosis patients. Plasma samples from multiple sclerosis patients and control subjects were assayed for Hsp60 by Western blot. A representative immunoblot is shown in (B).  $\beta$ -Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.

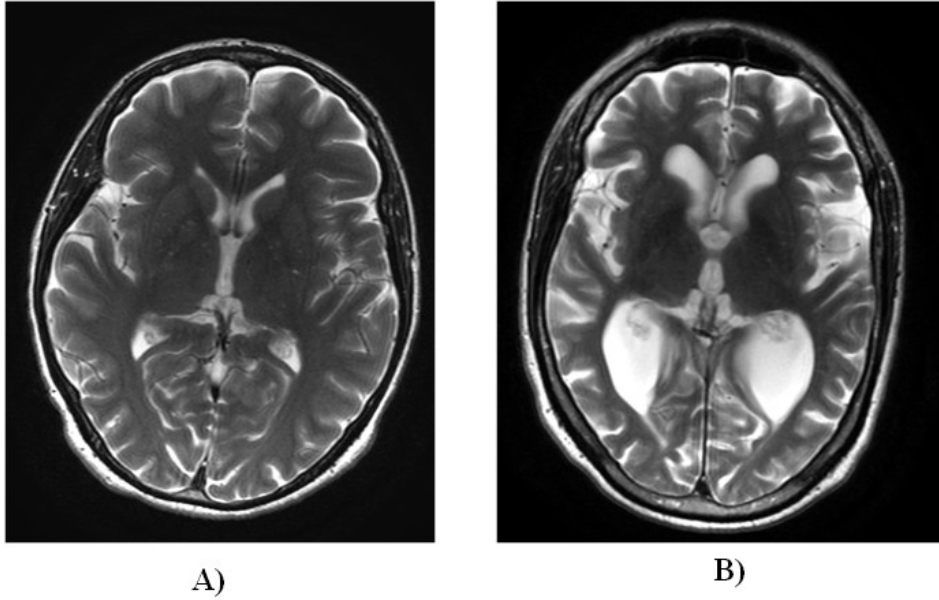




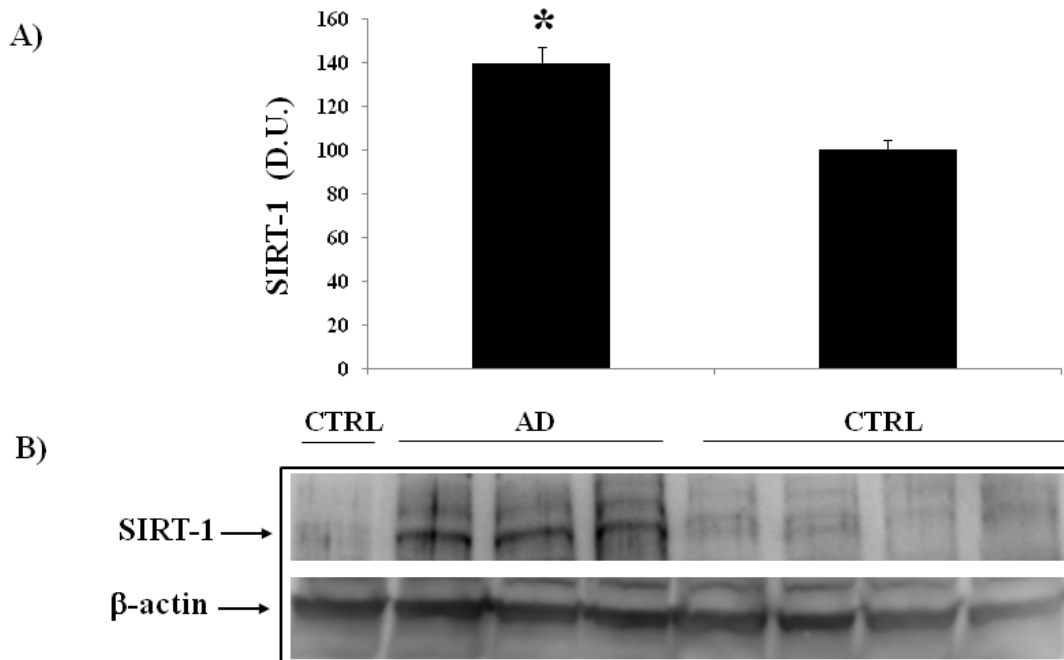
**Figure 26:** (A) Heat shock protein 72 (Hsp72) levels in plasma from multiple sclerosis patients. Plasma samples were assayed for Hsp72 by Western blot. A representative immunoblot is shown in (B).  $\beta$ -Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.



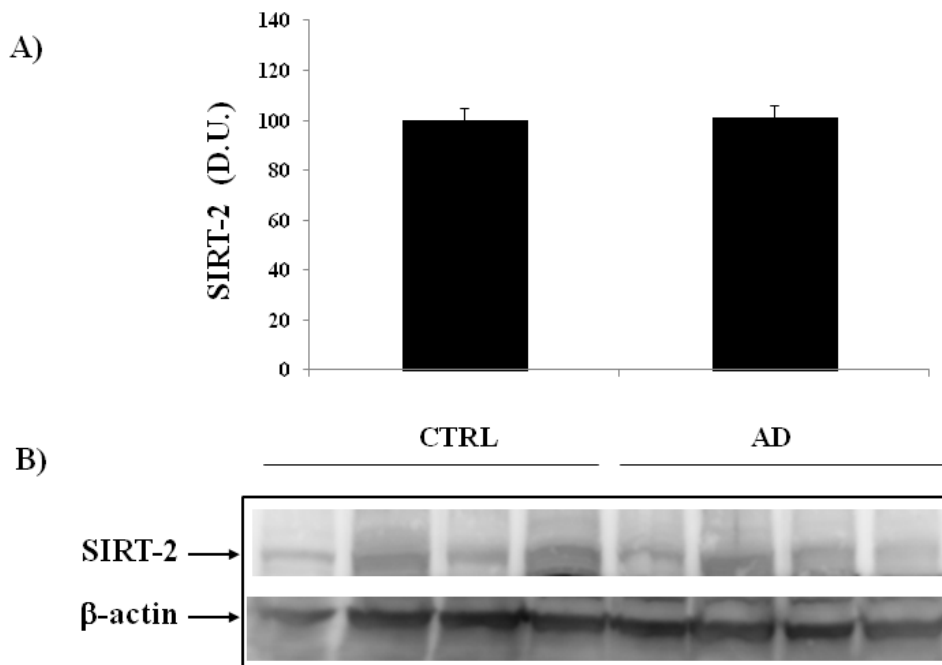
**Figure 27:** (A) Sirtuin-1 (Sirt-1) levels in plasma from multiple sclerosis patients. Plasma samples were assayed for Sirt-1 by Western blot. A representative immunoblot is shown in (B).  $\beta$ -Actin has been used as loading control. The bar graph shows the densitometric evaluation and values are expressed as mean standard error of mean of 3 independent analyses on twenty-two/twenty-six patients per group.  $P \leq 0.05$  vs control. D.U., densitometric units; MS, multiple sclerosis; CTRL, control.



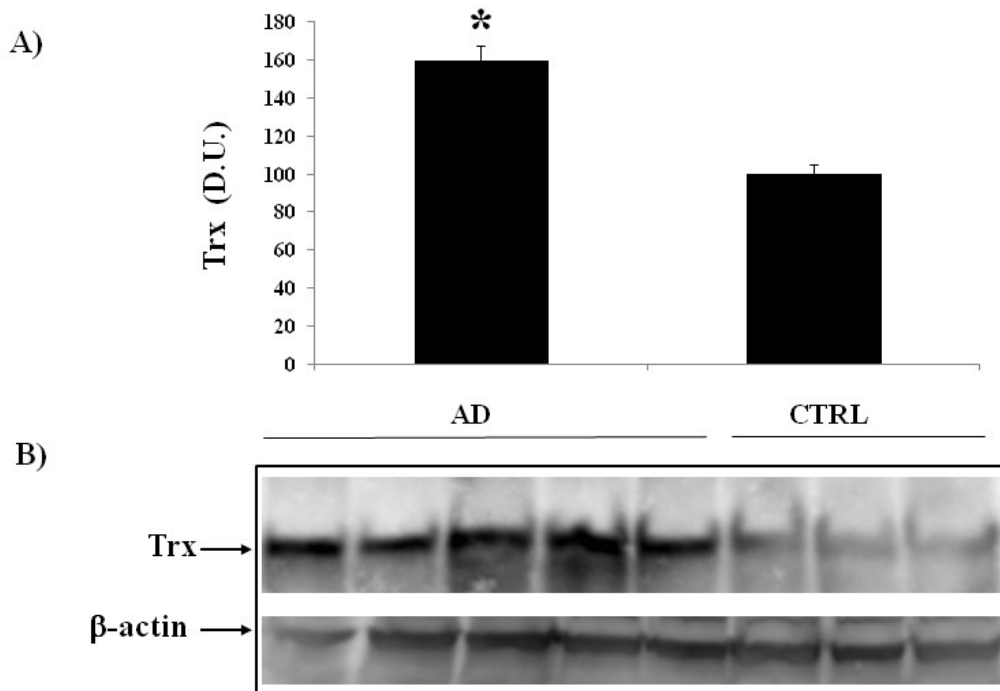
**Figure 28: Brain MRI.** Axial T2 images shows cerebral atrophy in patient with Alzheimer's disease (A) and normal brain in control patient of same age (B).



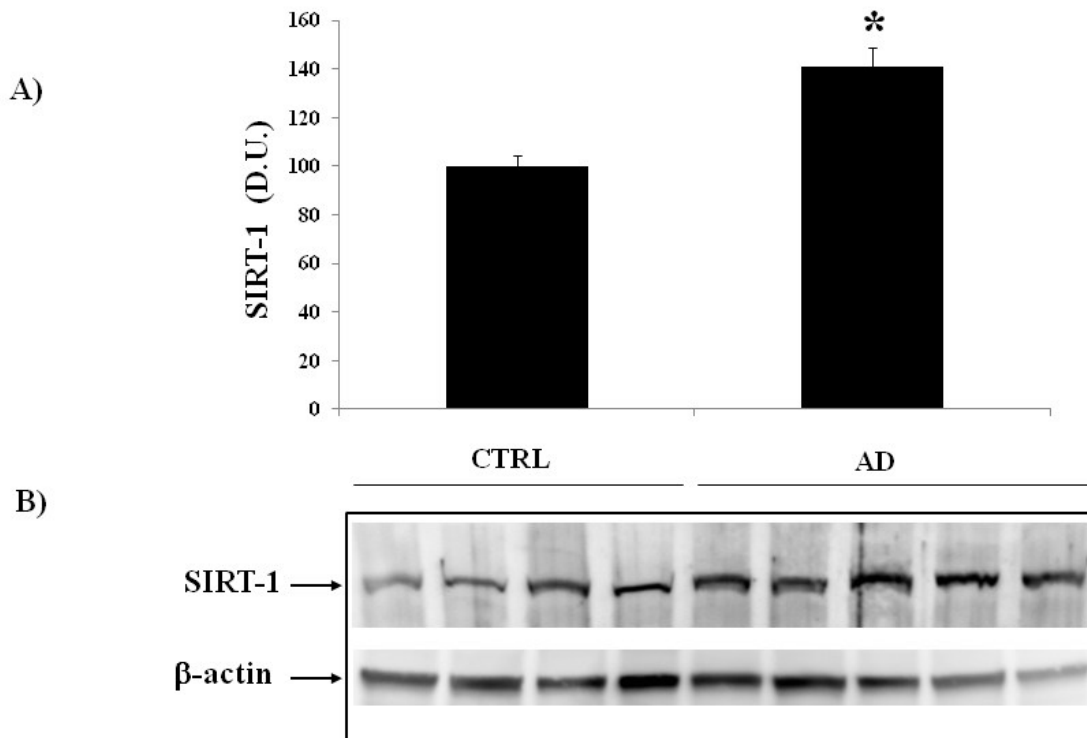
**Figure 29: Sirtuin-1 (Sirt-1) levels in AD lymphocytes.** Samples from control and AD were assayed for Sirt-1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.  $P \leq 0.05$  vs control. B) A representative immunoblot is shown.  $\beta$ -actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.



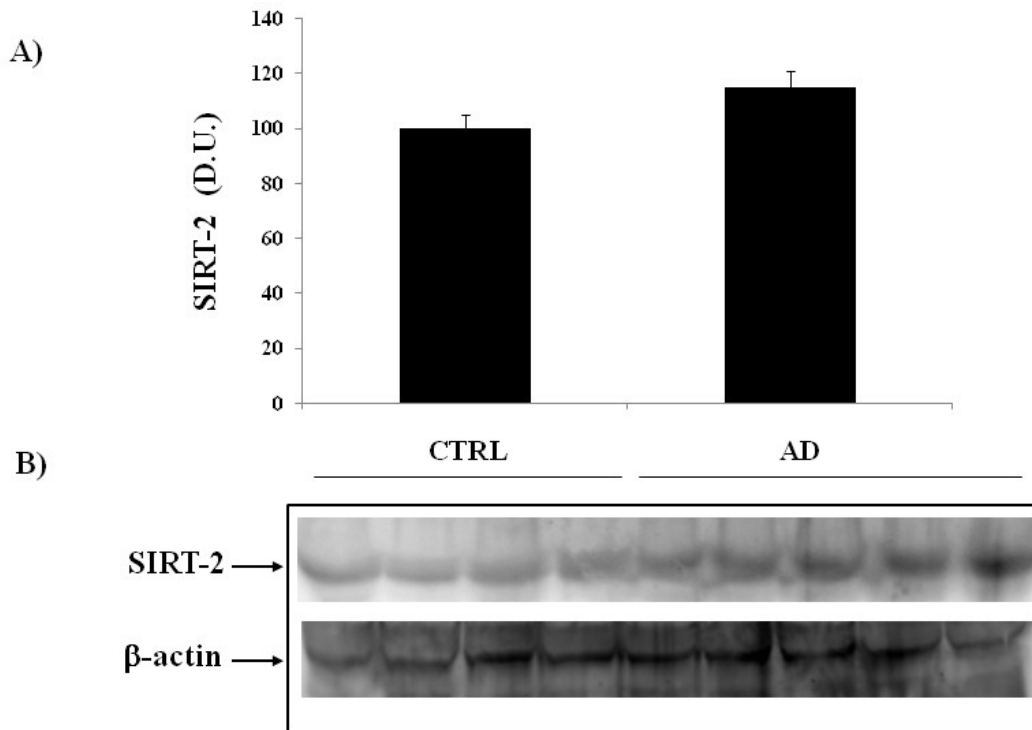
**Fig. 30: Sirtuin-2 (Sirt-2) levels in AD lymphocytes.** Samples from control and AD were assayed for Sirt-1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.  $P \leq 0.05$  vs control. B) A representative immunoblot is shown.  $\beta$ -actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.



**Fig. 31: Thioredoxin (Trx) levels in AD lymphocytes.** Samples from control and AD were assayed for Sirt-1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.  $P \leq 0.05$  vs control. B) A representative immunoblot is shown.  $\beta$ -actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.

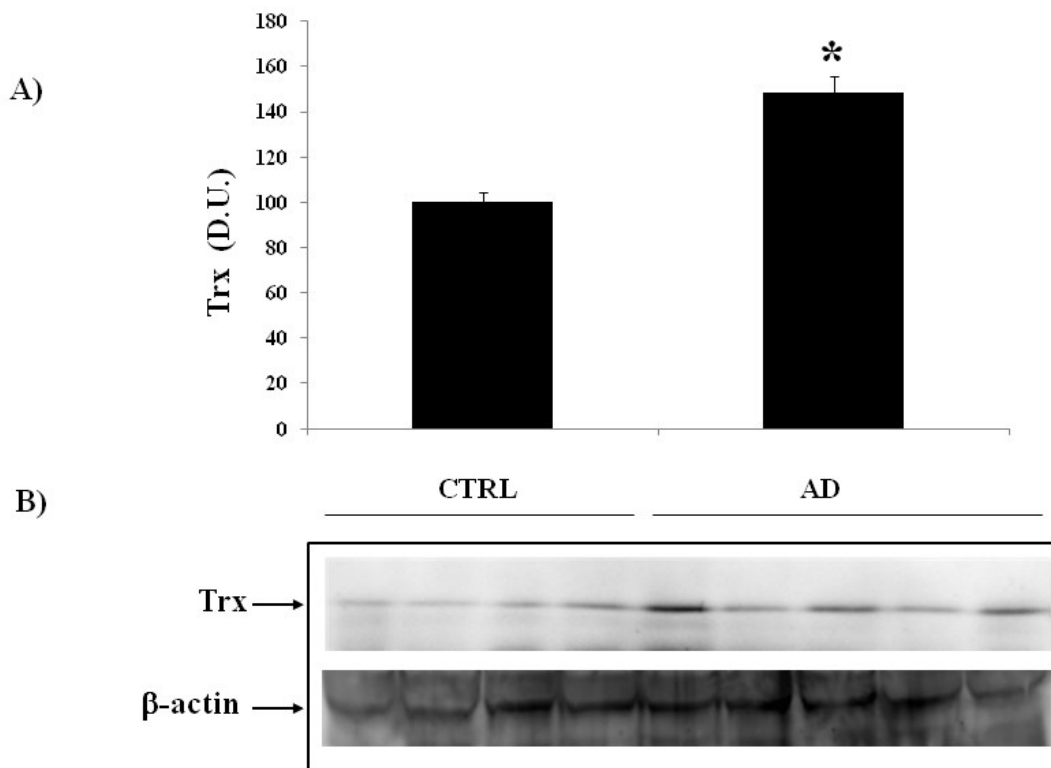


**Fig. 32: Sirtuin-1 (Sirt-1) levels in AD plasma.** Samples from control and AD were assayed for Sirt-1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.  $P \leq 0.05$  vs control. B) A representative immunoblot is shown.  $\beta$ -actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.



**Fig. 33: Sirtuin-2 (Sirt-2) levels in AD plasma.** Samples from control and AD were assayed for Sirt-1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.  $P \leq 0.05$  vs control. B) A representative immunoblot is shown.  $\beta$ -actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.





**Fig. 34: Thioredoxin (Trx) levels in AD plasma.** Samples from control and AD were assayed for Sirt-1 expression by Western blot. A) Densitometric evaluation: the bar graph shows the values are expressed as mean standard error of mean of 3 independent analyses.  $P \leq 0.05$  vs control. B) A representative immunoblot is shown.  $\beta$ -actin has been used as loading control. D.U., densitometric units; AD, Alzheimer's disease; CTRL, control.