U.PORTO
FACULDADE DE FARMÁCIA
UNIVERSIDADE DO PORTO

Dissertation of the 2nd Cycle of Studies Leading to Master's Degree in Analytical, Clinical and Forensic Toxicology

TRACE ELEMENTS IN HUMAN BRAIN:
AGE-RELATED CHANGES IN DIFFERENT ANATOMICAL REGIONS AND CHANGES RELATED WITH NEURODEGENERATIVE PROCESSES

Patrícia Sofia Rodrigues Ramos

Supervised by:
Professor Agostinho Almiro de Almeida
Faculdade de Farmácia da Universidade do Porto

Professor Agostinho José Carvalho dos Santos
Faculdade de Medicina da Universidade do Porto
Instituto Nacional de Medicina Legal e Ciências Forenses, I.P.

October 2012

AUTHOR'S DECLARATION

ACKNOWLEDGMENTS

I would like to express my gratitude to all the people who helped me and made this work possible. I would like to thank in particular:

To my supervisor, Prof. Agostinho Almeida, for his guidance and support during the course of this work. Thanks for the patience and help facing technical problems.

To my co-supervisor, Prof. Agostinho Santos, for his support, encouragement and sympathy demonstrated since the first day.

To all forensic pathologists and technicians involved in sample collection at Instituto Nacional de Medicina Legal. I would like to refer a special word to Ricardo and Nair, for their patience and extra care in samples collection procedure.

To my course colleagues. A special thank to Márcia and Ana, for sharing the last time anxiety and supported me in the last months of this journey. Thanks for the insanity moments shared together.

To all my friends that, even from distance, supported me in troubled times with motivational words. Alessa, Daniela, Gonçalo, Inês, Mariana and Pedro, thank you for being there when I needed.

To my family, for the support and unconditional love.

I am also grateful to University of Porto and Santander Totta for the financial support given through PP_IJUP2011-342 Project ("Trace elements in human brain: age-related changes and anatomic region specific differences").

ABSTRACT

Although its causes are not yet well defined, neurodegenerative diseases have been subject of intensive research in last decades in an attempt to understand the mechanisms underlying the neurodegeneration processes. The etiology of these diseases is multifactorial, being assumed that it involves a complex interaction between the (natural) aging, genetic predisposition and environmental factors. In many neurodegenerative diseases, one of the factors identified as responsible for their development is the alteration of homeostasis of some trace elements (TE) in certain areas of the brain. The main goal of the work reported in this dissertation was to study the age- and disease-related changes on TE levels in different anatomical regions of human brain.

It was found that distribution of TE within brain tissue is not homogeneous. Iron is the most abundant metal, followed by zinc and copper. Zn higher levels were found in hippocampus and middle temporal region; higher levels of the other TE were found in basal ganglia. Pons and medulla were the regions with lower levels of four of the TE studied (iron, copper, manganese and zinc). In specific areas, TE levels seem to be age-related. A positive correlation between Cu and Zn levels and age was found, namely in middle temporal, caudate, putamen and cingulated gyrus.

When compared with healthy people of the same age sub-group, TE levels from two individuals affected by Alzheimer's disease were found significantly altered in regions mainly related to memory and learning. In one Alzheimer's disease patient, 85 years old, high levels were found in hippocampus (Cu ↑2x; Fe ↑1,3x), middle temporal (Cu ↑1.4x), caudate (Fe ↑1.7x; Mn ↑1.6x; Cu ↑1.9%; Zn ↑1.5x), and pons (Mn ↑1.7x, Cu ↑1.93x, Zn ↑1.2x). In the other Alzheimer's disease patient, 73 years old, high levels of Fe in caudate (↑1,9x) and hippocampus (↑2,1x) were found. Low levels of Mn in hippocampus (↓0.6x) and cingulated gyrus (↓0.6x) and of Cu (↓0.4x) and Zn (↓0.7x) in cingulated gyrus were also found.

In Parkinson's disease, it was found that the regions affected by TE imbalances are mainly related to motor control but also to memory and learning: caudate (Fe ↑1.34x), putamen (Fe ↑1.5x; Mn ↑3.3x; Cu -↓0.6x), globus pallidus (Fe ↑2.3x; Mn ↓0.6x), cingulated gyrus (Fe ↑1.5x; Mn ↑1.6x; Cu ↓0.6x), hippocampus (Fe ↑1.3x; Mn ↑1.4x; Zn ↑1.2x; Cu ↓0.70x) and midbrain (Mn ↑1,5x; Zn ↓0.8x).

In amyotrophic lateral sclerosis, it were found lower Fe levels caudate (↓0.4x), putamen (↓0.4x) globus pallidus (↓0.6x), regions mainly connected to motor function.

Keywords: trace elements; human brain levels; aging; neurodegenerative diseases; atomic absorption spectrometry; inductively coupled plasma-mass spectrometry.

RESUMO

Apesar das suas causas ainda não estarem claramente definidas, nas últimas décadas as doenças neurodegenerativas tornaram-se um alvo de grande investigação, numa tentativa de compreender os mecanismos associados ao processo neurodegenerativo. A etiologia deste grupo de doenças é multifatorial, sendo assumido que envolve uma interação complexa entre fatores como envelhecimento natural, predisposição genética e fatores ambientais. Um dos fatores identificado como possivelmente envolvido na patologia das doenças neurodegenerativas é a alteração da homeostasia de alguns elementos vestigiais (EV) em determinadas regiões do encéfalo.

O objetivo principal do trabalho apresentado nesta dissertação foi o estudo das alterações nos níveis de diversos EV no encéfalo humano relacionadas com o envelhecimento e os processos degenerativos. Constatou-se que a distribuição dos elementos analisados no encéfalo não é homogénea. O Fe mostrou ser o metal mais abundante, seguindo-se o Zn e o Cu. Os níveis mais elevados de Zn encontraram-se na região do hipocampo e lobo temporal; os níveis mais elevados de Cu, Fe e Mn foram encontrados nos núcleos da base. A ponte e a medula são as regiões cerebrais com menor teor dos quatro elementos referidos. Em algumas regiões do encéfalo a acumulação dos EV estudados parece estar associada ao envelhecimento uma vez que se encontrou uma correlação positiva entre os seus níveis e a idade, nomeadamente nas zonas do lobo temporal médio, caudado, putamen e cíngulo.

Quando comparados com indivíduos da mesma faixa etária sem evidência de doença neurodegenerativa, observaram-se níveis de EV alterados em dois doentes de Alzheimer, nomeadamente nas regiões cerebrais associadas à memória. Num dos doentes, com 85 anos de idade, observou-se um aumento dos níveis de EV na zona do (Cu ↑2x; Fe ↑1,3x), temporal médio (Cu ↑1.4x), caudado (Fe ↑1.7x; Mn ↑1.6x; Cu ↑1.9%; Zn ↑1.5x), e ponte (Mn ↑1.7x, Cu ↑1.93x, Zn ↑1.2x). No outro doente, com 73 anos, foi também observado um aumento dos níveis de EV no caudado (↑1,9x) e hipocampo (↑2,1x). No hipocampo observou-se ainda uma redução dos níveis de Mn (↓0.6x). No cíngulo foram encontrados níveis reduzidos de Mn (↓0.6x), Cu (↓0.4x) e Zn (↓0.7x).

Num doente de Parkinson, encontraram-se níveis significativamente alterados dos EV no caudado (Fe ↑1.34x), putamen (Fe ↑1.5x; Mn ↑3.3x; Cu -↓0.6x), globus pallidus (Fe ↑2.3x; Mn ↓0.6x), giro cingulado (Fe ↑1.5x; Mn ↑1.6x; Cu ↓0.6x), hippocampus (Fe ↑1.3x; Mn ↑1.4x; Zn ↑1.2x; Cu ↓0.70x) e mesencéfalo (Mn ↑1,5x; Zn ↓0.8x).

No doente com esclerose lateral amiotrófica foi observada uma diminuição dos níveis de Fe no caudado (↓0.4x), putamen (↓0.4x) e globus pallidus (↓0.6x), regiões principalmente associadas a funções motoras.

Palavras-chave: elementos vestigiais; níveis no encéfalo humano; envelhecimento; doenças neurodegenerativas; espectrofotometria de absorção atómica; espectroscopia plasma-massa.

TABLE OF CONTENTS

INDEX OF FIGURES

INDEX OF TABLES

ABBREVIATIONS AND SYMBOLS LIST

Abbreviation	**Extension**
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
Aβ	Amyloid-β
CNS	Central nervous system
Co	Cobalt
CSF	Cerebrospinal fluid
Cu	Copper
Cu,Zn-SOD	Copper/zinc superoxide dismutase
DA	Dopamine
DMT-1	Divalent metal transporter-1
Fe	Iron
GABA	γ-aminobutyric acid
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
INMLCF	Instituto Nacional de Medicina Legal e Ciências Forenses
IREG1	Iron-regulated transporter 1
MAO	Monoamine oxidase
Mn	Manganese
Mn SOD	Manganese superoxide dismutase
Mo	Molybdenum
MT	Metallothionein
ND	Neurodegenerative disease
NE	Norepinephrine
NFT	Neurofibrillary tangles
$NO^{\bullet}$	Nitric oxide
ONOO-	Peroxynitrite anion
PD	Parkinson's disease
ROS	Reactive oxygen species

Se	Selenium
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SOD	Superoxide dismutase
TE	Trace Elements
Tf	Transferrin
TfR	Transferrin receptors
Zn	Zinc
$^{\bullet}OH$	Hydroxyl radical

INTRODUCTORY NOTE

In 2010, more than 500 million people were aged 65 or older, representing 8% of the world's population. By 2050, this number is expected to nearly triplicate to about 1.5 billion, representing 16% of the world's population [1, 2]. According to Instituto Nacional de Estatística data, until 2050 one third of Portuguese population will be ≥ 65 years old and one million will be ≥ 80 years old [3].

According to 2011 census, in Portugal, 60% of people aged ≥ 65 years live alone or with people with same age [3]. In addition, according to Polícia de Segurança Pública data, around 3000 elderly people were found dead at their homes in 2011 [4]. People with dementia living alone are exposed to risks that exceed the risks faced by people with dementia who live with family or a caregiver, including inadequate self-care, malnutrition, and lack of medical conditions, falls and accidental deaths.

The rate of death related to neurological disorders dramatically increased by one- to two fold between 1979 and 1997 in the major western world countries, illustrating the significance of brain-related disorders in actual society [5]. In addition, it is estimated that ''brain-related disorders'' represent 35% of the total economic burden of diseases in Europe, reaching a total of $386 billion, of which $135 billion are indirect medical costs [6].

Metal-induced oxidative stress has been considered to have a significant role in neurodegenerative diseases evolution, leading to the production of intracellular protein aggregates in neurons, and subsequent accumulation, reflecting cell incapacity to adequately respond to their formation.

Despite of the intensive work that has been done in last decades on the relationship between trace elements and neurodegenerative diseases, the evidence is still fragmentary and these diseases remain poorly understood, which is reflected by the lack of disease modifying therapies. It is clearly urgent to clarify the causes and mechanisms involved in age-related processes and neurodegenerative diseases in order to improve therapeutic measures to slow down the onset of these disorders and/or to enable an effective delay of their onset.

The present dissertation is structured in four main parts:

I. *Theoretical background*, reviewing key scientific publications related to natural aging, neurodegeneration and the involvement of main trace elements in these processes.

II. *Objectives*, presenting the specific goals of the work performed.

III. *Experimental research*, describing the experimental work in order to answer the questions formulated in the Objectives section.

IV. *Conclusions and future research*, where the deductions from data obtained will be taken in the context of the proposed objectives. Complementary studies will be also projected.

I. THEORETICAL BACKGROUND

THEORETICAL BACKGROUND

It is an old wisdom that metals are indispensable for life. However, the role of metals and their impact on life remained largely hidden until inorganic chemistry and coordination chemistry experienced a pronounced revival in the second half of the twentieth century [7]. The experimental and theoretical tools created in this period and their application to biochemical problems led to the development of *bioinorganic chemistry or inorganic biochemistry*, more recently also often named as *biological inorganic chemistry*.

The development of *biological inorganic chemistry* during the past decades was, and still is, driven by attempts to understand the interaction between metal ions and macromolecules and clarify biological processes, to understand the accumulation, transport, metabolism and toxicity of metal ions and to develop metal-based drugs.

The role of some metal ions was found to be particularly important in brain functions and has led to the introduction of *metalloneurochemistry*, the branch of *biological inorganic* chemistry dedicated to the study of metal ions in brain and nervous system at molecular level [8].

1.1. THE IMPORTANCE OF TRACE ELEMENTS IN BRAIN FUNCTION

About 50 of the known elements occur in measurable concentrations in the living systems but in humans and other mammals, only 23 elements have known physiological activities. For human health purposes, the essential elements can be sub-classified according to the concentration (trace or major) in which they are found in body fluids and tissues. According to the Clinical and Laboratory Standards Institute, elements are classified as *major* (concentrations above 10 mg/L in fluids; above 100 µg/g in tissues), *trace* (body content 0,01 to 100 µg/g; 10 to 10^4 µg/L) or *ultratrace* (body content less than 0,01 µg/g; less than 10 µg/L) [9]. For simplifying, in this work it will be consistently used the term *trace element* and no distinction will be made between the terms *ultratrace* and *trace*.

Of the elements with known physiological activities, 11 can be classified as trace elements (TE). Most of TE are in the period 4 of the Periodic Table (Figure 1), suggesting an optimal relationship of nuclei size/electron availability of the elements in this period to interact with organic molecules present in biological systems [10]. TE include the transition metals vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), copper (Cu), zinc (Zn), and molybdenum (Mo) and the non-metals selenium (Se), fluorine (F) , and iodine (I).

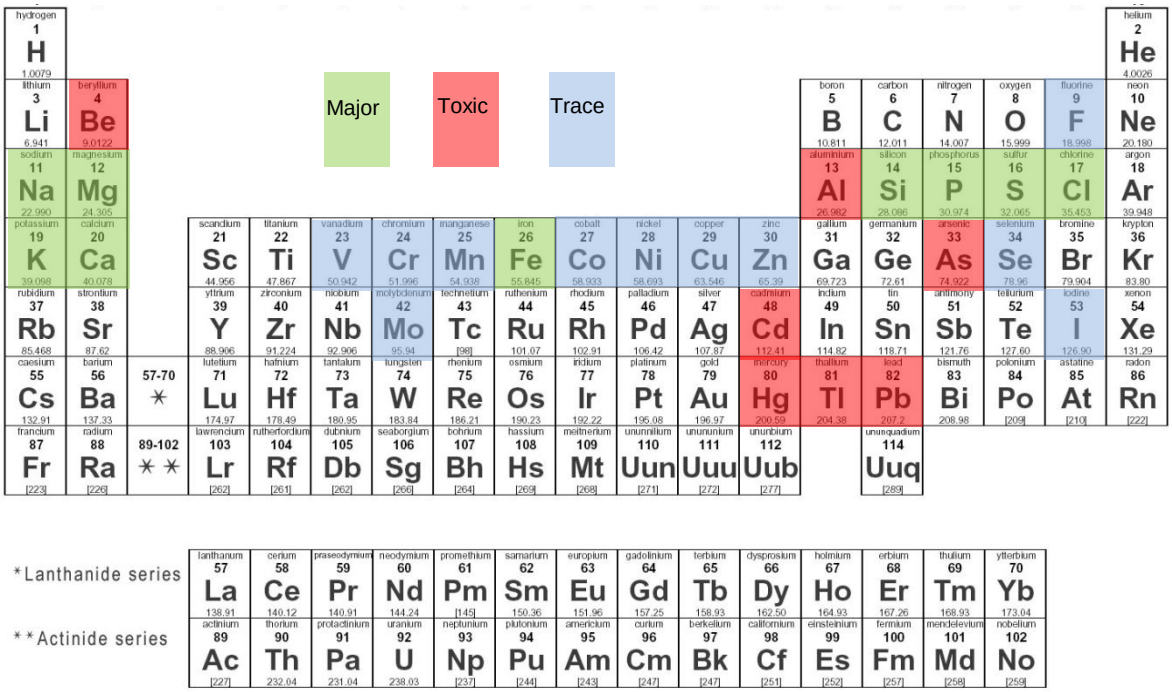

Figure 1: Classification of elements based on Clinical and Laboratory Standards Institute [5].

In biological systems, metallic TE are mostly conjugated or bound to proteins, forming metalloproteins, or to smaller molecules (e.g. phosphates, polyphenols and other chelating compounds). Most of the metals in metalloproteins are part of enzymatic systems, have structural functions, or use the protein to be transported to their target site in the organism. In enzymes, metals participate in catalytic processes as constituents of enzyme active sites or stabilizers of enzyme tertiary or quaternary structure. As constituents of active sites, metal cations with unpaired electrons mediate oxidation–reduction processes by reversible changes in their oxidation state, transferring or receiving electrons to or from the substrate and co-factor. For example, superoxide dismutases (SOD) reduce one superoxide anion ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2), and oxidize a second $O_2^{\bullet -}$ to generate molecular oxygen by either Cu or Mn present in the active site of the cytosolic or mitochondrial enzyme, respectively. The presence of metals bound to lipids, nucleic acids, and carbohydrates is well documented, but the biochemical functions of the metals present in these molecules is unclear [11].

1.1.1. IRON

Iron (Fe) is the most abundant transition metal in the human body and has many essential functions in brain and nervous system. It is not only involved in oxygen transport,

mitochondrial respiration, protein and DNA synthesis, but also plays an important role in the formation of myelin and the development of dendrites [12]. Further, iron is involved on the biosynthesis of neurotransmitters such as dopamine, in axonal growth and in receptor-mediated postsynaptic signal transduction [13].

Fe is found in four classes of proteins: Fe-heme proteins (e.g. hemoglobin, myoglobin, catalase, cytochromes); Fe–sulfur enzymes (e.g. aconitase, fumarate reductase); proteins for Fe storage and transport (transferrin, lactoferrin, ferritin, hemosiderin), and other Fe-containing or Fe-activated enzymes (e.g. NADH dehydrogenase, succinate dehydrogenase, alcohol dehydrogenase, cyclooxygenases) [12].

In vivo, Fe is present in both the ferrous (Fe^{2+}) and ferric (Fe^{3+}) valence states. The ease with which ion converts between Fe^{2+} and Fe^{3+} is critical for metabolism. Its uptake and transport across membranes, and release from transporter proteins such as transferrin (Tf), is dependent on reduction to Fe^{2+}. As the ferrous form is highly redox active (and thus more toxic), the majority of non-heme iron that is not immediately utilized is prevented from participating in harmful reactions through uptake, oxidation and storage in ferritin [14]. Free Fe is readily bound by Tf, and the 'supply and demand' response to the labile iron pool is thought to determine the expression of Tf receptors and ferritin to maintain iron homeostasis within the brain [12].

Fe is usually transported in plasma and other extracellular fluids bound to Tf and taken up by cells via the transferrin cycle (Figure 2). In plasma, at physiologic pH, holotransferrin (Tf-Fe (III)) binds to transferrin receptors (TfR) on the cell surface. The complexes localize to calathrine-coated pits, which invaginate to initiate endocytosis process. The calathrin coat is removed and specialized endosomes are acidified by an ATP-dependent proton pump. At the acidic pH the holotransferrin undergoes a conformational change and releases Fe (III), which is then converted to Fe (II) by an endosomal metalloreductase and transported out of the endosome into the cytosol by the divalent metal transporter-1 (DMT1). Apotransferrin (ApoTf) bounds to its receptor, TfR, and is exocytosed by a recycling endosome to the plasma cell membrane, where, at neutral pH, the complex dissociates and proteins are able to participate in further rounds of iron delivery. Fe (II) can be stored in ferritin in non-erythroid cells or incorporated into hemoglobin in erythroid cells [15-17].

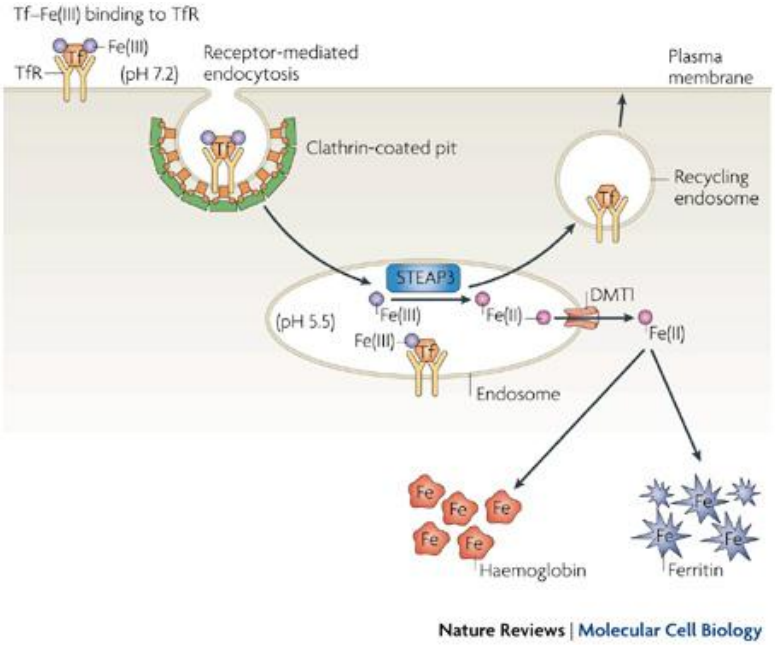

Figure 2: The transferrin cycle: iron transport and storage [17].

Brain is protected by a poorly permeable vascular membrane, the blood-brain barrier, composed of endothelial cells in small vessels throughout the brain that contain tight junctions, which regulate the access of nutrients and other molecules [18]. In addition, fenestrated blood vessels in the choroid plexus that produce cerebrospinal fluid (CSF) also regulate brain Fe [19]. Under normal physiological conditions, the brain stringently regulates Fe balance by, possibly, three systems: (i) via TfR-mediated transport or the non TfR mediated transport at brain barriers; (ii) the Fe storage in which the cellular sequestration is largely dependent upon availability of ferritin; and (iii) the efflux of Fe whose rate is controlled by bulk CSF flow and/or by the removal mechanism in the blood-CSF barrier back to the blood circulation [13]. Tf-bound Fe is the mainly primary species transported into the brain and this transport by blood-brain barrier may explain the unique Fe distribution pattern.

Available data suggest that normal Fe content within the brain varies significantly from one region to another: greater concentrations are found in substantia nigra (SN), basal ganglia, hippocampus and subcortical brain regions [20]. Studies show that regions of the brain associated with motor functions tend to have a higher Fe content than non-motor related regions, which highlight the link between movement disorders and iron loading [21].

Iron accumulation is a characteristic feature of several neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease, and also other rarer disorders like aceruloplasminemia, Hallervorden-Spatz disease (neurodegeneration with brain iron accumulation), Friedreich's ataxia or neuroferritinopathy. Iron has been implicated in these diseases for its capacity to increase oxidative stress, and chelators ameliorate Parkinson-like disease in animal models. However, its precise role in these disorders remains unclear. It is still not known whether abnormal iron metabolism leads to neurodegeneration or occurs as a consequence of neurons damage.

1.1.2. MANGANESE

Manganese (Mn) is associated with bone development and with amino acid, lipid and carbohydrate metabolism. Mn plays an important role in a number of physiologic processes as a constituent of multiple enzymes (e.g. mitocondrial Mn-SOD, glutamine synthetase and arginase) and as an activator of other enzymes (several hydrolases, transferases and carboxylases) [22].

Mitochondria consume over 90% of the oxygen used by cells, which make this compartment especially susceptible to oxidative stress since it is a primary site of production of free radicals. The radical superoxide anion ($O_2^{\bullet-}$) is one of the reactive oxygen species (ROS) produced in mitochondria during ATP synthesis. Mn-SOD is the principal antioxidant enzyme in the mitochondria and catalyzes the conversion of $O_2^{\bullet-}$ to H_2O_2, which can be further reduced to water by other antioxidant enzymes [12].

In the brain, glutamine synthetase, after activation by Mn, converts the amino acid glutamate to glutamine. Glutamate is an excitotoxic neurotransmitter and a precursor to an inhibitory neurotransmitter, γ-aminobutyric acid (GABA) [23].

Although the specific mechanisms for Mn absorption and transport have not been determined, some evidence suggests that Fe and Mn can share common absorption and transport pathways. Because of their chemical similarity they share and compete for many protein transporters, such as DMT-1. As such, individual Fe status can affect Mn bioavailability: during of low Fe uptake conditions, abnormal Mn accumulation occurs. In addition, when Mn concentrations are altered, the Fe and other transition metals homeostasis are disrupted. [24]

Mn intestinal absorption is increased during Fe deficiency, and increased Fe storage (ferritin levels) is associated with decreased Mn absorption [25]. Men generally absorb less Mn than women, which may be explained by the fact that men usually have higher Fe

stores than women [26]. Further, Fe deficiency has been shown to increase the risk of Mn accumulation in the brain, causing Parkinson-type syndrome, commonly referred as manganism or Parkinsonism [27, 28], mainly in areas that contain higher concentrations of nonheme iron, such as basal ganglia, SN and subthalamic nuclei [29].

Manganism is initially characterized by a psychiatric disorder (*locura manganica or* manganese madness) that may be due to Mn acute exposure, causing hyperactivity accompanied by elevated brain levels of catecholamines and their metabolites [30]. Symptoms include compulsive and violent behavior, emotional instability, disorientation and hallucinations. As exposure continues and the disease progresses, patients may develop prolonged muscle contractions, decreased muscle movement, rigidity, muscle tremors and memory loss [31]. As occurs in Parkinson's disease, these signs are associated with damage of dopaminergic neurons within brain structures associated to muscle movement control [32].

There has been some controversy about the alterations produced by Mn: some authors found that Mn modifies dopaminergic functions specifically in the basal ganglia and produces Parkinson-like disorder, while others indicate that Mn intoxication appears to be different from Parkinson's disease in both etiology and pathology particularly in the notable preservation of SNC dopaminergic somas [32].

1.1.3. COPPER

In humans, copper (Cu) is necessary for the development of connective tissue and bones, and for nerve myelination. It is also involved in the metabolism of neurotransmitters, and in regulation the balance of other metals in the body such as zinc and molybdenum [33].

Though the majority of the body's copper is in the Cu^{2+} form, in the human body copper shifts between the cuprous (Cu^{+}) and cupric (Cu^{2+}) forms [34]. Like other transition metals, the ability of Cu to easily accept and donate electrons explains its important role in oxidation-reduction reactions and free radicals scavenging.

Cu is a critical functional component of a number of essential enzymes such as Cu,Zn superoxide dismutase (Cu,Zn-SOD), lysil oxidase, dopamine hydroxylase, and several other oxidases that reduce molecular oxygen. As a cofactor in multiple redox reactions,

Cu is involved in the production of potentially damaging radical species through Fenton reaction [35].

Several reactions essential to normal function of the brain and nervous system are catalyzed by cuproenzymes. Dopamine-β-monooxygenase catalyzes the conversion of dopamine (DA) to norepinephrine (NE). Monoamine oxidase (MAO) plays a role in the metabolism of the neurotransmitters DA, NE and epinephrine. MAO also acts in the degradation of the neurotransmitter serotonin, which is the basis for the use of MAO inhibitors as antidepressants [36]. Cu acts as a reductant in cytochrome *c* oxidase, that plays a critical role in cellular energy production: it catalyzes the reduction of molecular oxygen (O_2) to water (H_2O), generating an electrical gradient used by the mitochondria to create ATP [37]. Cytochrome *c* oxidase activity is also involved on phospholipids synthesis, the principal constituent of myelin sheath.

After Cu has been taken up by the enterocytes it is exported to the bloodstream, in a process involving cytoplasmic transport to the membrane and exocytosis. Cu is then distributed to other tissues and is taken up by the cells. Once inside the cell, Cu homeostasis needs to be tightly regulated: the metal complexes with proteins to avoid the formation of deleterious hydroxyl radicals. Cu, bound either to albumin or histidine, crosses the plasma membrane via copper transport protein (Ctr1) or DMT-1. Inside the cell, Cu can suffer one of four possible fates: 1) it can directly enter the Cu metallothionein storage pool; 2) it can be transported to the mitochondria, for incorporation into the terminal oxidase of the respiratory chain, cytochrome c oxidase; 3) it can be incorporated into cytoplasmic Cu,Zn-SOD; or 4) it can be transported to the P-type ATPase located in the *trans* Golgi network for subsequent incorporation into ceruloplasmin [15, 38].

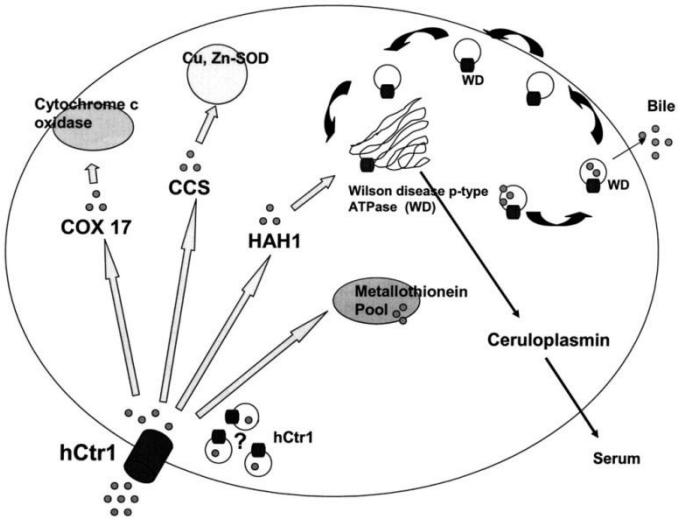

Figure 3: Model of copper transport in a hepatocyte [38].

Two copper-containing enzymes, ceruloplasmin and ferroxidase II have the capacity to oxidize ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), the form of iron that can be loaded onto transferrin for transport to the site of red blood cell formation. Although ferroxidase activity of these two cuproenzymes has not yet been proven to be physiologically significant, the fact that Fe mobilization from storage sites is impaired in Cu deficiency supports their role in iron metabolism [37]. Ceruloplasmin may function as an antioxidant in two different ways: binding Cu and preventing free Cu ions from catalyzing oxidative damage; and, once Ceruloplasmin has ferroxidase activity, the oxidation of ferrous iron facilitates Fe loading onto transferrin and may prevent free Fe^{2+} from participating in harmful free radical generating reactions [15].

Adequate Cu nutritional status appears to be necessary for normal Fe metabolism and red blood cell formation. Anemia is a clinical sign of Cu deficiency, and Fe has been found to accumulate in the liver of Cu deficient animals, indicating that Cu is required for Fe transport to the bone marrow for red blood cell formation. Infants fed with a high iron formula absorbed less copper than infants fed with a low iron formula, suggesting that high iron intake may interfere with copper absorption [39].

1.1.4. ZINC

Numerous aspects of cellular metabolism are zinc-dependent. Zinc (Zn) plays important roles in growth and development, immune response, neurological function and reproduction. Zinc is found at high levels in the brain, where it performs catalytic, structural and regulatory roles in cellular metabolism [15].

About a hundred of different enzymes (e.g. RNA polymerase, carbonic anhydrase, Cu,Zn-SOD, angiotensin I converting enzyme) depends on Zn for their ability to catalyze vital chemical reactions.

Zn plays an important role in the structure of proteins and cell membranes because its loss from biological membranes increases their susceptibility to oxidative damage and impairs their function [40] . A finger-like structure, known as zinc finger motif, stabilizes the structure of a number of proteins. For example, Cu provides the catalytic activity for the antioxidant enzyme Cu,Zn-SOD while Zn plays a critical structural role [39].

Zn finger proteins have been found to regulate gene expression by acting as transcription factors (binding to DNA and inducing specific genes transcription). Zn also plays a role in cell signaling and has been found to influence hormone release and nerve impulse

transmission. Zn has been found to play a role in apoptosis, a critical cellular regulatory process with implications for growth and development, as well as a number of chronic diseases [41].

Besides its structural and catalytic role in several enzymes, Zn has a unique location and function in CNS. According to previous studies, Zn is not uniformly distributed throughout the brain. It is found at higher concentrations in certain regions such as the hippocampus and cortex and in lower amounts in cerebellum [42]. Zn is bound to proteins but free zinc (10% of total brain Zn) is distributed thought the CNS in synaptic vesicles of glutamatergic neurons and performs a role in neurotransmission mediated by glutamate and GABA [12]. Upon stimulation, both glutamate and Zn are released into the synapse and Zn is thought to play a role in stabilizing the neurotransmitter. At the synapse level, Zn reaches high concentrations and enters post-synaptic neurons via calcium channels, competing with calcium [42].

Normally, when entering post-synaptic neurons, Zn will be captured by methallotionein (MT) proteins. The binding of Zn to MT is one of the most important mechanisms of Zn homeostasis.

Although there is a good correlation between Zn specific staining and neuronal death, the mechanism involved in Zn-induced neuronal degeneration is not completely elucidated. Increasing evidence suggests that Zn induces cell death by impairing proper mitochondrial function through different mechanisms, including inhibition of electron transport chain,O_2 consumption and energy production and induction of mitochondrial depolarization [43-46]. Other studies have reported that elevated intracellular Zn inhibits glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase, with consequent reduction of its downstream intermediates and a decrease in ATP intracellular concentrations [47]. All together, the evidences suggest that Zn decreases cell energy production.

Unlike Cu and Fe, Zn has no unpaired electrons which prevents its participation in redox reactions. Zn act as an antioxidant since it is a constituent of Cu,Zn-SOD (both intracellular and extracellular). Plus, Zn can act as an antioxidant by: (i) inducing MT synthesis, which can bind redox active metals and scavenge $^{\bullet}OH$ via their cysteine groups; and (II) binding to sites in the membrane that otherwise could bind redox active metals [42, 48].

High supplemental Zn intakes for extended periods of time may result in Cu deficiency, which may be explained because Zn increases the synthesis of MT in intestinal cells, and these proteins bind several metals and prevent their absorption by trapping them in

intestinal cells. MT has a stronger affinity for Cu than for Zn, so high levels of MT induced by Zn excess cause a decrease in Cu absorption. In contrast, high Cu dietary intakes have not been found to significantly affect Zn nutritional status [39].

Even short-term deficits of Zn have been shown to impair certain measures of mental and neurological function while long-term deficits of Zn, especially during gestation, results in malformation and deficits in attention, learning, memory or neuropsychological behavior [12].

1.2. TRACE ELEMENTS AND NEURODEGENERATIVE DISEASES

Although transition metals are important for life, it has been evidenced their direct or indirect involvement in neuronal damage in many neurodegenerative disorders. Neurodegenerative diseases (ND) associated with the disruption of brain metal homeostasis include Alzheimer's disease (AD),Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS), Acquired hepatocerebral degeneration, Hungtington's disease and Wilson's disease. It has been observed that patients with ND accumulate metals in their nervous system, suggesting a role of metals in those disorders. Furthermore, there is an increasing recognition that these diseases are associated with localized accumulation of metal ions and oxidative stress in disease-affected regions [49].

Abnormalities of metal ion biochemistry in neural tissue are associated with two key mechanisms: protein aggregation mediated by metal ions and oxidative reactions catalyzed by redox-active metals (Table 1) [7].

Table 1: Neurodegenerative disorders with possible metal-associated pathology.

Neurodegenerative disease	Implicated metal	Implicated proteins
Alzheimer's disease	Copper, iron, zinc	Amyloid-β, amyloid-β precursor protein
Parkinson's disease	Iron	α-synuclein, neuromelamin, ceruloplasmin, divalent cation transporter
Amyotrophic lateral sclerosis	Copper, zinc	Cu,Zn-Superoxide dismutase
Acquired hepatocerebral degeneration	Manganese	
Huntington's disease	Iron	Huntingtin
Wilson's disease	Copper	Ceruloplasmin, Wilson's protein

Biological systems have developed tightly mechanisms for metal regulation at both the systemic level of absorption and distribution as well as at the cellular level of storage, recycling and utilization, so that the organism meets metabolic demand without over accumulation [49]. Excess metal not correctly stored by store vesicles or bounded to protein transport, it becomes available for potentially toxic reactivity. One of the dangers of redox-active metals like Fe and Cu is their ability to promote the formation of highly toxic hydroxyl radicals that oxidize biomolecules, leading to cell death [49]. Even in the absence of redox activity, metal cations like Zn^{2+} may also cause damage by inducing abnormal protein aggregation [50].

As discussed early, transition metals such as Fe and Cu facilitate the generation of free radicals *in vivo* [51]. The reaction between reduced transition metals, typically Fe (II) or Cu (II), and H_2O_2 (Fenton reaction) is particularly harmful, as it yields the hydroxyl radical, a highly reactive oxidative species with a limited diffusion distance [52].

There are several factors inducing the pathological depositions, and, in general, neuronal death in neurological disorders appears to be multifactorial. However, it is clear that the underlying factor in neurological disorders is increased oxidative stress substantiated by findings showing that protein side-chains are modified either directly by ROS or reactive nitrogen species (RNS), or indirectly by products of lipid peroxidation [53].

The increased level of oxidative stress in AD brain is reflected by an increase of Fe and Cu brain content, both capable of stimulating free radical formation, increased protein and DNA oxidation, enhanced lipid peroxidation and decreased level of cytochrome c oxidase [53].

Various biochemical and physiological characteristics of the mammalian CNS are particularly vulnerable to redox-related damages, such as: I) the strong flux of molecular oxygen in normally-respiring neural tissues, II) excessive generation of ROS by mitochondria in aging post-mitotic neurons, III) susceptibility of CNS to lipid peroxidation accruing from its high cholesterol and unsaturated fat content, IV) the abundance of oxidizable (e.g. dopamine) and potentially-excitotoxic neurotransmitters (glutamate) [20].

Under normal conditions, free radicals are produced in the normal course of intermediary metabolism by some enzyme-catalyzed reactions, involving enzymes such as xanthine oxidase, cyclooxygenase or cytochrome P450, and are rapidly detoxified by defense systems.

Free radicals are molecules containing one or more unpaired electrons, which are responsible for its considerable degree of reactivity. The most important group of radicals

generated in living systems represents the derived from oxygen, including the $O_2^{\bullet-}$ [54]. The superoxide radical, considered as the ‘‘primary’’ ROS, is capable of further interaction with other molecules generating ‘‘secondary’’ ROS. This could be achieved either directly or prevalently through enzyme- or metal-catalyzed processes. The $O_2^{\bullet-}$ itself is not particularly reactive because it does not react directly with polypeptides, sugars or nucleic acids but it is the precursor of much more reactive radicals. The addition of a second electron to $O_2^{\bullet-}$ origins the peroxide ion O_2^{2-}, which is not a radical, but at neutral pH values it will be protonated, producing hydrogen peroxide (H_2O_2):

$$2\ O_2^{2-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{[Equation 1]}$$

The one-electron reduction of H_2O_2, also known as Fenton reaction, produces the highly reactive hydroxyl radical ($^{\bullet}OH$), which can react with a wide number of cellular constituents. The sum of the Fenton reaction (Equation 2) and reduction of Fe^{3+} reaction by superoxide (Equation 3) leads to the production of molecular oxygen,$^{\bullet}OH$ and hydroxyl anion (OH^-) (Haber-Weiss reaction; equation 4) in the presence of catalytic amounts of iron.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \qquad \text{[Equation 2]}$$

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \qquad \text{[Equation 3]}$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^- \qquad \text{[Equation 4]}$$

In addition to ROS, reactive nitrogen species (RNS) are also generated. Immune system cells produce both $O_2^{\bullet-}$ and NO during the oxidative burst triggered by inflammatory processes. Under these conditions, NO and $O_2^{\bullet-}$ may react together to produce significant amounts of peroxynitrite anion ($ONOO^-$), a much more oxidatively active molecule, which is an oxidizing free radical that can cause DNA fragmentation and lipid oxidation [53]:

$$NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^- \qquad \text{[Equation 5]}$$

1.2.1. ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common cause of dementia. It is characterized by a progressive and non-reversible neurological disorder causing cognitive and behavioral impairment. In the first stages of the disease, people experience memory loss and confusion, which may be mistaken with the memory changes that are sometimes associated with natural aging. The symptoms gradually lead to behavior and personality changes, a decline in cognitive abilities (such as decision-making and language skills, and people recognition ability) and motor functions [55].

According to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Association, the diagnosis of AD requires clinical evidence of memory impairment (learn new information or to recall previously learned information) and impairment of at least one other cognitive domain with evidence of disturbance in social or occupational function: loss of word comprehension ability (aphasia); loss of ability to perform complex tasks involving muscle coordination (apraxia); loss of ability to recognize and use familiar objects (agnosia); loss of ability to plan, organize, and execute normal activities) [56] .

Despite all the research performed, the precise physiological changes responsible for trigger the development of AD largely remain unknown. Exceptions are certain rare, inherited forms of the disease, caused by known genetic mutations. Since until today there is no effective prevention or treatment for AD, the increasing prevalence of dementia is a matter of public health and social concern [57].

1.2.1.1. EPIDEMIOLOGY

AD constitutes approximately 70% of all dementia cases [58]. Currently affects over 35 million people worldwide and its prevalence is estimated to nearly double every 20 years, reaching more than 55 million people in 2030 and 115 million in 2050 [59].

Incidence of AD increases with age, doubling every five to ten years: for persons aged 65–69, 70–74, 75–79, 80–84, and > 85 years the incidence of AD has been estimated as 0.6%, 1.0%, 2.0%, 3.3% and 8.4%, respectively [60]. Prevalence also increases exponentially with age, rising from 3% among in 65–74 years old individuals, to almost 50% on those aged 85 or older [60, 61]. The major risk factor for AD is age and the odds of receiving the diagnosis of AD after 85 years of age exceed one in three [62].

According to USA population data, more women than men have AD and other dementias, which is primarily explained by the fact that on average women live longer than men. Some studies on the age-specific incidence of AD or any other dementia have found no significant difference between sex at any given age [63]. Other studies support the theory that females are more susceptible to AD due to higher constitutive activity of the synaptic zinc transporter (ZnT3). Female mice exhibited age-dependent hyperactivity of this transporter, which was associated with increased amyloid-β, the main component of amyloid plaques associated with this disease [64]. Another hypothesis for higher prevalence in woman could be related to post-menopausal oestrogen deficiency, but the mechanism remains unclear [65]. Oestrogen seems to have anti-amyloidogenic effects, neurotrophic, neuroprotective and antioxidant properties.

1.2.1.2. ETIOLOGY

Sporadic AD accounts for approximately 95% of all AD cases and results from a complex array of biomolecular cascades that aggregate to produce cognitive decline, severe memory impairment, and ultimately death. These cascades initially occur in mediotemporal region, before spreading cortically outward to the neocortex [51]. The other 5% of total AD cases are related to genetic causes. This particular form of disease, also known as familial AD, is caused by rare mutations in the genes coding for three proteins – β-amyloid precursor protein (APP), presenilin-1 (PS-1) and presenilin-2 (PS-2) – associated with the abnormal production or clearance of Aβ [66] .

Despite differing in their causes and ages of onset (40–60 years old for the familial AD; ≥ 65 years old for the sporadic AD), their pathophysiological end-products are quite similar [55]. And in both cases, the disease causes progressive loss of memory and thinking ability, mood swings, personality changes and loss of independence.

The cause or causes of AD are not yet known. However, it is agreed that AD, like other common chronic diseases, develops as a result of multiple factors rather than a single cause. Age, family history of dementia, head trauma, environmental factors, genetic factors, female gender, low education level, cardiovascular disease and diet are risk factors assumed to be involved on AD onset [55, 63].

1.2.1.3. PATHOLOGY

Macroscopically, brains of individuals with AD are atrophic, about 8–15% smaller than age-matched controls, with widened sulci and narrowed gyri, due to massive loss of neurons and disruption of synaptic function [55]. It seems to exist a regional susceptibility since the pathological hallmarks of the disease are most frequently found within the association regions of the hippocampus and temporal lobe, which play a key role in formation of new memories [67]. Degeneration of selective hippocampal regions contributes to functional isolation of the hippocampus, resulting in to the marked short-term memory impairment evident in the early stages of the disease [55]. Neuronal damage may also affect the parietal and frontal lobes and the white matter volume is reduced, leading to an enlargement of the temporal horns of the lateral ventricles [68].

The most common and distinctive lesions present within the diseased brain are the senile plaques with β-amyloid peptide and neurofibrillary tangles. These findings are the chosen lesions to confirm the diagnosis of AD at autopsy exam [55]. Widespread oxidative stress, neuroinflammation, calcium dysregulation and mitochondrial malformation and amyloid-β oligomerization are found in AD patients [51]. Neuronal and dendritic loss, neuropil threads, dystrophic neurites, granulovacuolar degeneration, Hirano bodies and cerebrovascular amyloid angiopathy are also typical findings of the AD brain but are less specific findings [55].

β-amyloid (Aβ) peptides are natural products of metabolism consisting of 39 to 43 amino acids cleaved from the C-terminal region of the amyloid precursor protein (APP) by the sequential enzymatic actions of β-site amyloid precursor protein–cleaving enzyme 1 (BACE-1) and γ-secretase [69]. Monomers of Aβ40 are more abundant than the aggregation-prone and neurotoxic Aβ42. An imbalance between production and clearance, and aggregation of peptides causes Aβ accumulation, which can be responsible for the onset of AD. This is is the so-called “amyloid hypothesis”.

Neurofibrillary tangles (NFTs) are filamentous inclusions in pyramidal neurons which main component is an abnormally hyper-phosphorylated and aggregated form of Tau protein. Normally an abundant soluble protein in axons, Tau promotes the assembly and stability of microtubules and vesicle transport. Hyperphosphorylated Tau is insoluble, lacks affinity for microtubules, and self-associates into paired helical filament structures. As Aβ oligomers, intermediate aggregates of Tau molecules are cytotoxic and impair axonal transport and, consequently, cognition [69].

The accumulation of these misfolded proteins in the aging brain results in oxidative and inflammatory damage, which in turn leads to energy failure and synaptic dysfunction [62].

Despite an extensive understanding of each of these phenomena individually occurs within the cell, an explanation for their origin, interaction and evolution as related to AD is lacking.

It is known that oxidative stress undoubtedly plays a critical role, as evidence for its impact at a molecular level exists very early in the disease progression and Aβ confers striking neurotoxicity to neurons and is certainly an important aspect of the disease [70, 71]. Aβ is toxic for mitochondria, affecting particularly the synaptic pool [72]. In AD, exposure to Aβ inhibits key mitochondrial enzymes, as cytochrome c oxidase, in the brain [73]. Consequently, electron transport, ATP production, oxygen consumption and mitochondrial membrane potential all become impaired. The increase in mitochondrial $O_2^{\bullet -}$ formation and conversion into H_2O_2 cause oxidative stress, release of cytochrome c and apoptosis.

Notably, oxidative stress and Aβ accumulation share a common starting factor: reactive transition metal aberration. Increased oxidative stress, the impaired protein-folding function of the endoplasmic reticulum, and deficient proteasome-mediated and autophagic mediated clearance of damaged proteins, all of which are also associated with aging, accelerate the accumulation of amyloid and Tau proteins in AD [74].

1.2.1.4. TRACE ELEMENTS INVOLVEMENT IN ALZHEIMER'S DISEASE

Imbalances in Al, Si, Pb, Hg, Zn, Fe and Cu have been reported in AD, and the latter three are known to be elevated in AD neuropil, suggesting their importance in disease pathogenesis [75]. Furthermore, *in situ* iron detection has revealed a marked association between redox-active Fe and both NFTs and Aβ-rich senile plaques [2]. The association between Aβ and transition metals such as Fe and Cu may also lead to the generation of H_2O_2, exacerbating the oxidative damage [76].

There is evidences suggesting that also altered Cu homeostasis exists in AD and that such alteration can lead to a redox imbalance by altering the functioning of important enzymes like Cu,Zn-SOD, cytochrome c oxidase and ceruloplasmin [57]. Zn is also known to accumulate in high concentrations within the core and peripheral areas of senile plaques such that tissue exposure to metal-selective chelators prevents lesion detection [52].

Differences in brain metal homeostasis between individuals could therefore influence risk of developing AD via a number of routes: such as by increasing Aβ production, by facilitating Aβ aggregation and fibrillation, by altering the availability of metals for

catalyzing essential enzyme reactions, or conversely generating toxic reactive oxygen species [65].

There is evidences suggesting that altered Cu homeostasis also exists in AD and that such alteration can lead to a redox imbalance by altering the functioning of important enzymes like Cu,Zn-SOD, cytochrome c oxidase and cerulopasmin [57]. Zn is also known to accumulate in high concentrations within the core and peripheral areas of senile plaques such that tissue exposure to metal-selective chelators prevents lesion detection [52].

Differences in brain metal homeostasis between individuals could therefore influence risk of developing AD via a number of mechanisms: increasing Aβ production, facilitating Aβ aggregation and fibrillation, altering the ability of metals for catalyzing essential enzymatic reactions; or conversely generating toxic reactive oxygen species [65].

1.2.1.4.1. COPPER HOMEOSTASIS IN ALZHEIMER'S DISEASE

Evidence suggests that AD development involves an altered Cu homeostasis. Some studies support the idea that Cu participates in the development of AD as a noxious metal, since high levels of Cu have been found in amyloid plaques from AD patients [75, 77]. Other studies suggest that Cu may prevent both the formation and the accumulation of βA plaques *in vitro*, reducing amyloidogenesis [78, 79]. In the transgenic murine model of AD, exposure to Cu in the drinking water produced the exacerbation of Aβ and Tau pathologic consequences [80], suggesting that Cu may be influencing not only the senile plaque, but also the neurofibrillary tangles. It should be noted that the Aβ involved in the pathogenesis of AD show high affinity for Cu [81]. Cu binding promotes Aβ toxicity through the formation of H_2O_2 and the subsequent generation of free radicals through the Fenton reaction [82, 83] involving Cu (II) reduction by Aβ [76, 82]. Consequently, a cascade of events related to oxidative stress, ROS production and subsequent neuronal death occurs (Figure 4). It has been reported that the Aβ inhibits the cytochrome c oxidase complex of the mitochondrial electron transport chain and other studies indicate that this inhibition may be further increased by the presence of Cu (II) ions. This effect may be due to the ability of the Aβ-Cu complex to generate H_2O_2 and also to the formation of an intermediate reactive product that interacts with cytochrome c oxidase [84].

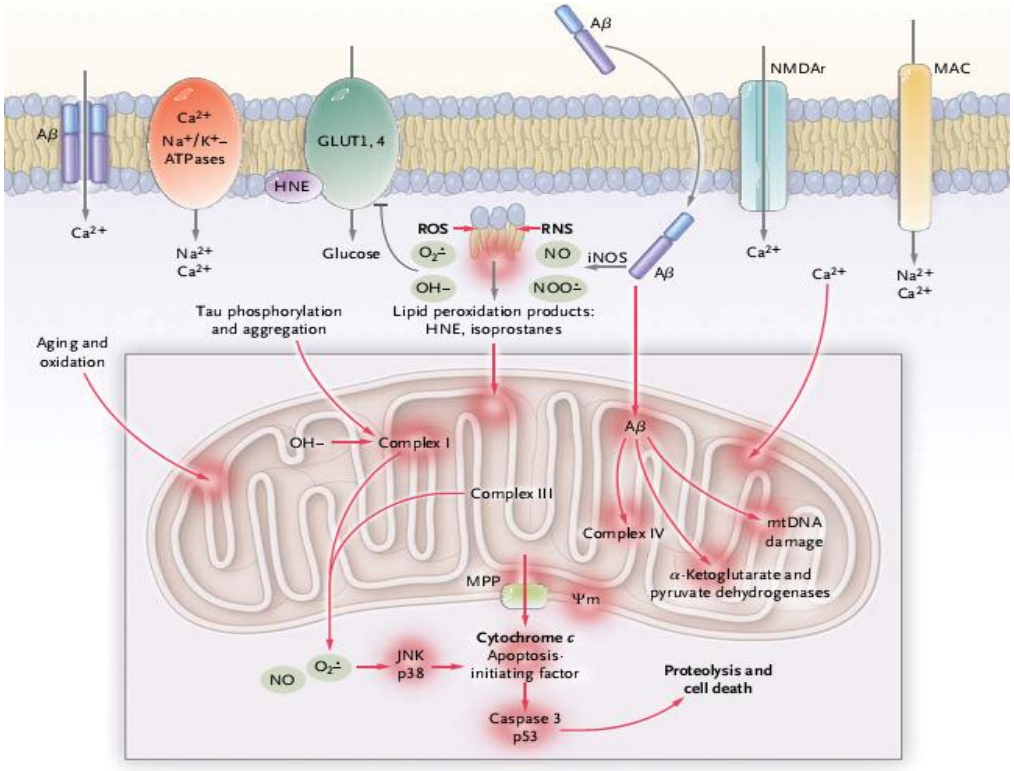

Figure 4: Oxidative stress and mitochondrial failure [69].

Aβ is linked to ROS and RNS production. Their peroxidative attack on cell and organelle membrane lipids yields mitochondrial toxins such as hydroxynonenal (HNE) and malondialdehyde. Oxidative damage to membrane-bound, ion-specific ATPases and stimulation of calcium (Ca^{2+}) entry mechanisms cause cytosolic and mitochondrial Ca^{2+} overload. Cellular Aβ directly attacks electron transport complex IV (cytochrome c oxidase) and key tricarboxylic acid cycle (α-ketoglutarate and pyruvate dehydrogenase) and damages mitochondrial DNA (mtDNA), leading to its fragmentation. Lipid peroxidation products also promote Tau phosphorylation and aggregation, which in turn inhibit complex I. High amounts of ROS and RNS are generated at complexes I and III. As the mitochondrial membrane potential (MPP) collapses and permeability-transition pores (ψm) open, caspases are activated. Aβ also induces the stress-activated protein kinases p38 and c-jun. Substrate deficiencies, notably NADH and glucose, combine with electron transport uncoupling to further diminish ATP production.

In glutamatergic neurotransmission Cu is co-released with glutamate and substantial amounts of Cu ions, susceptible to bind to Aβ (coming from the action of secretases on APP), can be found in the synaptic space [85], enhancing Aβ oligomerization and precipitation and consequently the formation of senile plaques (Figure 5).

Although its function is not completely known, it has been suggested that APP-Aβ may act as a Cu carrier system since APP knockout mice showed an increased Cu content in the cortex as compared to wild-type animals [86], what could explain why Cu deficiency down-regulates APP transcription [87] and the Aβ's high affinity for copper [81]. Cu also interacts with APP in an electron transfer reaction that reduces Cu (II) to Cu (I), enhancing the production of hydroxyl radical [77].

In contrast, evidence supporting a Cu deficiency in AD is based on findings showing Cu concentration in the cerebrospinal fluid (CSF) from AD patients inversely correlated to Aβ [88]. In addition, it has been demonstrated that Cu deficiency can elevate βA peptide secretion by either influencing APP cleavage or inhibiting its degradation [89].

Cu deficiency may also influence the activity of Cu-binding proteins in AD. It has been observed a reduced Cu,Zn-SOD activity in CSF from AD patients when compared to controls, as well as the activity of cytochrome c oxidase [90]. Ceruloplasmin, a multicopper ferroxidase necessary for the oxidation of Fe^{2+} to Fe^{3+} and subsequent binding of Fe to transferrin, could be an important factor in AD because in this protein converge both Cu and Fe homeostasis [57]. However, there are conflicting reports in the literature. Some studies report significantly increased Ceruloplasmin levels in most brain regions of AD patients compared to elderly controls [91], whereas, decreased levels were found in temporal cortex [57]. Serum levels of both Cu and Ceruloplasmin were significantly higher in AD patients and both Ceruloplasmin ferroxidase and Cu,Zn-SOD activities have a tendency to be decreased in CSF when compared with controls [92]. Cu deficiency also results in higher ROS production and oxidative damage to proteins.

Although many questions remain unanswered, it is widely accepted that in AD there is an abnormal brain Cu distribution with accumulation in amyloid plaques and a deficiency of Cu in adjacent cells [78] but further investigations are needed to fully understand its dual role in AD. All the evidence suggests that altered Cu homeostasis is present in AD and that such alteration can lead to a redox imbalance by altering the functioning of important enzymes, namely Cu,Zn-SOD and Ceruloplasmin.

1.2.1.4.2. IRON HOMEOSTASIS IN ALZHEIMER'S DISEASE

Fe is involved in the pathophysiology of AD as suggested by its presence in senile plaques and neurofibrillary tangles of AD patients brain [75]. Fe has been associated with free radical generation through Fenton reaction, leading to the formation of the highly reactive hydroxyl radical ($^{\bullet}OH$) which damages cell molecules such as membrane lipids, proteins and nucleic acids. Hydroxyl radical also seems to contribute to Aβ aggregation by promoting covalent binding between peptide monomers [93]

.

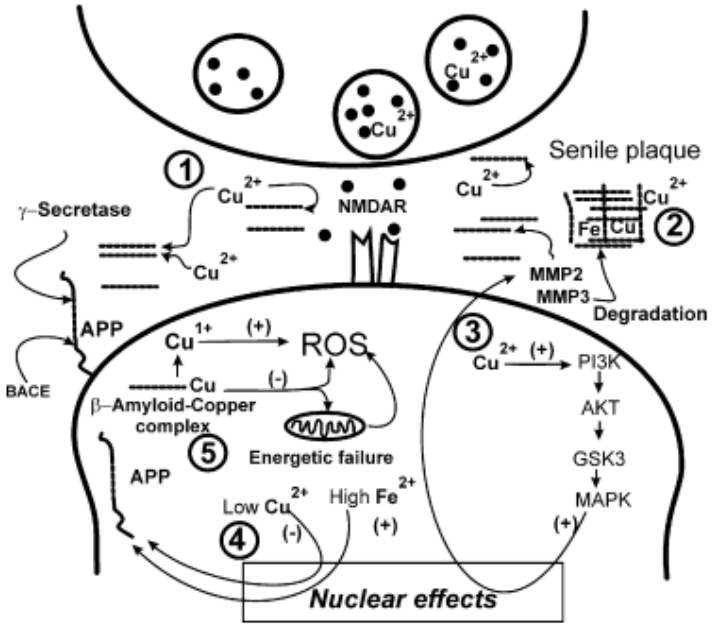

Figure 5: Cu and Fe homeostasis in Alzheimer's disease [57].

(1) Cu ions in the synaptic space are co-released with glutamate. Aβ, produced from APP by secretases, has high affinity for Cu. Aβ and Cu ions coincide in synaptic space, possibly inducing the precipitation of Aβ. (2) Cu is suspected to consolidate the formation of senile plaques by catalyzing new covalent bonds among polypeptides. Cu is found in high quantities in amyloid plaques. (3) Intracellular Cu is involved in the expression of matrix metalloproteinase responsible for the cleavage of Aβ. (4) High levels of Fe are involved in increased expression of APP through an Iron Response Element (IRE), whereas depletion of Cu decreases the expression of this protein. (5) Cu binding to Aβ increases the production of free radicals by itself and by inhibition of mitochondrial respiratory chain.

The role of Fe in this disorder is supported by studies linking hereditary hemochromatosis to AD [94] and the presence of an Iron Responsive Element in the untranslated region of the APP gene [95]. Additionally, studies show that ferritin is also present in the brain senile plaques of patients with AD [57]. It was also found a positive correlation between Fe levels in the hippocampus and mental state of AD patients, supporting an important role for Fe in this ND [96].

1.2.2. PARKINSON'S DISEASE

Parkinson's disease (PD) is a neurodegenerative brain disorder that progresses slowly in most people. It is characterized by motor manifestations (e.g., tremor, bradykinesia and muscle rigidity) but cognitive and behavioral abnormalities also occur [97, 98]. Aside from classical motor deficits, various non-motor symptoms including autonomic dysfunction, sensory and cognitive impairments as well as neuropsychiatric alterations, pain and sleep disturbances are common in PD. Some of these non-motor symptoms can even manifest before motor problems [99].

1.2.2.1. EPIDEMIOLOGY

PD is the second most common neurodegenerative disease after Alzheimer's disease, affecting 7 to 10 million people worldwide [100]. PD has a prevalence of 0.5-1% among people older than 65 years of age which increases to 1-3% in the population over 80 years of age [101]. Incidence of PD increases with age, but an estimated 4% of people with PD are diagnosed under 50 years of age [100].

Statistics indicate that PD seems to affect man at a higher rate (1,5 times higher) than woman [100]. Possible reasons for this increased PD risk in man are toxicant exposure, head trauma, neuroprotection by oestrogen, mitochondrial dysfunction or X-linkage of genetic risk factors [15].

1.2.2.2. ETIOLOGY

Although the etiology of this selective neurodegeneration is still unknown, several genetic and environmental factors such as occupational exposure to transition metals and pesticides, have been implicated on the development of PD [97, 102, 103].

Several studies support the role of brain transition metal accumulation in the pathophysiology of PD that may be independent of the environmental exposure, suggesting that metal homeostasis is altered in PD [57].

1.2.2.3. PATHOLOGY

The pathology of PD is not fully understood. Between the fifth and the ninth decade of life the number of nigral cells in normal brain is reduced by 4.7-6% per decade, but this is not sufficient to cause PD itself [104].

Macroscopically, the SN and the locus coeruleus of PD brains are pale, reflecting loss of the normal pigment-bearing neurones. Microscopically, in addition to neuronal loss, large eosinophilic neuronal inclusions (Lewy bodies) can be seen displacing neuromelanin in these regions [68]. At the cellular level, severe loss of dopaminergic nerve cells in the substantia nigra pars compacta (SNpc) in the midbrain region and in the striatum (caudate nucleus and putamen) is linked with PD [105]. When manifestations of PD are fully developed, around 80% of dopaminergic neurons in the SN are already lost, resulting in reduced synthesis and release of DA from the striatal nerve terminals [106].

The specific death of dopaminergic neurons in SNpc has also drawn attention towards a melanin like pigment, neuromelanin (NM), present in these cells. NM can bind metal ions, thus playing a protective role against cellular damage resulting from oxidative processes, which is compromised in PD, leading to death of dopaminergic cells in SN [105].

Besides dopamine neuronal degeneration, PD is characterized by cytoplasmic intrusions in some surviving nigral dopaminergic neurons, the Lewy bodies. The principal protein component of the Lewy bodies is α-synuclein, a small cytosolic protein ubiquitously expressed throughout the brain. Recent studies have shown that α-synuclein is able to interact with Zn, Cu and Fe, and that these interactions lead to protein aggregation and crosslinking [107].

1.2.2.4. TRACE ELEMENTS IN PARKINSON'S DISEASE

The scenario of the PD is still not entirely clear, but a consensus is rising on oxidative injury co-factor of the neurodegenerative process. In this context, metals cannot be disregarded because of their key role in the intracellular oxidative equilibrium both as pro-oxidant agents and as part of the antioxidant enzymes.

In fact, the SN of PD subjects showed a high content of Fe, which is considered to catalyze, via Fenton reaction, the production of free radicals and to increase the rate of dopamine auto-oxidation, as well as the deposition of intracellular inclusion bodies, both characteristic features of the disease [57]. Cu and Mn can participate in the same reaction and displace Fe from its complex with NM, thereby increasing free Fe in the cytoplasm [108]. Although the neurotoxic potential of Fe has been consistently reported, evidence of Cu toxicity is not completely conclusive [57].

Zn is also reported to be increased and Cu decreased in SN of PD patients. In addition, Al, Cd, Cr and Hg are able to increase the susceptibility to the oxidative damage or interfere with detoxifying enzymes.

1.2.2.4.1. Iron HOMEOSTASIS in Parkinson's disease

Post-mortem analysis of PD brains showed total Fe levels to be increased in SNpc but not in cerebellum, caudate nucleus, putamen or cerebral cortex [109], suggesting that the underlying mechanisms of Fe accumulation maybe specific for SNpc. In contrast, levels of this metal were found reduced in globus pallidus [57]. However, non-significant difference in Fe content has been found in brain tissue showing moderate neurodegeneration [94];

thus, it could be considered that Fe accumulation in PD may be the consequence of the underlying mechanisms of neuronal death. Otherwise its levels should be expected to be increased since the early stages of the disorder; thus, some mechanisms may initiate neuronal death at the early stages of the disorder and lead to Fe accumulation that may potentiate oxidative damage [57].

Brain Fe levels increase during normal ageing and it is associated with a reduced motor performance [110]. Although Fe accumulation in PD is not explained by ageing itself, ageing seems to modulate Fe neurotoxicity [57]. Adult mice fed with Fe during the neonatal period showed reduced striatal dopamine levels while young mice have unchanged dopamine levels following the same treatment [97]. However, although brain Fe accumulation in young animals is not likely to produce neuronal death by itself, early postnatal Fe administration potentiates dopamine depletion during adulthood, suggesting that Fe accumulation in young animals may lead to neurotoxicity [111].

Fe brain levels in PD are higher than the expected by normal ageing and the underlying mechanism for such accumulation remains to be elucidated. Excitotoxicity may be involved in PD and may lead to Fe accumulation. Fe uptake is enhanced by N-Methyl-D-Aspartate (NMDA) receptor activation through NO signaling [112]. Both glutamate and NO are involved in excitotoxic death suggested in PD (Figure 6); thus, Fe accumulation in this disorder may be associated with neuronal death through glutamate receptors [112].

Since Fe levels are increased in PD, it can enhance neuronal death through oxidative stress, induce lipid peroxidation and increase ROS production [113, 114]. Fe also stimulates the formation of intracellular aggregates of α-synuclein and promotes oxidative damage. It is known that autosomal dominant PD is related to mutations in α-synuclein that enhance aggregation of the protein; therefore, those individuals with mutations in α-synuclein could be more susceptible to Fe-induced oxidative damage [57, 115].

Several of the regulators of intestinal Fe absorption have also been localized in CNS, suggesting a role for these transporters in brain Fe uptake. Among them, DMT1 and ferroportin (IREG1) has been identified. In addition, studies report ferroportin in pre-synaptic vesicles of the striatum, thalamus, brain stem, cerebral cortex and cerebellum, suggesting that Fe is transported into vesicles by ferroportin and released as free Fe into the synaptic cleft [116].

1.2.2.4.2. COPPER HOMEOSTASIS IN PARKINSON'S DISEASE

Several studies have shown that Cu may lead to both toxic and protective effects, depending on the experimental conditions. Since brain Cu content has been reported to be decreased in PD [117].

Since its concentration has been found altered in the brain and CSF from PD patients, Cu has been implicated in the pathophysiology of the disease [118]. Several studies found reduced Cu levels in SNpc and caudate nucleus, but not in cerebellum, globus pallidus, putamen or dorsolateral prefrontal cortex from PD patients [91, 119]. In post-mortem study, divergent results were found: Cu content was shown to be significantly higher in the reticular formation in PD cases [119]. Concerning Cu levels in CSF, while total Cu concentration is not changed in PD patients compared to controls, free Cu is increased and positively correlated to both disease severity and duration. [120]. It is possible that free Cu leads to oxidative stress and neuronal death in PD through Fenton reaction; also, free Cu may be increased due to the uncoupling from its binding sites in antioxidant proteins (Ceruloplasmin and SOD), leading to oxidative stress.

The neuroprotective effect of Cu may be related to Fe transport modulation (Figure 6). Cu reduces Fe uptake possibly through neuronal DMT1 [121]. Decreased DMT1 expression is associated with neuronal survival following Fe overload [122]; thus, an inhibitory effect of Cu on Fe uptake is also expected to be neuroprotective. Both oral and intracerebroventricular Cu administration are neuroprotective against dopaminergic degeneration; oral Cu administration may lead to this effect by decreasing Fe levels since Cu competes with Fe for intestinal absorption [121], thus decreasing Fe uptake, and consequently, the brain content of this metal.

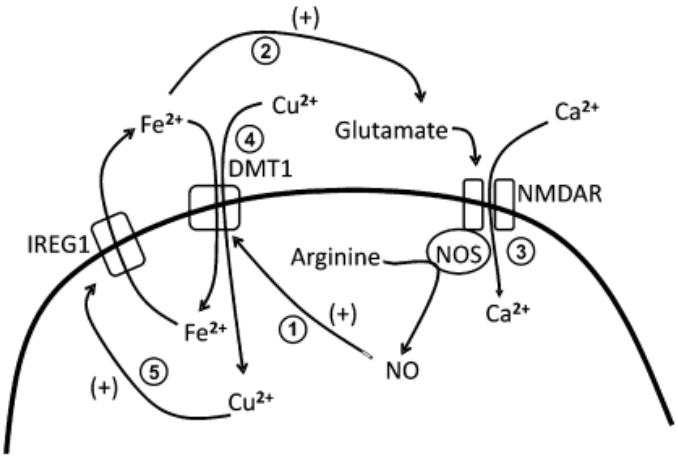

Figure 6: Reciprocal modulation of Fe and Cu and its further association with excitotoxicity in PD. [57].

(1) Glutamate increases Fe uptake through a NO-dependent mechanism involving DMT1. (2) Fe may increase glutamate release. (3) Excessive glutamate induces excitotoxicity through a NMDA-mediated mechanism. (4) Cu can protect neurons against Fe overload by competing for transport through DMT1 and (5) favoring Fe efflux through IREG1.

Modulation of Fe transport through ferroportin (IREG-1) may also be involved in the neuroprotective effect of Cu since this protein mediates Fe efflux in neurons and astrocytes [121, 122]. Increased ferroportin expression is associated with neuronal survival after Fe overload [121]. Cu-deficient diets reduce ferroportin expression in the rat liver [123], possibly leading to Fe accumulation. In patients with non-alcoholic fatty liver disease, low hepatic Cu content is associated with a decreased ferroportin expression, contributing to Fe accumulation in those patients [123]. According to those studies, Fe accumulation may be the consequence of Cu deficiency.

1.2.3. AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurological disease with unknown pathogenesis, whose rapid progress leads to paralysis and premature death.

The diagnosis of ALS usually is based on clinical and neurological examination which confirms the presence of symptoms characteristic of the combination of upper and lower motor neurons degeneration in different anatomical regions of the body (bulbar, cervical, thoracic, lumbar regions) [124]. The clinical symptoms of the disorders, such as muscle atrophy, weakness or fasciculations, spasticity, speech and swallowing disturbances are used for diagnostic procedure [125]. However, appearance of these symptoms reflects a far gone neurodegenerative process with significant degeneration of neurons [124].

1.2.3.1. EPIDEMIOLOGY

ALS is one of the most common neurodegenerative disorder, with an incidence of 4-6 per 100 000 [15]. The onset of disease usually occurs between 50 and 70 years of age but it may occur from the teenage years to the late 80's [124].

Most cases of the disease have no genetic linkage (sporadic form) while familial amyotrophic lateral sclerosis (FALS) showing dominant autosomal inheritance only represents 5-10% of the cases [57]. Mean age of onset of sporadic ALS is 65 years and mean age of onset of familial ALS is 46 years [126]. Over 110 FALS-linked mutations throughout the Cu,Zn-SOD gene are related to approximately 10-20% of the FALS cases [57]. In FALS, Cu,Zn-SOD acquires a toxic function as demonstrated by transgenic models showing that mice overexpressing the human mutant enzyme G93A exhibit features of ALS [127] while those lacking the enzyme do not develop the disease [57] .

The incidence of ALS is 1.5 to 2 times higher in men than in women. After 65-70 years of age, incidence seems to be equal for both genders [126] .

1.2.3.2. ETIOLOGY

Beside the genetic mutations in Cu,Zn-SOD associated to FALS, until today the etiology of ALS remains unknown. However, some risk factors have been identified: age at menopause (women), diet, family history of ALS or other ND, smoking, previous poliomyelitis infection [126]. Most authors support the hypothesis of a complex genetic-environmental interaction as the causal factor for motor neuron degeneration in ALS.

1.2.3.3. PATHOLOGY

Clinically, ALS is characterized by the progressive loss of motor neurons of the anterior horns in the spinal cord, bulb and cortex [57].

The exact molecular pathway causing motor neuron degeneration in ALS is unknown, but as with other ND, it is likely to be a complex interplay between multiple pathogenic cellular mechanisms which may not be mutually exclusive. These include: excitotoxicity, oxidative stress, mitochondrial impaired axonal transport, neurofilament, protein aggregation and inflammatory dysfunction [126].

Cytoplasmic aggregates such as Bunina bodies and Lewy body-like inclusions have been found in motor neurons from both sporadic and familial ALS patients and in transgenic mice models of ALS. The association between Cu,Zn-SOD mutations and FALS suggest that oxidative injury is involved in this disorder [57].

1.2.3.4. Trace Elements in Amyotrophic Lateral Sclerosis

1.2.3.4.1. Iron homeostasis in Amyotrophic Lateral Sclerosis

It has been suggested that Fe accumulation may be due to increased uptake of the metal, since lactoferrin [57] is also increased in motoneurons affected by ALS. The increased spinal cord Fe levels may be involved in oxidative damage through the Fenton reaction [128].

The prevalence of hemochromatosis (HFE) gene mutation in ALS patients supports the involvement of Fe in this disorder [129]. HFE gene interacts with TfR to sense Fe levels. HFE gene mutations are associated with a Cu,Zn-SOD decreased expression, suggesting that polymorphisms in ALS are associated an altered Fe homeostasis and, consequently, with oxidative damage [130].

1.2.3.4.2. Copper homeostasis in Amyotrophic Lateral Sclerosis

Since Cu and Zn are constituents of Cu,Zn-SOD, altered levels of these metals have been associated with ALS.

Altered Zn and Cu levels could be the consequence of structural changes in Cu,Zn-SOD. The FALS Cu,Zn-SOD proteins can be divided into two groups according to their metal content [131] and the position of the specific mutation: metal content in wild-type-like (WTL) mutant SOD is nearly identical to that found in the wild-type protein, whereas mutations at the metal-binding region (MBR) or at the electrostatic and Zn loop elements [132] lead to a deficiency in Zn and Cu content [131]. WTL Cu,Zn-SOD mutants show high reactivity with hydrogen peroxide and produce site-specific oxidative damage to the MBR, compromising metal binding, while MBR mutants appear to aggregate with no further modification. Hence, the aggregation of both types of mutants may involve metal uncoupling [132, 133].

Evidence suggests that Cu,Zn-SOD stability is dependent on its metal-binding state. Some hypotheses hold that the balance between normal and toxic Cu,Zn-SOD functioning depends on Zn binding at the active site of the enzyme (Figure 7); experimental models have shown that in the absence of Zn the catalytic reaction of Cu,Zn-SOD runs backwards, producing ROS [134].

It has been observed that even wild-type human Cu,Zn-SOD, in its metal-free state, may form large, stable, soluble, amyloid-like protein oligomers under relatively mild conditions,

suggesting that the gain of a toxic Cu,Zn-SOD function in ALS may be related to the inability of this protein to achieve or maintain the metallated state [135].

The loss of Cu and Zn from Cu,Zn-SOD facilitates the reduction of the intersubunit disulfide bound between two residues at the Zn loop, leading to the dissociation of subunits and promoting the formation of insoluble aggregates [136]. Cu liberated from Zn deficient SOD could potentially initiate apoptosis (Figure 7).

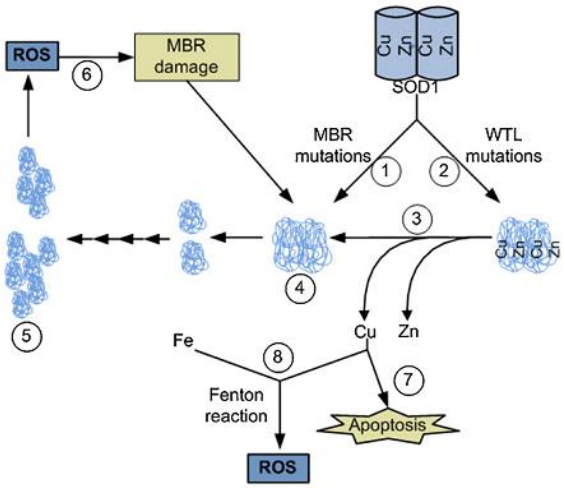

Figure 7: The role of SOD metal uncoupling in ALS [57].

SOD mutations can be 1) MBR-mutations) or 2) WTL-mutations. MBR-mutant SOD has an altered metal content, while WTL-mutant SOD conserves its normal metal content. WTL-mutant SOD is susceptible to oxidative damage, compromising metal binding (3). Therefore, both types of mutations can conduce to alterations in SOD structure (4), involving protein aggregation (5), gain of toxic function, oxidative damage and apoptosis. ROS generated by dysfunctional SOD can potentiate damage to MBR (6). Cu released from SOD could be implicated in apoptosis (7). Additionally, Cu and Fe can promote ROS generation through Fenton reaction (8).

1.3. TRACE ELEMENTS ANALYSIS

As previously referred, TE are present in biologic matrices at so low concentrations (10 µg/L to 10^4 µg/L) that they could not be detected at the beginning of the development of instrumental methods of analysis. However, the remarkable development of analytical technologies observed in last decades made possible a reliable quantification of these chemical species and contributed to the current understanding of the importance of trace elements in human health and disease. The development of increasingly sensitive analytical methods based on atomic spectrometry has had a major influence on the establishment of reference intervals for both essential and toxic elements in human populations.

One of the first important contributions of atomic spectrometry to clinical chemistry was the introduction of the flame photometer, based on atomic emission spectrometry, which significantly improved sodium and potassium determination [135]. Later, the development of atomic absorption spectrometry with flame atomization allowed faster determinations of several important metals (e.g., Ca, Mg, Fe, Cu, Zn), with greater accuracy and precision, in small sample volumes. However, the range of analyzable metals was reduced due to its poor sensitivity (high detection limits). This limitation was overtaken in the 1970s with the introduction of the graphite furnace as atomization system, which greatly improves the efficiency of the atomization process [136]. The development of absorption spectrophotometry with graphite furnace (or electrothermal) atomization allowed to decrease by about 10 to 100 times the limits of detection for most metallic elements, making possible its quantification at the parts per billion (ppb) level [137] .

1.3.1. ATOMIC ABSORPTION SPECTROMETRY

In flame atomic absorption spectrometry (FAAS), an air/acetylene or a nitrous oxide/acetylene flame is used to evaporate the solvent and dissociate the sample into its component atoms. When light from a hollow cathode lamp (characteristic of the element to be determined) passes through the cloud of atoms, the atoms of interest absorb the light from the lamp.

Each element absorbs its own characteristic wavelength of light, but a monochromator is needed to isolate the particular wavelength of interest from other wavelengths emitted at the flame. The detector is a photomultiplier tube which converts the stream of photons into an amplified electrical signal which can be easily measured and used to calculate the element concentration in the original sample (solution). The use of a flame limits the

excitation temperature to a maximum of approximately 2600 °C (with the N_2O/acetylene flame) with detection limits in the sub-ppm range [138].

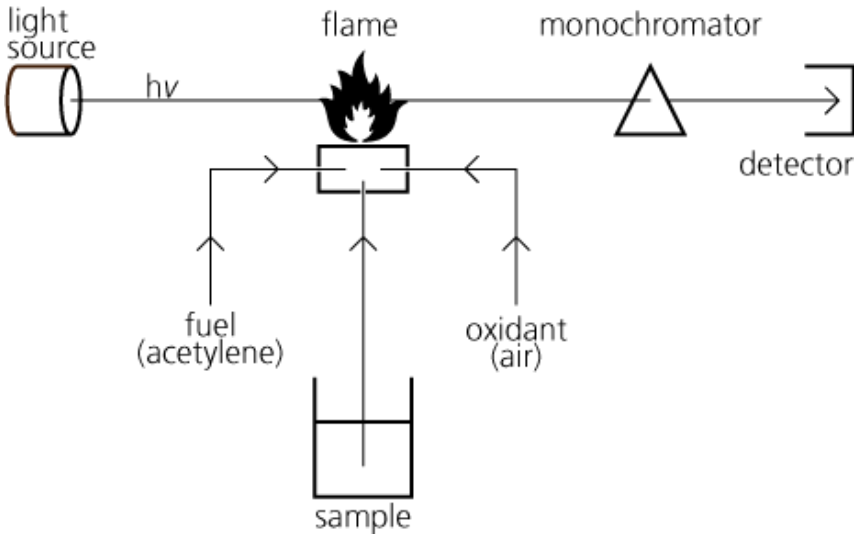

Figure 8: Diagram of a flame atomic absorption spectrometer [139].

Graphite furnace atomic absorption spectrometry (GFAAS) is essentially similar to FAAS, however the flame is replaced by a small, electrothermally heated graphite tube, which reaches temperatures up to 3000°C to generate the cloud of atoms. The higher atom density and longer residence time of the atoms in the tube when compared with the flame improve detection limits by a factor of up to 1000x, which decrease down to the sub-ppb range [138].

The heating program in GFAAS comprises four main steps: 1) drying (solvent evaporation), performed at temperatures around 100 ºC–120 ºC; 2) pyrolysis, performed at about 1000 ºC, which volatilizes the organic and several inorganic matrix compounds (but not the analyte); 3) atomization (ca. 2000 ºC), which leads to the production of free gaseous atoms of the analyte of interest, in the ground state, so that the light absorption by the cloud of atoms can be measured; and cleaning, at high temperatures (2500 ºC), to remove all the residues from the graphite tube.

GFAAS is sensible to matrix interferences, i.e., interferences caused by matrix components that inhibit formation of free analyte atoms during the atomization step. This problem can be minimized by using chemical “matrix modifiers”, which either stabilize the analyte or react with the interfering compounds, leaving the analyte free for quantification.

1.3.2. Inductively Coupled Plasma – Mass Spectrometry

Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) is a multi-element analytical technique that uses a plasma source to dissociate the sample into its constituent atoms. Due to the very high temperature of the plasma (6000-10,000 ºC), a significant fraction of the atoms are converted into monovalent positive ions. These ions are then separated and detected.

The sample (solution) is introduced into the plasma as an aerosol, by aspirating it through a nebulizer. The larger droplets are removed and the remaining reaches the plasma (produced at the ICP torch). Here they are completely desolvated and the elements present in the sample are converted first into gaseous atoms and then, by losing and electron, ionized to monovalent cations. These positive monovalent ions are then forwarded to the mass spectrometer via the interface cones, where they are separated by their mass-to-charge ratio. In the most common instruments this separation is made with a quadrupole (see Fig. 9) [138]. The high number of ions produced, combined with the very low background signal, provides the best detection limits available for most elements, typically in the parts-per-trillion range.

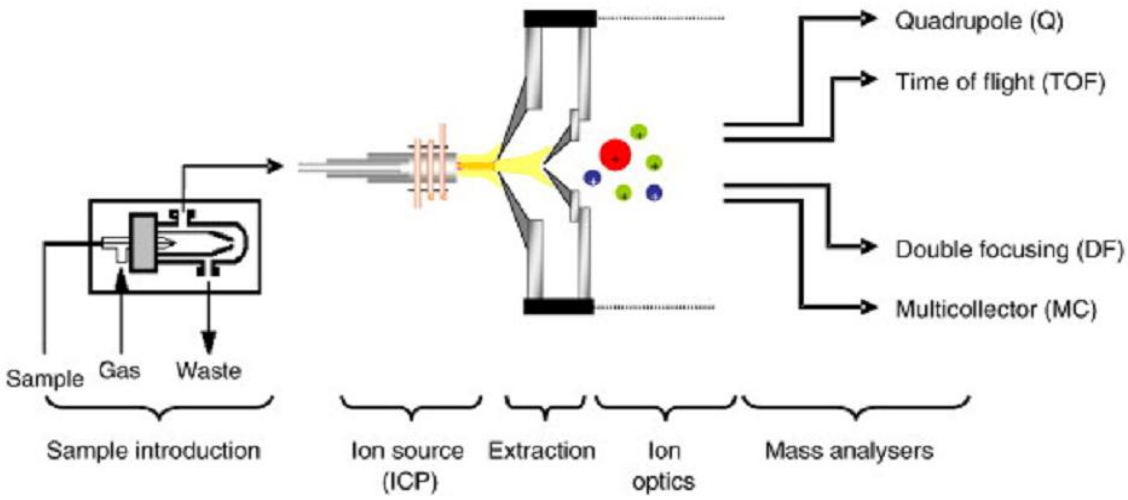

Figure 9: Diagram of ICP-MS instruments [140].

However, ICP-MS is not free of limitations. In clinical samples (blood, serum, urine), the high concentration of organic and inorganic constituents often results in matrix interferences and/or spectral interferences from polyatomic ions. Moreover, the biological matrix can clog the nebulizer and the interface cones during long analytical runs. A combination of sample dilution, specialized nebulizers and low aspiration flow rates can generally reduce clogging of the nebulizer and matrix deposition on the plasma torch and sampler/skimmer cones [136].

Today, because of its much lower acquisition and operation costs, GFAAS continues to be the technique of choice for single trace element analysis [136]. For multielement analysis ICP-MS is the most suitable technique because of its rapidity, low detection limits and low sample volume required for analysis.

II. OBJECTIVES

OBJECTIVES

For a better understanding of the disease-related changes of TE levels in human brain and their potential role in neurodegenerative processes, a previous knowledge of their physiological (normal) distribution, i.e., their distribution within the brain of healthy subjects is essential.

However, most of the current information about the relationship between TE and human brain functioning is an extrapolation based on animal studies or relies on determinations in cerebrospinal fluid and blood.

On the other hand, it is well known that ND are strongly age-related and there is a specific region of the CNS that seems to be particularly affected in each disease

In this context, the main objectives of our research work were to study, directly in human brain samples:

1) the regional anatomic differences of TE levels within the brain;

2) the changes on TE levels in relation to age; and

3) the differences in TE levels between individuals with and without evidence of ND.

III. EXPERIMENTAL RESEARCH

EXPERIMENTAL RESEARCH

3.1. MATERIAL AND METHODS

3.1.1. LABORATORY WARE

Glass and metal materials were avoided in order to reduce the risk of contamination. Whenever possible, polypropylene made laboratory ware (pipette tips, volumetric flasks, tubes, autosampler cups) was used.

All material was previously washed with 10% (v/v) HNO_3 and rinsed with ultrapure water (resistivity > 18.2 MΩ cm at 25 °C) obtained by de-ionization with mixed bed ion-exchange resins and further purification in a Milli-Q RG water-purification system (Millipore, Italy).

3.1.2. SUBJECTS

This study was conducted in brain samples from individuals (n=44) submitted to medico-legal autopsy exam at *Instituto Nacional de Medicina Legal e Ciências Forenses, I.P.* – Delegação do Norte (INMLCF, I.P.) during first semester of 2012.

Brain samples were collected from individuals submitted to medico-legal autopsy by the cooperating forensic pathologists at INMLCF, I.P. Only individuals with a post-mortem interval not superior to 72 hours were considered. In order to fulfill all current legal regulations regarding human tissue collection for scientific research purposes, it was assured that the name of the individual was not in Registo Nacional de Não Dadores (RENNDA) database.

Two groups of individuals were studied:

Group A – "Normal" individuals. Clinical criteria were used for case selection. This was based on the absence of neurodegenerative, neurological or psychiatric disorders history and/or injuries involving CNS before death and macroscopically normal tissues.

Individuals from this group were divided in the following age sub-groups: 20-30 (n = 2), 50-60 (n = 10); 60-70 (n = 10); 70-80 (n = 10); 80-90 (n = 9) and >90 (n = 3) years old.

Group B – Individuals with evidence of AD (n = 2), PD (n = 1) and ALS (n = 1).

Clinical inclusion criteria were defined and case selection was an absence of neurodegenerative, neurological or psychiatric disorders history and/or injuries involving the central nervous system before death and macroscopically normal tissues. Medical status was evaluated on the examination of medical files and the standard questionnaires to relatives. Individuals with autopsy findings involving brain hemorrhage (e.g. hemorrhagic cerebrovascular accident, head trauma, brain aneurysm rupture) were not included in this study.

3.1.3. SAMPLE COLLECTION

After removal of the brain from the cranium, the excess of blood was cleansed with ultrapure water. Meninges were removed with plastic tweezers and the brain was washed again to minimize contamination from blood or cerebrospinal fluid.

Since there are studies showing no statistical significant differences between brain hemispheres [141], the right one was sampled for systematic reasons. (Exceptionally, due to non-recent stroke areas on right hemisphere, the left one was sampled in some subjects, as described in Attachment 1).

Using a long-bladed knife, a single horizontal cut was made through the midbrain at the level of the superior colliculus to the origin of the third cranial nerve, separating the brainstem and cerebellum from the cerebral hemispheres. The cerebellar hemispheres were separated from the brainstem by cutting through the superior, middle and inferior cerebellar peduncles, and sliced in the coronal plane. Starting at the frontal pole, the hemispheres were sectioned at 1 cm intervals in the coronal plane.

As suggested by Paine and Lowe [68], samples (approximately 1 cm^3) were collected at the following brain areas (Figure 10): (1) frontal cortex; (2) (A) superior and (B) middle temporal gyri; (3) the basal ganglia including (A) caudate nucleus, (B) putamen and (C) globus pallidus; (4) cingulated gyrus; (5) hippocampus; (6) inferior parietal lobule; (7) visual cortex of the occipital lobe; (8) midbrain, including the SN to the level of the third nerve; (9) pons – locus coeruleus; (10) medulla and (11) cerebellum – dentate nucleus.

Samples were collected with plastic knives and stored in polypropylene tubes. After transport to laboratory in refrigerated conditions, the samples were stored at -4 ºC until analysis.

Note: To reduce the risk of contamination, all stainless steel material (long-bladed knife and scalpel) used by INMLCF cooperating forensic pathologists and all the material that contacted with the sample during collection procedure (teflon board, plastic knives, tweezers, polypropylene tubes) was previously decontaminated by immersion during 24 hours on 10% (v/v) HNO_3 solution followed by thorough rinsing with ultrapure water.

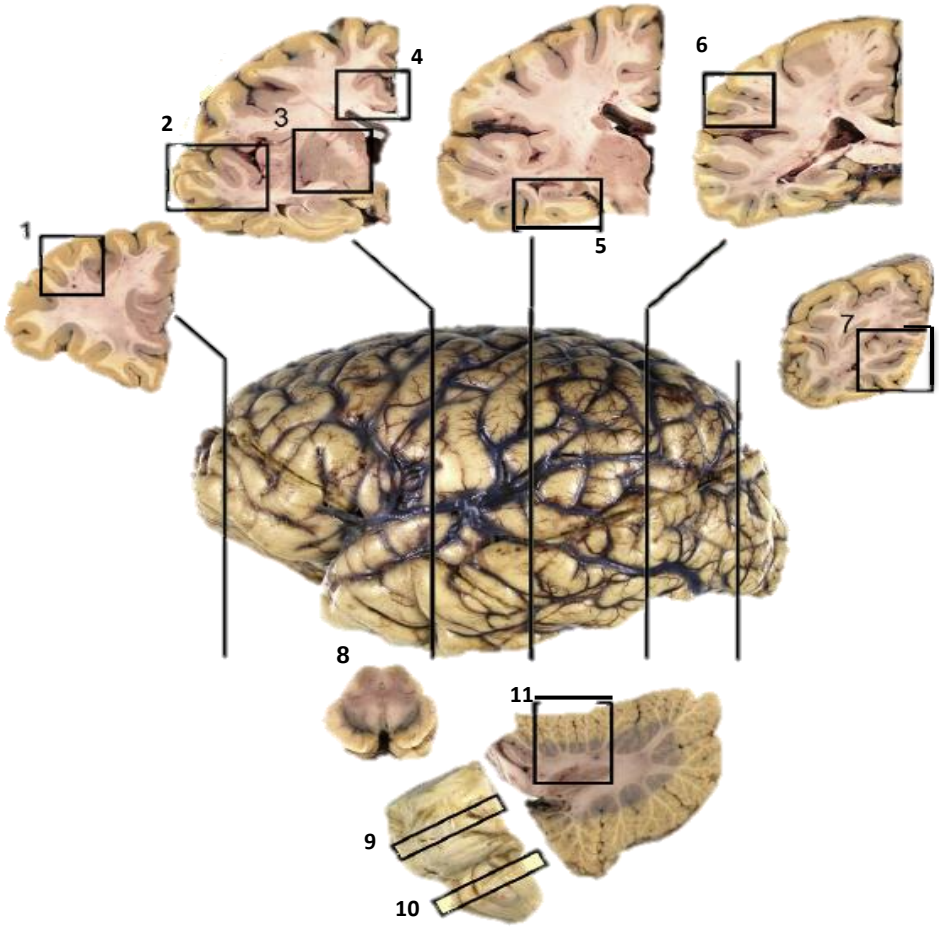

Figure 10: The suggested regions to be sampled when a neurodegenerative disease is suspected: 1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons (locus coeruleus); 10 – medulla; and 11 – cerebellum (dentate nucleus) [68].

3.1.4. SAMPLE PREPARATION

Before analysis, brain samples were washed several times with ultrapure water and placed in an incubator, at 110 ºC, until constant weight (approximately 24 hours). Between 100 and 500 mg of dried tissue was weighted in the Teflon microwave oven digestion vessels previously washed with ultrapure water. Samples were digested using 2.5 mL of concentrated (≥ 65%) HNO_3 (*Trace*SELECT® Ultra, Sigma-Aldrich, France) and 1.0 mL of concentrated (≥ 30%) H_2O_2 (*Trace*SELECT® Ultra, Sigma-Aldrich) in the closed vessels of a Milestone MLS-1200 Mega microwave oven equipped with an HPR-1000/10 S rotor (Milestone, Italy). The microwave oven program is described in Table 2.

Table 2: Microwave oven program for samples acid digestion.

Step	*Power (W)*	*Time (min.)*
1	250	1
2	0	2
3	250	5
4	400	5
5	600	5

After cooling, the vessels content was transferred to decontaminated polypropylene tubes and diluted to 50 mL with ultrapure water. All solutions were stored at 4 °C until analysis.

3.1.5. SAMPLE ANALYSIS

3.1.5.1. FE DETERMINATION

Fe determination was performed using a PerkinElmer® (Überlingen, Germany) 4100 ZL atomic absorption spectrometer equipped with longitudinal Zeeman background correction**,** a transversely heated graphite atomizer (THGA) (end-capped graphite tubes with integrated L'vov platform, PerkinElmer Part.No. B3 000653, were used) and an AS-70 autosampler. An Intensitron™ hollow cathode lamp (PerkinElmer, Norwalk, CT, USA) was used as light source (λ = 248,3 nm). High purity (99.9999%) argon (Gasin, Matosinhos, Portugal) was used as internal gas. A commercial magnesium nitrate solution (Fluka,

Switzland) was used to prepare the matrix modifier solution (5 µL = 15 µg Mg). Instrumental conditions and graphite furnace program used are described in Table 3.

Table 3: Instrumental conditions and graphite furnace program for Fe determination.

Instrumental parameter	
Wavelength (nm)	248,3
Hollow cathode lamp current (mA)	30
Slit with (nm)	0,2
Inert gas	Argon
Flow rate (ml min^{-1})	250 ("0" at atomization step)
Background correction	Longitudinal Zeeman-effect
Sample injection volume (µL)	15
Matrix modifier injection volume (µL)	5
Injection temperature (ºC)	20
Pipette speed (%)	40
Measurement mode	Integrated absorbance (As)
Integration time (seconds)	5
Baseline Offset Correction (BOC) time (seconds)	2

Graphite furnace program			
Step	**Temperature (ºC)**	**Ramp time (s)**	**Hold time (s)**
Dry	110	1	30
Dry	130	15	30
Pyrolysis	1400	10	20
Atomization	2100	0	5
Cleaning	2450	1	3

Samples were diluted (100x) with acidified (0.2% v/v HNO_3) ultra-pure water before injection into the graphite tube. Standard solutions were also prepared by diluting a commercial multi-element aqueous solution (PerkinElmer Pure GFAAS mixed standard Lot #2-232YP) with acidified 0.2% v/v HNO_3. ultra-pure water. Calibration curve was obtained with six standard solutions of Fe concentrations equally spaced within the calibration range (10-60 µg/L).

The limit of detection and quantification were calculated as the concentration corresponding to 3 and 10 standard deviations of 10 repeated determinations of the blank, respectively. Expressed as µg/L in real samples, the typical Fe detection / quantification limits achieved were 1.0 and 3.4, respectively.

3.1.5.2. Cu, Mn and Zn determination

Cu, Zn and Mn determination was performed using a VG Elemental (Winsford, UK), PlasmaQuad 3 ICP-MS instrument, equipped with a Meinhard® type A pneumatic concentric nebulizer, a quartz water-cooled impact-bead spray chamber, a standard quartz tube torch and nickel sample and skimmer cones. Both the spray chamber and sampling interface were cooled to 12 ºC by circulating water. High purity (99.9999%) argon (Gasin) was used as nebulizer and plasma gas. For sample introduction, a Gilson (Villiers le Bel, France) model M312 peristaltic pump was used. A 2% (v/v) HNO_3 solution was used in the washing steps. The main operating conditions for the ICP-MS determinations are summarized in Table 4.

The elemental isotopes (m/z ratios) 55Mn, 65Cu and 66Zn (as analytical masses) and 45Sc, 89Y, 115In, 159Tb and 209Bi (as internal standards) were monitored. The limit of detection and quantification were calculated as the concentration corresponding to 3 and 10 standard deviations of 10 repeated determinations of the blank, respectively. Expressed as µg/L in real samples, the typical detection limits achieved in routine operation during the samples analysis were: 0.2 (Mn); 0.4 (Cu); 2.9 (Zn). Quantification limits achieved were: 0.7 (Mn); 1.3 (Cu); 9.5 (Zn) respectively.

A 10 µg/L tuning solution was prepared by dilution of a commercial multi-element aqueous solution (AccuTrace™, ICP-MS 200.8-TUN-1; AccuStandards Inc, New Haven, CT, USA). The instrument was daily tuned for maximum signal sensitivity and stability using 115In as the target isotope.

Internal standard (Sc, Y, In, Tb and Bi) solution was prepared by dilution of a commercial solution (AccuTrace™ Reference Standard, ICP-MS 200.8-IS-1; AccuStandard Inc.). It was added to the samples and standards solutions in order to obtain a 10 µg/L final concentration.

Table 4: Main instrumental and operating conditions for ICP-MS.

Instrument parameter	*Condition*
Detector	Sequential
Detection mode	Pulse counting
Acquisition mode	Continuous
Pre-scan	Yes
Setup of the ICP	
Rf power (W)	1350
Nebulizer gas flow (L/min)	0.78
Auxiliary gas flow rate (L/min)	0.60
Cool gas flow (L/min)	13.0
Setup for main run	
Acquisition mode	Peak jumping
Sweeps	200
Dwell time (ms)	10
Channels per mass	1
Channel spacing	0.02
Replicates	2

ICP-MS calibrating solutions were prepared by dilution of a commercial multi-element aqueous solution (AccuTrace™ Reference Standard, ICP-MS 200.8-CAL1R-1; AccuStandards material, S.L., Madrid. Spain). Calibration curve was obtained with six solutions of element concentrations within the 1-200 µg/L range.

All the solutions were prepared with 2% (v/v) HNO_3.

3.1.6. QUALITY CONTROL

Since brain tissue is not available as certified reference materials for, DOLT-4 Dogfish liver and DORM-3 Fish Protein Certified Reference Materials for Trace metals (National Research Council Canada, Canada) and independent standard solutions were used to validate accuracy. CRMs were subjected to the same sample pre-treatment and employed at the same concentration range as the analyte content in the sample.

For contamination control during microwave-assisted acid digestion, a sample blank was performed in each digestion cycle (10 samples).

All the samples, after adequate dilution, were analyzed twice (two determinations in the same analytical run). For results with relative standard deviation ≥ 10 % another two determinations were performed.

3.1.7. STATISTICAL EVALUATION

Statistical analysis of the data was performed using GraphPad Prism 5 for Windows (version 5.04) statistical software (San Diego, California, USA).

For each brain region, of each age sub-groups, element concentration (μg/g) is expressed as the mean ± SD from two determinations of each sample. Descriptive statistical parameters (mean, median, standard deviation, minimum and maximum) were calculated for each element within each brain region.

3.2. RESULTS AND DISCUSSION

3.2.1. REGION DISTRIBUTION OF TRACE ELEMENTS IN NORMAL HUMAN BRAIN

It was found that brain distribution of TE is not homogeneous. Independently from age, Fe is the most abundant metal, followed by Zn and Cu. Zn higher levels were found in hippocampus and middle temporal region; higher levels of the other TE were found in basal ganglia. Pons and medulla were the regions with lower levels of the four TE studied. To illustrate the regional distribution of TE within brain, the mean [TE] (µg/g) of all adult individuals of each brain regions was used.

3.2.1.1. IRON

During adult life, Fe highest levels were found in (3B) putamen (range: 409 – 1155 µg/g), (3C) globus pallidus (577 – 1062 µg/g) and (3A) caudate nucleus (299 – 743 µg/g), while Fe lowest levels were found in (10) medulla (44 – 71 µg/g) and (9) pons (64 – 116 µg/g). These data are consistent with previous studies, either *in vivo*, using magnetic resonance imaging techniques [142, 143], and *post-mortem* studies, using atomic absorption spectrometry techniques [144, 145] .

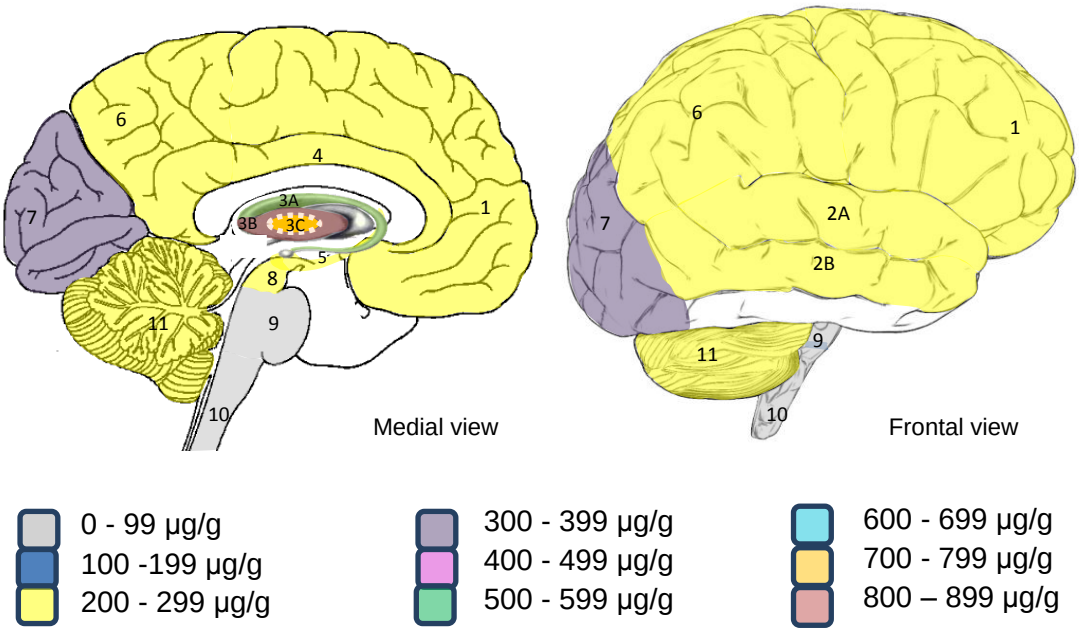

Figure 10: Normal Fe distribution in different regions of adult human brain.

The specific distribution of Fe in the basal ganglia, co-localized with dopaminergic neurons, suggests a special role for this element. It can be concluded from the Fe preferential accumulation in these areas that there are certain metabolic processes

requiring great amounts of Fe. Cerebellum is also involved in motor coordination but the Fe levels found were not as high as in basal ganglia. The reason for this selective Fe distribution in different areas of brain related to motor function is yet unknown [146]. Putamen is the motor part of the striatum, whereas the caudate is mainly connected with more associative cortical regions, not directly involved in motor control [143].

3.2.1.2. COPPER

In agreement with previous *post-mortem* studies [144, 145], Cu highest levels were found in (3B) putamen (30 – 34 µg/g), while Cu lowest levels were found in (9) pons (5 – 22 µg/g) and (10) medulla (8 – 13 µg/g).

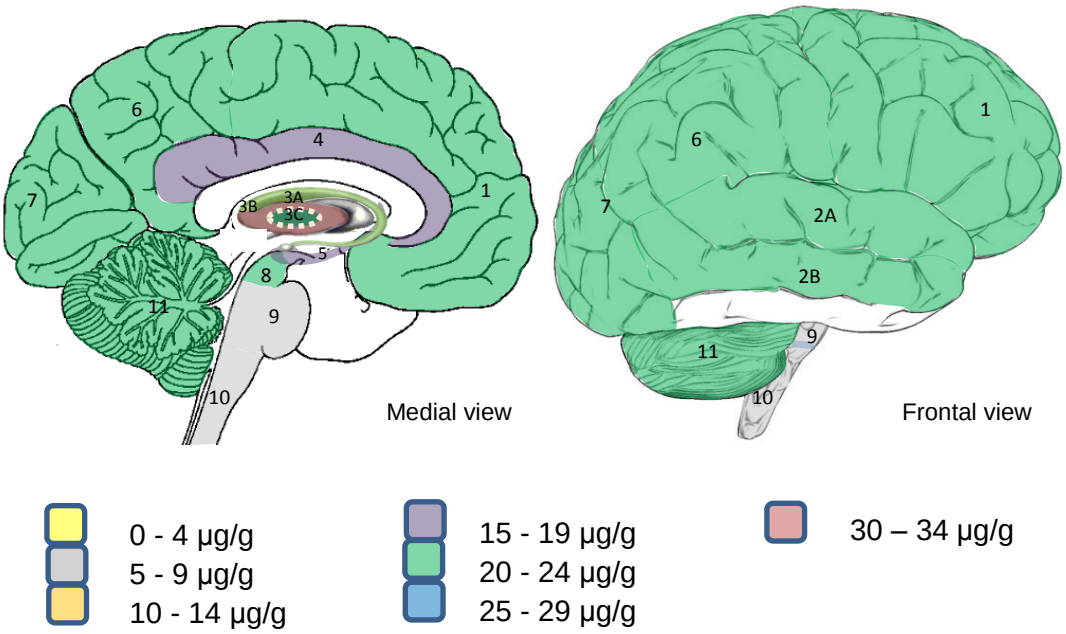

Figure 11: Normal Cu distribution in different regions of adult human brain..

As also described in literature [147], Cu brain distribution was found not homogeneous. Being a cofactor of numerous enzymes, Cu is essential for normal CNS function. High Cu levels in certain regions most likely reflect higher metabolic demands for these areas.

3.2.1.3. MANGANESE

Among the four TE studied, Mn was the less abundant in human brain, whose levels found ranged from 0.8 µg/g in (9) pons and (10) medulla, to 2.8 µg/g in (3B) putamen. Mn regional distribution found (Figure 13) is concordant with previous *post-mortem* studies referring (3B) putamen and (3A) caudate nucleus as Mn richer regions [148, 149].

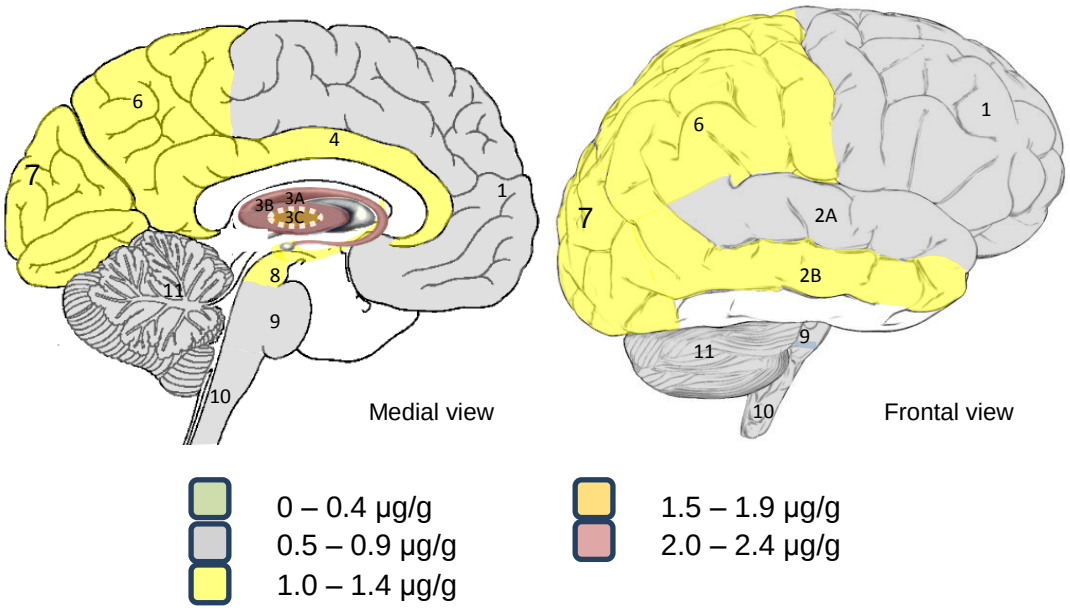

Figure 12: Normal Mn distribution in different regions of adult human brain.

3.2.1.4. ZINC

Zn was found to be the second most abundant TE in normal human brain. Highest levels were found in (5) hippocampus (66 – 81 µg/g) and (2B) middle temporal (62 – 74 µg/g) and lowest levels were found in (9) pons (28 – 37 µg/g), (10) medulla (30 – 44 µg/g) and (11) cerebellum (34 – 38 µg/g). The distribution found is concordant with literature data, which refers that higher zinc levels are found in forebrain regions, including hippocampus, amygdala and neocortex [57].

Since hippocampus and middle temporal are regions related to memory formation, it is suggested that Zn plays a role in memory function.

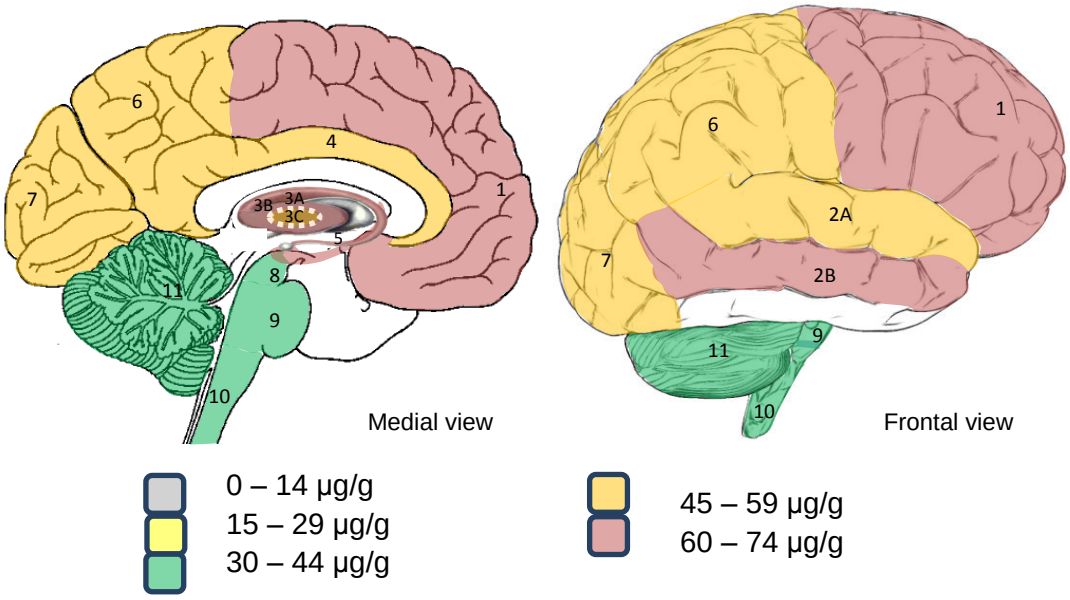

Figure 13: Normal Zn distribution in different regions of adult human brain..

Middle temporal and hippocampus, the regions with higher Zn content, are coincident with Fe moderate levels; consequently they are probably involved in different metabolic reactions [146].

3.2.2. AGE-RELATED CHANGES IN HUMAN BRAIN

It seems to be a trend to elderly people has high levels of the four TE in specific brain regions. However, the difference between age-groups was found not significant due to substantial heterogeneity of population.

In summary, these results corroborate previous reported data from studies on TE deposition in human brain, suggesting that accumulation seems to be related to neuronal degeneration proceeding in a retrograde way to neuronal cell death with disease progression [150]. However, a causative role cannot be implicated due insufficient subjects with neurodegenerative diseases for testing causal relationships.

3.2.2.1. IRON

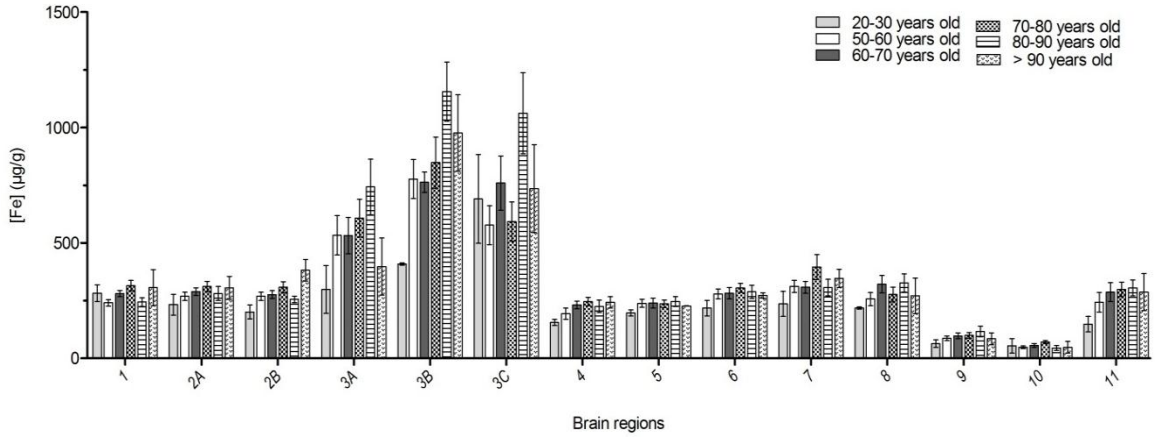

Figure 14: Fe levels (µg/g) age-related changes in different regions of human brain.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

Post-mortem and *in vivo* studies have demonstrated that, in normal individuals, Fe levels increase over lifetime in subcortical and some cortical gray matter regions [143, 151]. In addition, Xu et al. [143] confirmed significant effects of age on brain iron levels. Age-related increased iron deposition was found in putamen (3B in Figure 15), red nucleus and frontal white matter from 22 years to over 70 years, reaching maximal values at about the sixth decade. In the globus pallidus (3C), SN and caudate (3A), Fe concentration was found unchanged with age. Bilgic et al. [144] also found that elderly people (64 to 86 years old) had significantly higher Fe levels in striatal regions of the putamen (3B), caudate nucleus (3A), globus pallidus and SN than younger group (21 to 29 years old).

Motor disturbances in elderly people can be related with this age-related increasing in Fe levels, since it induces free toxic radical production, which leads to tissue oxidative stress and neuronal cell death.

3.2.2.2. COPPER

According to Figure 16, no significant correlation between Cu levels and age seems to exist, except for cerebellum (11), where Cu levels seems to be negative correlated with age. However, due to high variability between individuals, differences are not significant.

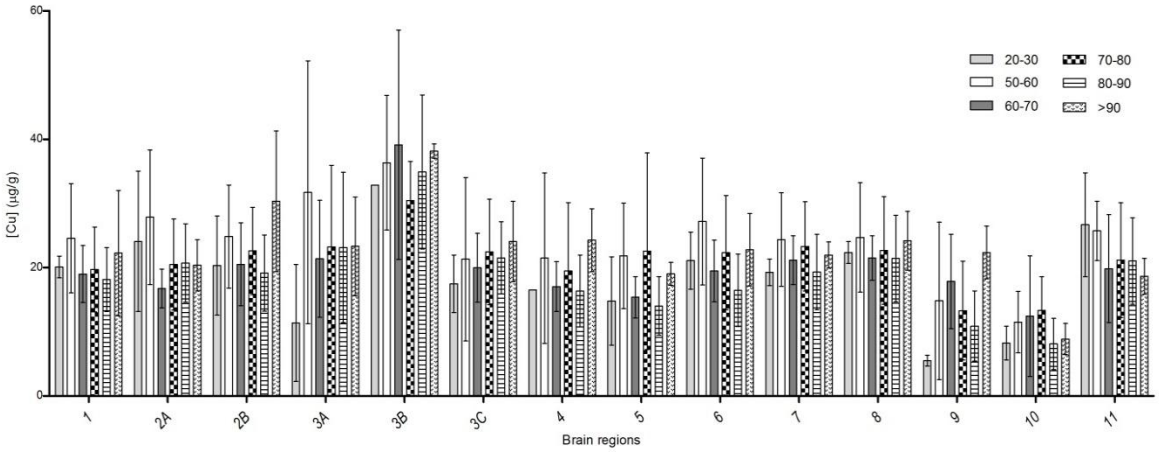

Figure 15: Cu levels (µg/g) age-related changes in different regions of human brain.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

3.2.2.3. MANGANESE

Mn distribution in the brain varies during development and aging and its alteration might be associated with brain maturation and/or functions. Data from literature repot high Mn levels in globus pallidus, putamen and caudate [148].

Our data regarding a Mn distribution (Figure 17) shows a trend for accumulation in brain during lifetime in putamen (3B) and globus pallidus (3C). In the other regions Mn levels remain quite constant.

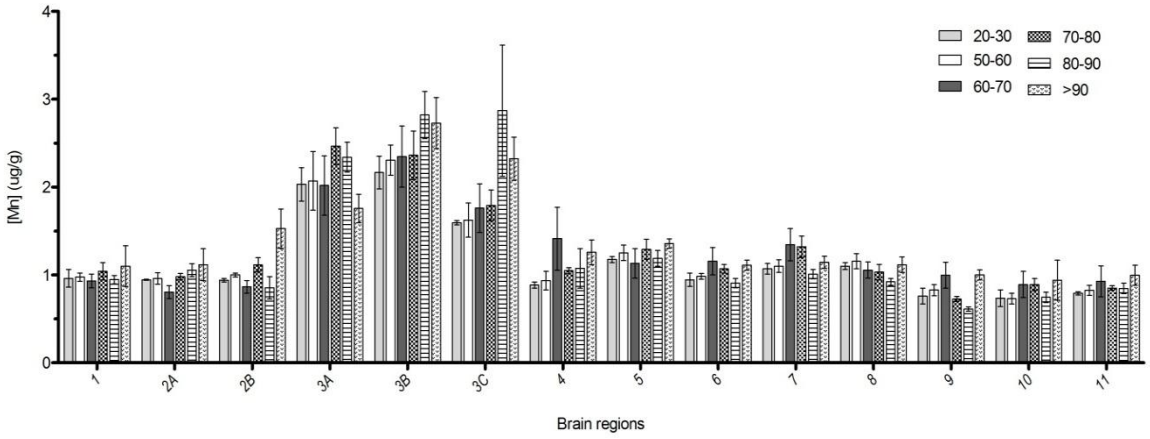

Figure 16: Mn levels (µg/g) age-related changes in different regions of human brain.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

3.2.2.4. ZINC

Zn showed to increase with age in middle temporal (2B), hippocampus (5), cingulated gyrus (4) and inferior parietal (6) (Figure 18). In the other regions it remains quite unchanged.

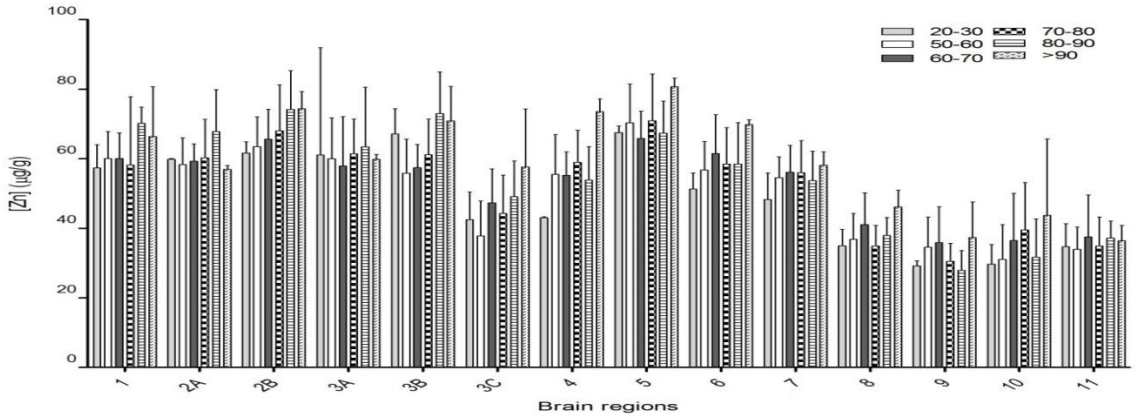

Figure 17: Zn levels (µg/g) age-related changes in different regions of human brain.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

Discrepant data can be found in literature. Deibel et al. [152] reported that brain Zn remains relatively constant throughout adult life but Panayi et al. [153] found positive correlations with age for subjects older than 65 years.

3.2.3. DISEASE-RELATED CHANGES IN HUMAN BRAIN

3.2.3.1. ALZHEIMER'S DISEASE

When compared with non-diseased people of the same age sub-group, TE levels from individuals affected by Alzheimer's disease (n=2) were found significantly altered in regions mainly related to memory and learning.

Although the regions are coincident in the two individuals, the values found were not concordant. Explanation for divergent findings could be related to different stages of neurodegeneration at the time of death and/or with involvement of other factors contributing to the pathology of the disease.

3.2.3.1.1. IRON

In one AD patient, 73 years old, a statistical significant increase of Fe levels was found in several brain regions: 1.9x in caudate, 1.4x in globus pallidus, 2.1x in hippocampus. Decreased Fe levels were found in frontal cortex (0.6x) and medulla (0.8x) (Figure 19).

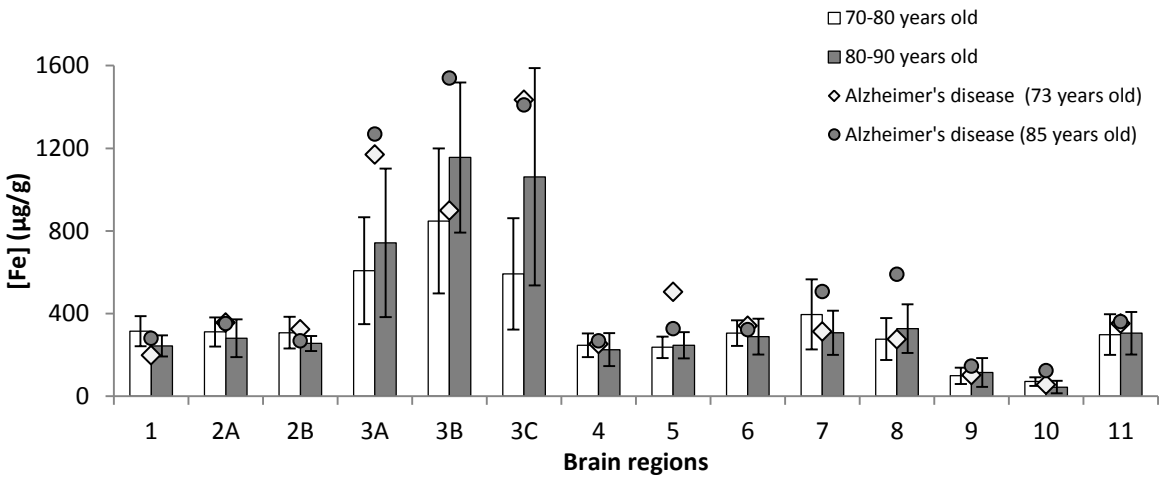

Figure 18: Fe levels (µg/g) in different brain regions of Alzheimer's disease patients and age-matched control.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

In the other patient, it was found a significant increase in Fe levels: around 1.3x in superior temporal, 1.7x in caudate, 1.3x in putamen, 1.3x in hippocampus, 1.7x in occipital cortex, 1,8x in midbrain and 2.8x in medulla (Figure 19).

According to Andrási et al. [146], significant differences were expected for Fe in putamen (3B), caudate nucleus (3A) and occipital cortex (7), with Fe levels higher in the AD group, but not in other studied brain regions (frontal and parietal cortex, globus pallidus and thalamus).

One remarkable features of AD is the accumulation of Fe in neurofibrillary tangles and senile plagues that was found to be specifically associated with the lesions of AD [146]. Excess Fe could mobilize metals (e.g., Al and Mn) and thus increases the chance of neurological damage of CNS, such as dementia [57].

3.2.3.1.2. COPPER

It seems that Cu distribution is particularly affected in AD patients' brain. Significant changes in almost all sampled regions were found for both AD cases.

In one AD patient, 73 years old, significant lower Cu levels were found in all regions: frontal cortex (0.6x), superior temporal (0.7x), middle temporal (0.6x), putamen (0.7x), cingulated gyrus (0.4x), hippocampus (0.6x), parietal inferior (0.7x), occipital cortex (0.7x), midbrain (0.6x) and medulla (0.6x) (Figure 20).

In the other AD patient, however, a significant increase of Cu levels was found in all brain regions: frontal cortex (2.0x, $p<0.0001$), superior temporal (1.6x), middle temporal (1.4x), caudate (1.9x), putamen (1.5x), caudate (1.5x), cingulated gyrus (1.5x), hippocampus (2.0x), parietal inferior (2.1x), occipital cortex (1.8x), midbrain (1.8x), pons (1.9x), medulla (2.5x) and cerebellum (1.3x) (Figure 20).

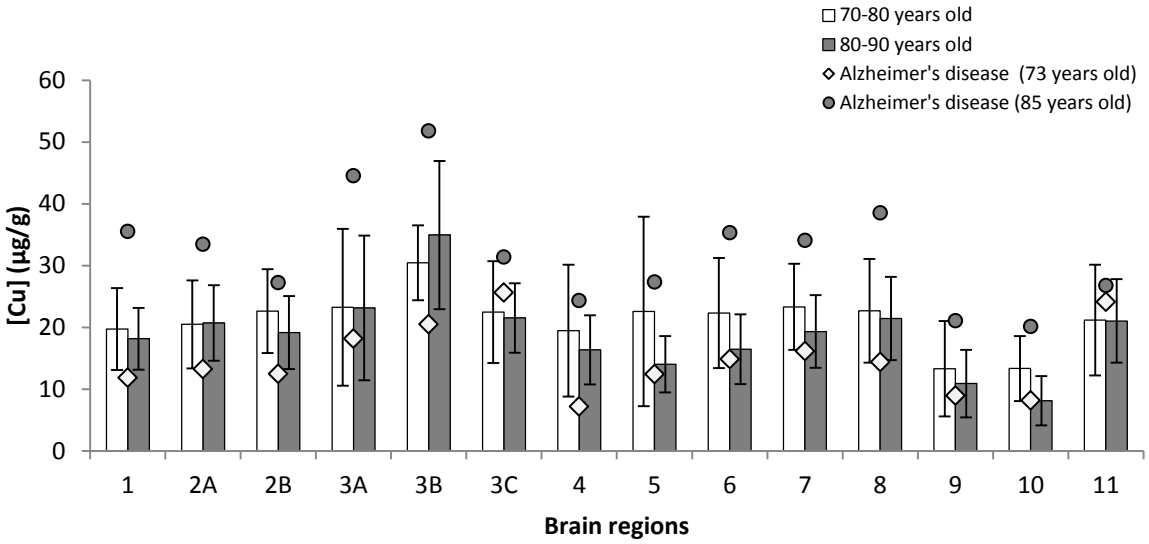

Figure 19: Cu levels (µg/g) in different brain regions of Alzheimer's disease patients and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

Previous studies on the role of copper in AD development and progression are also inconsistent and occasionally contradictory. Deibel et al. [152] showed a significant decrease of Cu in hippocampus. Platin et al. [154] also found significantly decrease of Cu levels in frontal, temporal, parietal and occipital lobes in AD compared with controls.

The involvement of Cu in AD development is complex and multilayered. Extracellular amyloid plaques, one of the two key pathologies present in AD brains, are enriched with Cu [155]. However, the brain tissues of AD patients are paradoxically Cu-deficient [124–127]. Cu complexes with Aβ, which is linked to ROS-related neurotoxicity, Aβ aggregation and formation of amyloid plaques. Copper–Tau interaction has been also hypothesized to induce the development of intracellular NFTs, the other characteristic hall-mark of AD [57].

3.2.3.1.3. MANGANESE

In one AD patient Mn levels were found to be significantly reduced in frontal cortex (0.7x), superior temporal (0.8x), caudate (0.8x) and hippocampus (0.6x).

In other hand, in the older AD patient higher Mn levels were found in frontal cortex (1.2x), caudate (1.6x), putamen (1.6x), hippocampus (1.3x), inferior parietal (1.7x), occipital cortex (1.6x), midbrain (2.0x), pons (1.7x), and medulla (1.8x) (Figure 21).

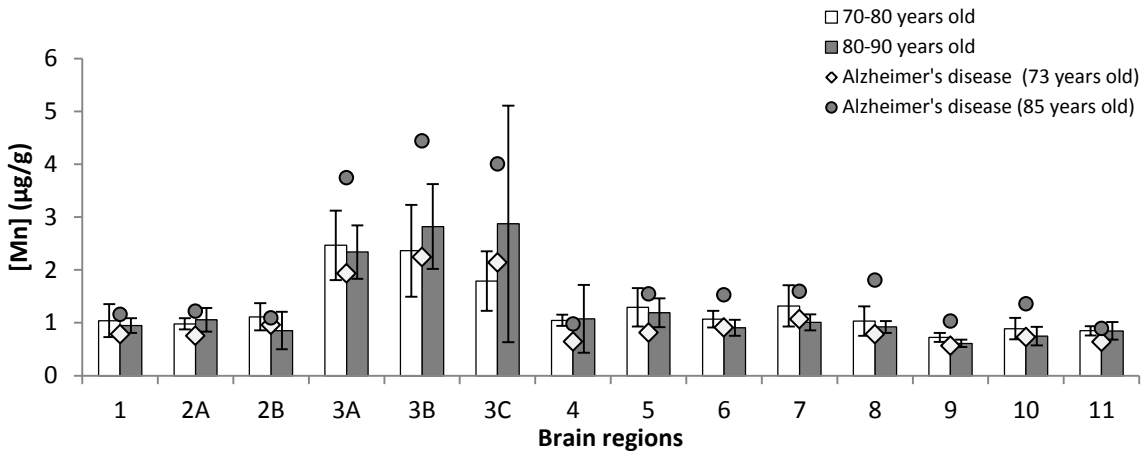

Figure 20: Mn levels (µg/g) in different brain regions of Alzheimer's disease patients and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

Until recently, the majority of the studies examining the neurological consequences of chronic Mn exposure were focused on Mn effects on basal ganglia, due to its effects on motor function [156]. Last decade, a number of human studies have demonstrated Mn-induced deficits in attention and cognitive impairment that may persist long after exposure [157, 158]. It has been suggested that Mn effects on the frontal cortex and subcortical structures may be associated with these cognitive and executive function deficits [159], inducing diffuse amyloid-beta plaques in frontal cortex, potentially supporting a link between advanced-stage manganism and dementia.

In accordance with the above cited study, we found significantly increased Mn levels in cortical regions, which may be a contributing factor for AD pathogenesis. But, since there is a lack of evidence linking Mn with AD, further studies are needed.

3.2.3.1.4. ZINC

As already described for other TE, changes found in AD patients were not concordant: in one patient Zn levels were found to be significantly increased in all brain regions, but not on the other one.

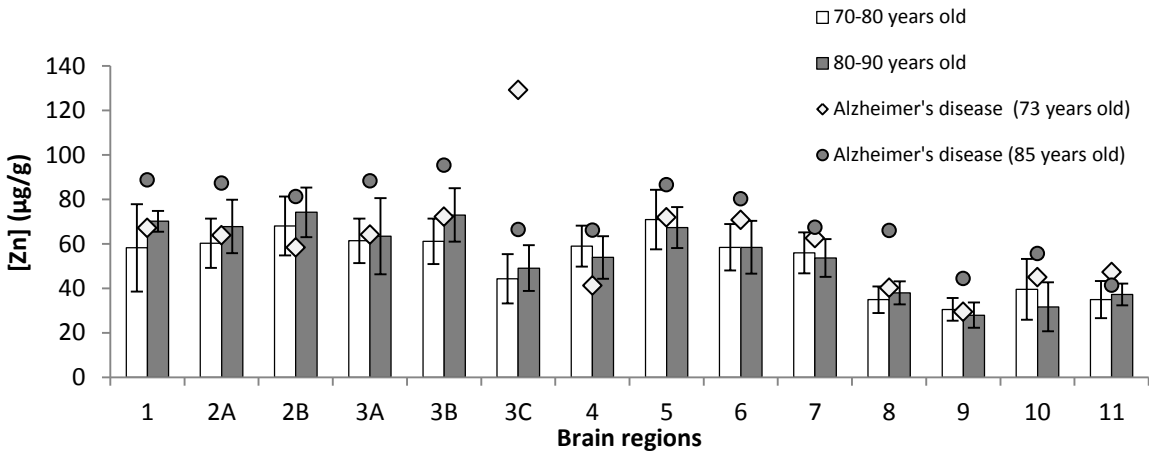

Figure 21: Zn levels (µg/g) in different brain regions of Alzheimer's disease patients and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

Regarding Zn levels and AD, there are contradictory reports in literature. Several authors report increased levels of Zn in AD subjects compared to normal groups: Deibel et al. [154] found increased levels in hippocampus; Cornett et al. [153] found Zn increased concentrations in frontal, temporal, parietal lobes, hippocampus and cerebellum.

The consequences of high levels of Zn in neurodegenerative diseases such as AD have been discussed. Bush et al.[160] showed that Aβ specifically bind to Zn and, at high concentrations, this metal accelerates protein aggregation suggesting a role in the pathogenesis in AD.

Contrarily, several authors reported decreased levels of Zn in AD subjects in the basal ganglia, central cortex, putamen and hippocampus [144, 153]. There is lesser information regarding the role of low levels of Zn in AD. Some authors suggest that amyloid accumulation allows metals to enter the brain via disrupted BBB, but that is a displacement and loss of Zn, which leads to neurofibrillary tangles and dementia [153]. Lovell et al.[75] refer low Zn levels as protective against Aβ toxicity due to the enhancement of Na+/K+ ATPase activity, which prevents consequences of Aβ aggregation such as the disruption of calcium homeostasis and cell death.

Furthermore, no significant differences were found for Zn levels in cortex, temporal, parietal and frontal areas [153].

The heterogeneity of these results could be due to different stage of neurodegeneration at the time of death, insufficient number of subjects, dietary, geographical and environmental variations between subjects, differences in tissue sampling, handling protocols and different sensibility methods.

3.2.3.2. PARKINSON'S DISEASE

3.2.3.2.1. IRON

In the PD patient, significantly higher Fe levels were in globus pallidus (2,3x), cingulated gyrus (1,5x) and hippocampus (1,3x). In inferior parietal cortex it was observed a 46% reduction of Fe levels.

In normal brain, Fe was found predominantly in the extrapyramidal system, in particular the globus pallidus, SN and putamen. Fe deposition normally increases with age but the accumulation observed in PD patient exceeds the increasing found in normal elderly individuals in basal ganglia (3 A-C; Figure 23). Previous studies also report similar data: increased levels on SN and globus pallidus [103, 150, 151]. Moreover, Fe deposits seems to be correlated with the severity of the disease [103, 152, 153].

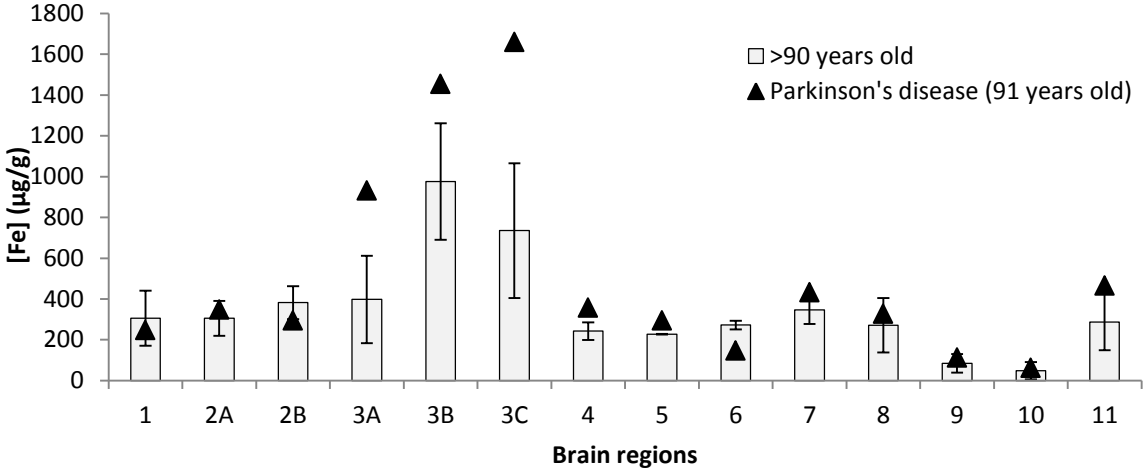

Figure 22: Fe levels (µg/g) in different brain regions of Parkinson's disease patients and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

The most characteristic hallmark in PD is the loss of dopaminergic neurons in the *pars compacta* region of the SN. SN is the source of the striatal input of the neurotransmitter

dopamine, which plays an important role in basal ganglia function [161]. Injury of dopaminergic projections from SN to the caudate nucleus and putamen is the cause of the main motor symptoms on PD patients such as rest tremor, rigidity, bradykinesia and postural instability.

3.2.3.2.2. COPPER

Reduced Cu levels in PD brain were found in some regions, namely putamen (0.6x), hippocampus (0.7x), inferior parietal (0.7x) and pons (0.2x) (Figure 24). In literature, contradictory results can be found: several studies found decreased total Cu levels in SNpc and caudate nucleus, but not in cerebellum, globus pallidus or putamen of PD brains [91, 119], but increased levels are also reported [119].

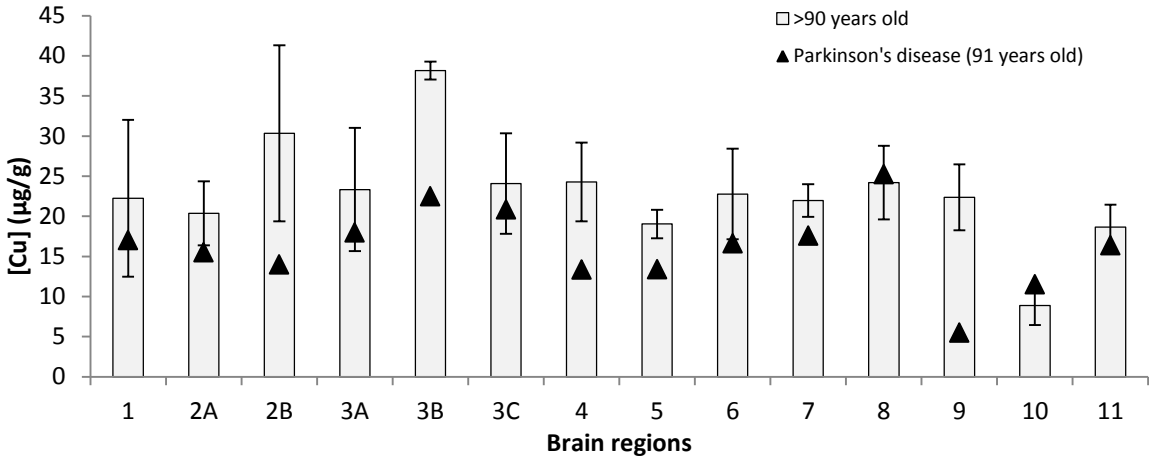

Figure 23: Cu levels (µg/g) in different brain regions of Parkinson's disease patients and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

Some authors suggest that Cu may lead to both toxic and protective effects depending on the experimental conditions. However, since brain Cu levels have been reported to be decreased in PD [117], the toxic effects that occur at high concentrations are not likely to be involved in the pathophysiology of the disorder. Due to non-concordant results available in literature, further research is needed to clarify the dual role of Cu in PD.

3.2.3.2.3. MANGANESE

Mn levels was found significantly increased in particular brain areas of the PD patient, namely in putamen (3.3x), cingulated gyrus (1.6x), hippocampus (1.4x), inferior parietal (1.5x), occipital cortex (1.4x), midbrain (1.5x) and medulla (2.1x).

Manganese toxicity is clearly associated with damage of basal ganglia structures, as observed in animal and human studies [162]. The exact mechanism of Mn neurotoxicity and the reason for the globus pallidus selective vulnerability is still unknown. It is thought that its toxicity is mediated by excessive oxidative stress, enhanced free radical formation and decreased glutathione levels, leading to cell death [162].

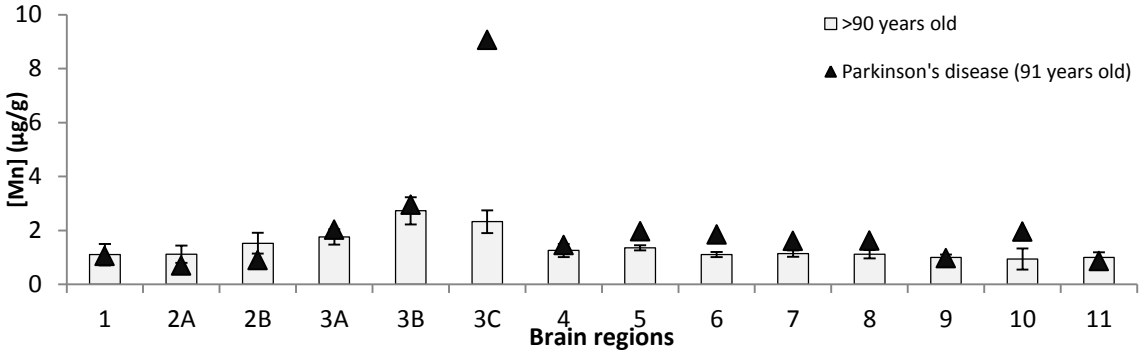

Figure 24: Mn levels (µg/g) in different brain regions of Parkinson's disease patients and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

Various mechanisms mediated by Mn could converge on α-synuclein *in vivo*, potentially connecting Mn to PD [163]. Mn can directly increase fibril formation and to act jointly with α-synuclein in neuronal cell death inducing [164]. Overexpression of α-synuclein in human cells seems to facilitate Mn-induced neurotoxicity and possibly plays a role in dopaminergic cell death [165].

3.2.3.2.4. ZINC

In PD-affected brain, Zn levels were found increased in (5) hippocampus (1.2x), but they were decreased in (1) frontal cortex (0.5x), and (3A) caudate (0.9x).

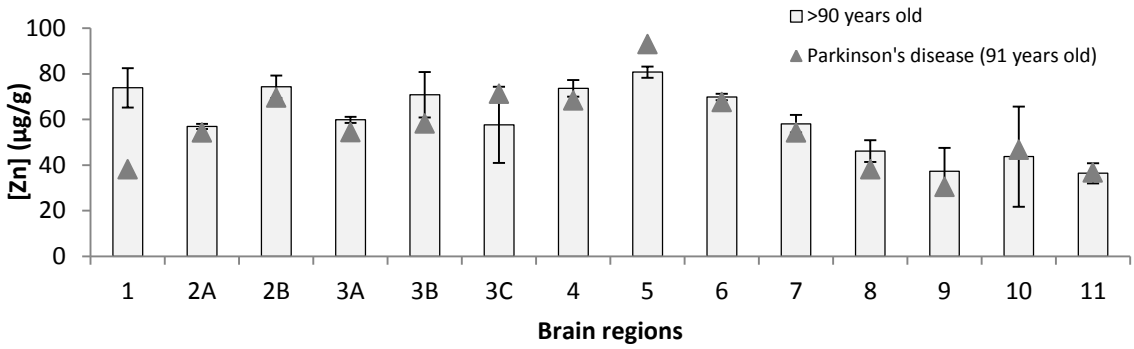

Figure 25: Zn levels (µg/g) in different brain regions of Parkinson's disease patients and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

Previous studies found significantly decreased levels of Zn in putamen, caudate nucleus, globus pallidus and SN [117], suggesting that a Zn disrupted homeostasis could be also involved in PD.

3.2.3.3. AMYOTROPHIC LATERAL SCLEROSIS

3.2.3.3.1. IRON

Fe levels were found decreased in some brain regions of a ALS patient, namely: frontal cortex (-22%), superior temporal (-41%), middle temporal (-23%), caudate (-58%), globus pallidus (-42%), inferior parietal (-32%) and pons (-57%).

Increased spinal cord Fe levels were reported in ALS but there is a lack of studies in brain tissue [166]. Although lower Fe levels were found in regions related to motor functions, further studies are needed in order to elucidate Fe involvement in this disease.

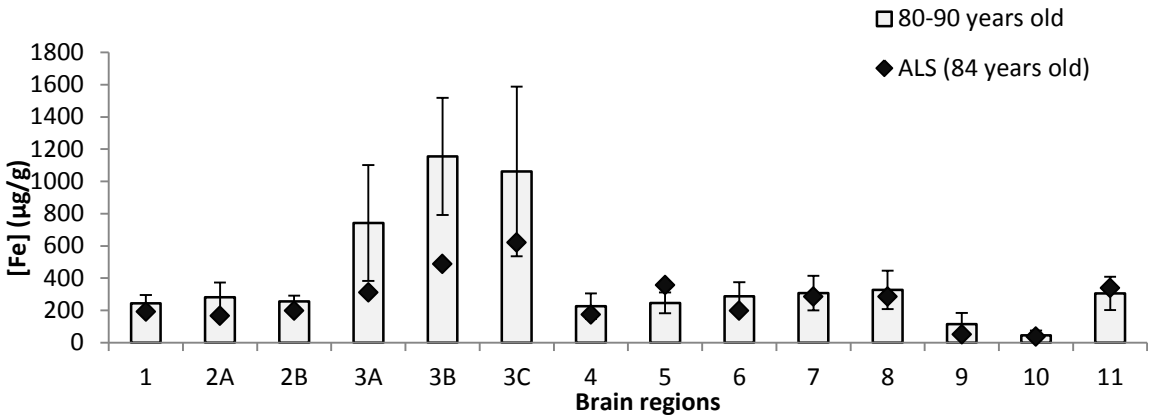

Figure 26: Fe levels (µg/g) in different brain regions of amyotrophic lateral sclerosis patient and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

3.2.3.3.2. COPPER

Compared with age-matched control, Cu levels were not found significantly altered in ALS brain, with the exception of cerebellum, where a 1.4x increase was observed. These data suggest that, in this subject, Cu homeostasis was not altered and it was not a major contributing factor for ALS pathology.

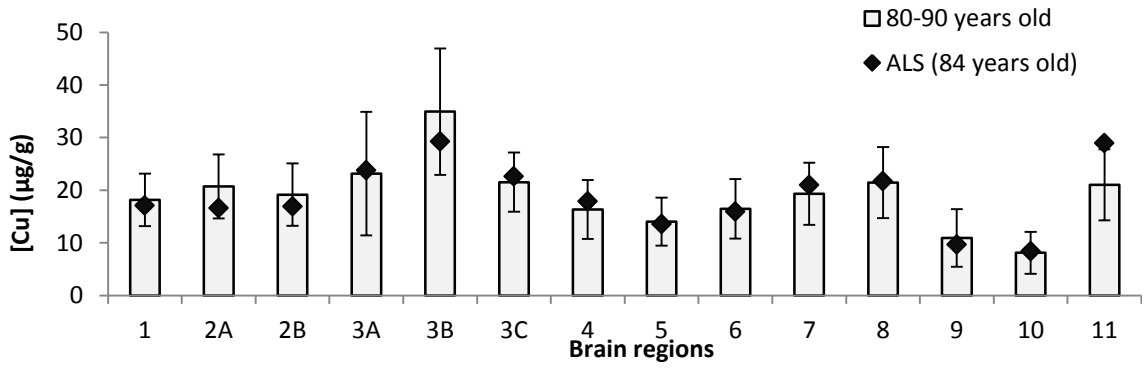

Figure 27: Cu levels (µg/g) in different brain regions of amyotrophic lateral sclerosis patient and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

3.2.3.3.3. MANGANESE

Mn was found reduced in superior temporal (0.6x), caudate (0.7x) and inferior parietal (0.4x). In hippocampus and midbrain, a slight increase was observed (1.2x and 1.1x, respectively).

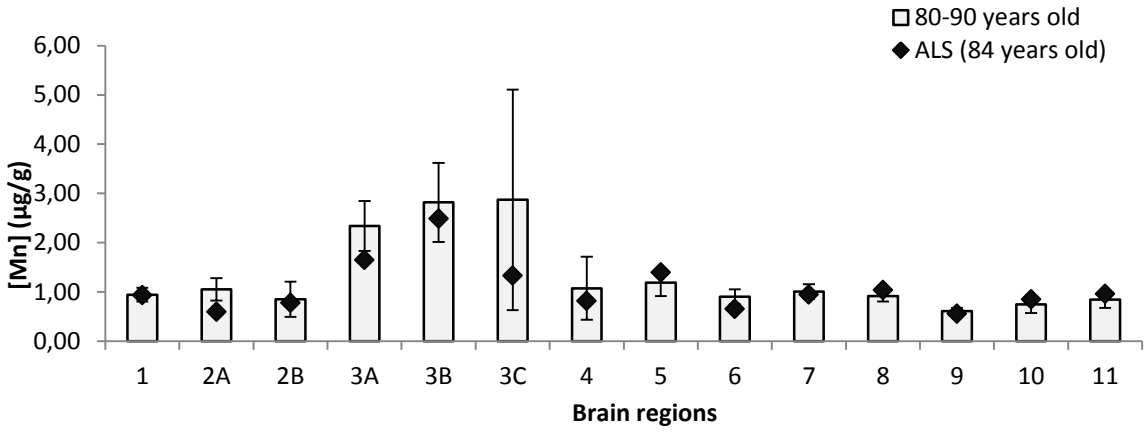

Figure 28: Mn levels (µg/g) in different brain regions of amyotrophic lateral sclerosis patient and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

Mn overload has been implicated in ALS. There is documented evidence that Mn smelters and welders who developed occupational manganism also showed some neurological signs of motor neuron disease and bulbar ALS [31]. In addition, a significant percentage of ALS patients show neuroradiological signs compatible with Mn overload (T1-weighted hyperintensity) in areas related to the motor system [167], but to date no studies have directly quantified Mn levels in these regions in ALS patients. Further, Mn overload induces apoptosis, which contributes to motor neuron disease [31].

Until today, there is not a definitive proof linking Mn to ALS, these observations collectively indicate that in certain conditions the metal can cause or trigger motor neuron disease in some individuals [31] .

In the ALS patient studied, Mn levels were not significantly different from age-matched controls. Brain regions linked to motor control (putamen, globus pallidus or caudate nucleus) showed no changed levels of Mn, but it was noticed a slightly increase in hippocampus and midbrain.

3.2.3.3.4. ZINC

When compared with age-matched control, Zn levels were found to be decreased in superior temporal (0.7x), middle temporal (0.7x), caudate (0.8x), globus pallidus (0.8x) and inferior parietal (0.7x). Zn levels in midbrain and cerebellum were found to be increased 1.3x and 1.2x, respectively.

Since there is an association between Cu,Zn-SOD mutations and ALS, altered levels of those metals have been associated with ALS pathology, suggesting that oxidative injury is involved in this disorder [57]. As previously described, TE altered levels in CSF and serum are reported but there is a lack of data of direct determinations in brain tissue [57].

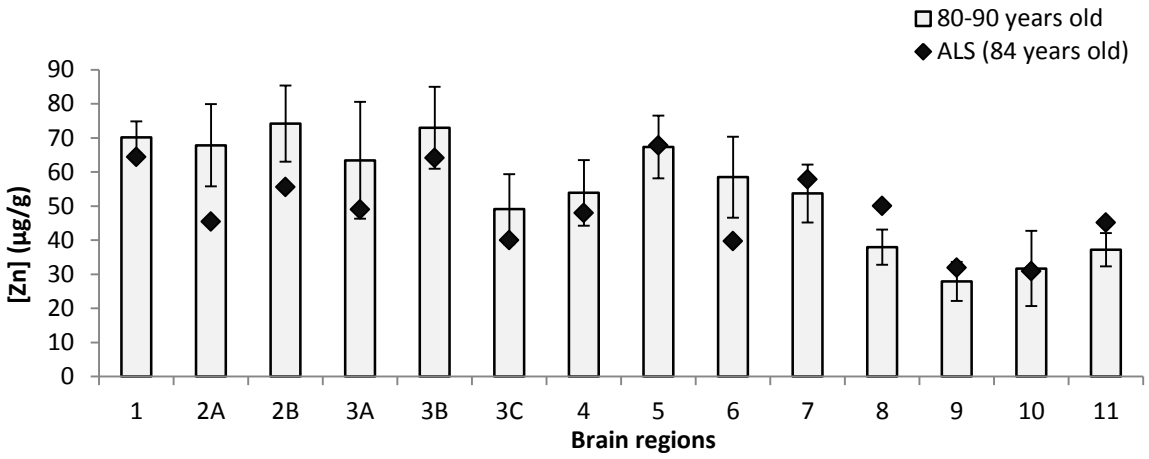

Figure 29: Zn levels (µg/g) in different brain regions of amyotrophic lateral sclerosis patient and age-matched controls.

1 – frontal cortex; 2 – (A) superior and (B) middle temporal gyri; 3 – basal ganglia, including the (A) caudate, (B) putamen and (C) globus pallidus; 4 – cingulated gyrus; 5 – hippocampus; 6 – inferior parietal lobule; 7 – visual cortex of the occipital lobe; 8 – midbrain, including the substantia nigra at the level of the third nerve; 9 – pons 10 – medulla; and 11 – cerebellum .

IV. CONCLUSIONS AND FUTURE RESEARCH

CONCLUSIONS AND FUTURE RESEARCH

Since no updated and comprehensive data about trace elements in human brain are available, this study may be a relevant scientific contribution for establishing “normal” human brain levels, allowing future comparisons of levels found in brains affected by neurodegenerative diseases, in an attempt to clarify trace elements role in the disease process.

Data obtained clearly show that there is an inhomogeneous distribution of trace elements within the brain tissue. This can be regarded as a proof of an important physiologic role of trace elements in brain functioning.

On the other hand, age-related changes are not so clear. Globally, there is not evident any significant increase or decrease. However, in specific regions, significant changes were observed, which also supports a role for trace elements in age-related motor and cognitive problems.

Due to the limited number of subjects with ND and the population heterogeneity, the presented results should be considered as preliminary. However, they seem to corroborate the hypothesis that trace elements play an important role in neurodegenerative diseases, since significant differences were observed regarding their distribution in specific brain regions. Further investigation of regional distribution of trace elements in brain tissue, with particular focus on the correlation with health and disease status, in order to understand age- and disease-related motor slowing and other selective cognitive decline [142].

Given the multifactorial nature of neurodegenerative diseases, it is becoming evident that the next generation of therapies must have multiple functions to combat multiple mechanisms of disease progression [49]. Since metals have gained increased importance regarding a potential involvement in ND, inhibiting metal-promoted damage by use of small chelating agents could be a potential strategy for treating diseases associated with localized metal accumulation [49]. However, the current understanding of metal homeostasis in CNS is still very limited, which difficult the interpretation of the effects of manipulating metals within the brain [51]. It is crucial to clarify the mechanisms involved in oxidative stress and other factors responsible by neuronal degeneration, since it is essential to find new effective therapeutic approaches in order to improve not only the effective treatment of neurodegenerative diseases but also to delay their onset.

V. REFERENCES

REFERENCES

1.National Institute on Aging, National Institutes of Health; Global Health and Aging Report. [cited 2012 21st October]; Available from: http://www.nia.nih.gov.
2.Smith MA, Harris PL, Sayre LM, Perry G. Iron accumulation in Alzheimer disease is a source of redox-generated free radicals. Proceedings of the National Academy of Sciences of the United States of America. 1997 Sep 2;94(18):9866-8.
3.Instituto Nacional de Estatística; Censos 2011 – Resultados Pré-definitivos 2012 [cited 2012 21st October]; Available from: www.ine.pt.
4.Rádio Renascença. [cited 2012 21 October]; Available from: http://rr.sapo.pt/informacao_detalhe.aspx?fid=25&did=48980.
5.Pritchard C, Baldwin D, Mayers A. Changing patterns of adult (45-74 years) neurological deaths in the major Western world countries 1979-1997. Public health. 2004 Jun;118(4):268-83.
6.Gustavsson A, Svensson M, Jacobi F, Allgulander C, Alonso J, Beghi E, et al. Cost of disorders of the brain in Europe 2010. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology. 2011 Oct;21(10):718-79.
7.Sigel A, Sigel H, Sigel RK. Metal Ions in Life Science. 1 ed: John Wiley & Sons; 2006.
8.Burdette SC, Lippard SJ. Meeting of the minds: metalloneurochemistry. Proceedings of the National Academy of Sciences of the United States of America. 2003 Apr 1;100(7):3605-10.
9.NCCLS. Control of Preanalytical Variation in Trace Element Determinations; Approved Guideline. National Committee for Clinical Laboratory Standards (NCCLS) Document C38-A. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087 USA1997.
10.Fraga CG. Relevance, essentiality and toxicity of trace elements in human health. Molecular Aspects of Medicine. 2005;26(4–5):235-44.
11.Anatoly V S. Bioelementology as an interdisciplinary integrative approach in life sciences: Terminology, classification, perspectives. Journal of Trace Elements in Medicine and Biology. 2011;25, Supplement 1(0):S3-S10.
12.Fraga CG. Relevance, essentiality and toxicity of trace elements in human health. Molecular aspects of medicine. 2005 Aug-Oct;26(4-5):235-44.
13.Zheng W, Monnot AD. Regulation of brain iron and copper homeostasis by brain barrier systems: implication in neurodegenerative diseases. Pharmacology & therapeutics. 2012 Feb;133(2):177-88.
14.Fraga CG, Oteiza PI. Iron toxicity and antioxidant nutrients. Toxicology. 2002;180(1):23-32.
15.Crichton RR, Ward RJ. Metal-based neurodegeneration : from molecular mechanisms to therapeutic strategies. Chichester ; Hoboken, NJ: J. Wiley & Sons; 2006.
16.Andrews NC. Iron homeostasis: insights from genetics and animal models. Nat Rev Genet. 2000;1(3):208-17.
17.De Domenico I, McVey Ward D, Kaplan J. Regulation of iron acquisition and storage: consequences for iron-linked disorders. Nature reviews Molecular cell biology. 2008 Jan;9(1):72-81.
18.Crichton R, Ward R. Metal-based neurodegeneration. 1st ed. Oxford: John Wiley & Sons, Lda; 2011.
19.Sipe JC, Lee P, Beutler E. Brain iron metabolism and neurodegenerative disorders. Developmental neuroscience. 2002;24(2-3):188-96.
20.Schipper HM. Neurodegeneration with brain iron accumulation - clinical syndromes and neuroimaging. Biochimica et biophysica acta. 2012 Mar;1822(3):350-60.
21.Koeppen AH. The history of iron in the brain. J Neurol Sci. 1995 Dec;134 Suppl:1-9.
22.Nielsen FH. Ultratrace minerals mythical elixirs or nutrients of concern? Boletin de la Asociacion Medica de Puerto Rico. 1991 Mar;83(3):131-3.
23.Albrecht J, Sonnewald U, Waagepetersen HS, Schousboe A. Glutamine in the central nervous system: function and dysfunction. Frontiers in bioscience : a journal and virtual library. 2007;12:332-43.
24.Fitsanakis VA, Zhang N, Garcia S, Aschner M. Manganese (Mn) and iron (Fe): interdependency of transport and regulation. Neurotoxicity research. 2010 Aug;18(2):124-31.
25.Finley JW. Manganese absorption and retention by young women is associated with serum ferritin concentration. The American journal of clinical nutrition. 1999 Jul;70(1):37-43.
26.Finley JW, Johnson PE, Johnson LK. Sex affects manganese absorption and retention by humans from a diet adequate in manganese. The American journal of clinical nutrition. 1994 Dec;60(6):949-55.

27.Aschner M, Dorman DC. Manganese: pharmacokinetics and molecular mechanisms of brain uptake. Toxicological reviews. 2006;25(3):147-54.
28.Aschner M. Manganese: brain transport and emerging research needs. Environmental health perspectives. 2000 Jun;108 Suppl 3:429-32.
29.Aschner M. The transport of manganese across the blood-brain barrier. Neurotoxicology. 2006 May;27(3):311-4.
30.Zhang D, Kanthasamy A, Anantharam V. Effects of manganese on tyrosine hydroxylase (TH) activity and TH-phosphorylation in a dopaminergic neural cell line. Toxicology and applied pharmacology. 2011 Jul 15;254(2):65-71.
31.Bowman AB, Kwakye GF, Herrero Hernández E, Aschner M. Role of manganese in neurodegenerative diseases. Journal of Trace Elements in Medicine and Biology. 2011;25(4):191-203.
32.Sanchez-Betancourt J, Anaya-Martínez V, Gutierrez-Valdez AL, Ordoñez-Librado JL, Montiel-Flores E, Espinosa-Villanueva J, et al. Manganese mixture inhalation is a reliable Parkinson disease model in rats. Neurotoxicology. 2012;33(5):1346-55.
33.Rashed MN. The role of trace elements on hepatitis virus infections: a review. Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements. 2011 Jul;25(3):181-7.
34.Linder MC, Hazegh-Azam M. Copper biochemistry and molecular biology. The American journal of clinical nutrition. 1996 May;63(5):797S-811S.
35.Cerpa W, Varela-Nallar L, Reyes AE, Minniti AN, Inestrosa NC. Is there a role for copper in neurodegenerative diseases? Molecular aspects of medicine. 2005 Aug-Oct;26(4-5):405-20.
36.Halbach O, Dermietzel R. Neurotransmitters and Neuromodulators - Handbook of Receptors and Biological Effects. 2nd ed. Germany: WILEY-VCH Verlag GmbH & Co; 2006.
37.Uauy R, Olivares M, Gonzalez M. Essentiality of copper in humans. The American journal of clinical nutrition. 1998 May;67(5 Suppl):952S-9S.
38.Shim H, Harris ZL. Genetic defects in copper metabolism. The Journal of nutrition. 2003 May;133(5 Suppl 1):1527S-31S.
39.Trumbo P, Yates AA, Schlicker S, Poos M. Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. Journal of the American Dietetic Association. 2001 Mar;101(3):294-301.
40.O'Dell BL. Role of zinc in plasma membrane function. The Journal of nutrition. 2000 May;130(5S Suppl):1432S-6S.
41.Truong-Tran AQ, Ho LH, Chai F, Zalewski PD. Cellular zinc fluxes and the regulation of apoptosis/gene-directed cell death. The Journal of nutrition. 2000 May;130(5S Suppl):1459S-66S.
42.Oteiza PI, Mackenzie GG, Verstraeten SV. Metals in neurodegeneration: involvement of oxidants and oxidant-sensitive transcription factors. Molecular aspects of medicine. 2004 Feb-Apr;25(1-2):103-15.
43.Dineley KE, Votyakova TV, Reynolds IJ. Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. Journal of neurochemistry. 2003 May;85(3):563-70.
44.Brown AM, Kristal BS, Effron MS, Shestopalov AI, Ullucci PA, Sheu KF, et al. Zn2+ inhibits alpha-ketoglutarate-stimulated mitochondrial respiration and the isolated alpha-ketoglutarate dehydrogenase complex. The Journal of biological chemistry. 2000 May 5;275(18):13441-7.
45.Sensi SL, Yin HZ, Carriedo SG, Rao SS, Weiss JH. Preferential Zn2+ influx through Ca2+-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production. Proceedings of the National Academy of Sciences of the United States of America. 1999 Mar 2;96(5):2414-9.
46.Lorusso M, Cocco T, Sardanelli AM, Minuto M, Bonomi F, Papa S. Interaction of Zn2+ with the bovine-heart mitochondrial bc1 complex. European journal of biochemistry / FEBS. 1991 Apr 23;197(2):555-61.
47.Sheline CT, Behrens MM, Choi DW. Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of NAD(+) and inhibition of glycolysis. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2000 May 1;20(9):3139-46.
48.Zago MP, Oteiza PI. The antioxidant properties of zinc: interactions with iron and antioxidants. Free radical biology & medicine. 2001 Jul 15;31(2):266-74.
49.Perez LR, Franz KJ. Minding metals: tailoring multifunctional chelating agents for neurodegenerative disease. Dalton transactions. 2010 Mar 7;39(9):2177-87.
50.Faller P, Hureau C. Bioinorganic chemistry of copper and zinc ions coordinated to amyloid-beta peptide. Dalton transactions. 2009 Feb 21(7):1080-94.

51.Bonda DJ, Lee HG, Blair JA, Zhu X, Perry G, Smith MA. Role of metal dyshomeostasis in Alzheimer's disease. Metallomics : integrated biometal science. 2011 Mar;3(3):267-70.
52.Sayre LM, Perry G, Harris PL, Liu Y, Schubert KA, Smith MA. In situ oxidative catalysis by neurofibrillary tangles and senile plaques in Alzheimer's disease: a central role for bound transition metals. Journal of neurochemistry. 2000 Jan;74(1):270-9.
53.Jomova K, Vondrakova D, Lawson M, Valko M. Metals, oxidative stress and neurodegenerative disorders. Molecular and cellular biochemistry. 2010 Dec;345(1-2):91-104.
54.Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. Free radical biology & medicine. 2000 Aug;29(3-4):222-30.
55.Castellani RJ, Rolston RK, Smith MA. Alzheimer disease. Disease-a-month : DM. 2010 Sep;56(9):484-546.
56.Association As. [cited 2012 21st October]; Available from: www.alz.org.
57.Rivera-Mancia S, Perez-Neri I, Rios C, Tristan-Lopez L, Rivera-Espinosa L, Montes S. The transition metals copper and iron in neurodegenerative diseases. Chemico-biological interactions. 2010 Jul 30;186(2):184-99.
58.Fratiglioni L, De Ronchi D, Aguero-Torres H. Worldwide prevalence and incidence of dementia. Drugs & aging. 1999 Nov;15(5):365-75.
59.Alzheimer's Disease International. World Alzheimer Report 2009. London: Alzheimer's disease International. 2009.
60.Hebert Le SPABLA, et al. Age-specific incidence of Alzheimer's disease in a community population. Journal of the American Medical Association. 1995;273(17):1354-9.
61.Zhu CW, Sano M. Economic considerations in the management of Alzheimer's disease. Clinical interventions in aging. 2006;1(2):143-54.
62.Querfurth HW, LaFerla FM. Alzheimer's Disease. New England Journal of Medicine. 2010;362(4):329-44.
63.Alzheimer's A. 2012 Alzheimer's disease facts and figures. Alzheimer's & dementia : the journal of the Alzheimer's Association. 2012;8(2):131-68.
64.Lee JY, Cole TB, Palmiter RD, Suh SW, Koh JY. Contribution by synaptic zinc to the gender-disparate plaque formation in human Swedish mutant APP transgenic mice. Proceedings of the National Academy of Sciences of the United States of America. 2002 May 28;99(11):7705-10.
65.Maynard CJ, Cappai R, Volitakis I, Cherny RA, Masters CL, Li QX, et al. Gender and genetic background effects on brain metal levels in APP transgenic and normal mice: implications for Alzheimer beta-amyloid pathology. Journal of inorganic biochemistry. 2006 May;100(5-6):952-62.
66.Selkoe DJ, Podlisny MB. Deciphering the genetic basis of Alzheimer's disease. Annual review of genomics and human genetics. 2002;3:67-99.
67.Rauk A. The chemistry of Alzheimer's disease. Chemical Society reviews. 2009 Sep;38(9):2698-715.
68.Paine SML, Lowe JS. Approach to the post-mortem investigation of neurodegenerative diseases: from diagnosis to research. Diagnostic Histopathology. 2011;17(5):211-6.
69.Querfurth HW, LaFerla FM. Alzheimer's disease. The New England journal of medicine. 2010 Jan 28;362(4):329-44.
70.Lee HG, Casadesus G, Zhu X, Takeda A, Perry G, Smith MA. Challenging the amyloid cascade hypothesis: senile plaques and amyloid-beta as protective adaptations to Alzheimer disease. Annals of the New York Academy of Sciences. 2004 Jun;1019:1-4.
71.Castellani RJ, Lee HG, Siedlak SL, Nunomura A, Hayashi T, Nakamura M, et al. Reexamining Alzheimer's disease: evidence for a protective role for amyloid-beta protein precursor and amyloid-beta. Journal of Alzheimer's disease : JAD. 2009;18(2):447-52.
72.Mungarro-Menchaca X, Ferrera P, Moran J, Arias C. beta-Amyloid peptide induces ultrastructural changes in synaptosomes and potentiates mitochondrial dysfunction in the presence of ryanodine. Journal of neuroscience research. 2002 Apr 1;68(1):89-96.
73.Hauptmann S, Keil U, Scherping I, Bonert A, Eckert A, Muller WE. Mitochondrial dysfunction in sporadic and genetic Alzheimer's disease. Experimental gerontology. 2006 Jul;41(7):668-73.
74.Lopez Salon M, Morelli L, Castano EM, Soto EF, Pasquini JM. Defective ubiquitination of cerebral proteins in Alzheimer's disease. Journal of neuroscience research. 2000 Oct 15;62(2):302-10.
75.Lovell MA, Robertson JD, Teesdale WJ, Campbell JL, Markesbery WR. Copper, iron and zinc in Alzheimer's disease senile plaques. J Neurol Sci. 1998 Jun 11;158(1):47-52.
76.Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, et al. The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. Biochemistry. 1999 Jun 15;38(24):7609-16.

77.Zhu X, Su B, Wang X, Smith MA, Perry G. Causes of oxidative stress in Alzheimer disease. Cellular and molecular life sciences : CMLS. 2007 Sep;64(17):2202-10.
78.House E, Mold M, Collingwood J, Baldwin A, Goodwin S, Exley C. Copper abolishes the beta-sheet secondary structure of preformed amyloid fibrils of amyloid-beta(42). Journal of Alzheimer's disease : JAD. 2009;18(4):811-7.
79.Borchardt T, Camakaris J, Cappai R, Masters CL, Beyreuther K, Multhaup G. Copper inhibits beta-amyloid production and stimulates the non-amyloidogenic pathway of amyloid-precursor-protein secretion. The Biochemical journal. 1999 Dec 1;344 Pt 2:461-7.
80.Kitazawa M, Cheng D, Laferla FM. Chronic copper exposure exacerbates both amyloid and tau pathology and selectively dysregulates cdk5 in a mouse model of AD. Journal of neurochemistry. 2009 Mar;108(6):1550-60.
81.Atwood CS, Scarpa RC, Huang X, Moir RD, Jones WD, Fairlie DP, et al. Characterization of copper interactions with alzheimer amyloid beta peptides: identification of an attomolar-affinity copper binding site on amyloid beta1-42. Journal of neurochemistry. 2000 Sep;75(3):1219-33.
82.Huang X, Cuajungco MP, Atwood CS, Hartshorn MA, Tyndall JD, Hanson GR, et al. Cu(II) potentiation of alzheimer abeta neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction. The Journal of biological chemistry. 1999 Dec 24;274(52):37111-6.
83.Opazo C, Huang X, Cherny RA, Moir RD, Roher AE, White AR, et al. Metalloenzyme-like activity of Alzheimer's disease beta-amyloid. Cu-dependent catalytic conversion of dopamine, cholesterol, and biological reducing agents to neurotoxic H(2)O(2). The Journal of biological chemistry. 2002 Oct 25;277(43):40302-8.
84.Crouch PJ, Barnham KJ, Duce JA, Blake RE, Masters CL, Trounce IA. Copper-dependent inhibition of cytochrome c oxidase by Abeta(1-42) requires reduced methionine at residue 35 of the Abeta peptide. Journal of neurochemistry. 2006 Oct;99(1):226-36.
85.Schlief ML, Craig AM, Gitlin JD. NMDA receptor activation mediates copper homeostasis in hippocampal neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005 Jan 5;25(1):239-46.
86.White AR, Reyes R, Mercer JF, Camakaris J, Zheng H, Bush AI, et al. Copper levels are increased in the cerebral cortex and liver of APP and APLP2 knockout mice. Brain research. 1999 Sep 25;842(2):439-44.
87.Bellingham SA, Lahiri DK, Maloney B, La Fontaine S, Multhaup G, Camakaris J. Copper depletion down-regulates expression of the Alzheimer's disease amyloid-beta precursor protein gene. The Journal of biological chemistry. 2004 May 7;279(19):20378-86.
88.Strozyk D, Launer LJ, Adlard PA, Cherny RA, Tsatsanis A, Volitakis I, et al. Zinc and copper modulate Alzheimer Abeta levels in human cerebrospinal fluid. Neurobiology of aging. 2009 Jul;30(7):1069-77.
89.Cater MA, McInnes KT, Li QX, Volitakis I, La Fontaine S, Mercer JF, et al. Intracellular copper deficiency increases amyloid-beta secretion by diverse mechanisms. The Biochemical journal. 2008 May 15;412(1):141-52.
90.Maurer I, Zierz S, Moller HJ. A selective defect of cytochrome c oxidase is present in brain of Alzheimer disease patients. Neurobiology of aging. 2000 May-Jun;21(3):455-62.
91.Loeffler DA, LeWitt PA, Juneau PL, Sima AA, Nguyen HU, DeMaggio AJ, et al. Increased regional brain concentrations of ceruloplasmin in neurodegenerative disorders. Brain research. 1996 Nov 4;738(2):265-74.
92.Avramovich-Tirosh Y, Amit T, Bar-Am O, Zheng H, Fridkin M, Youdim MB. Therapeutic targets and potential of the novel brain- permeable multifunctional iron chelator-monoamine oxidase inhibitor drug, M-30, for the treatment of Alzheimer's disease. Journal of neurochemistry. 2007 Jan;100(2):490-502.
93.Barnham KJ, Haeffner F, Ciccotosto GD, Curtain CC, Tew D, Mavros C, et al. Tyrosine gated electron transfer is key to the toxic mechanism of Alzheimer's disease beta-amyloid. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2004 Sep;18(12):1427-9.
94.Moalem S, Percy ME, Andrews DF, Kruck TP, Wong S, Dalton AJ, et al. Are hereditary hemochromatosis mutations involved in Alzheimer disease? American journal of medical genetics. 2000 Jul 3;93(1):58-66.
95.Rogers JT, Randall JD, Cahill CM, Eder PS, Huang X, Gunshin H, et al. An iron-responsive element type II in the 5'-untranslated region of the Alzheimer's amyloid precursor protein transcript. The Journal of biological chemistry. 2002 Nov 22;277(47):45518-28.

96.Ding B, Chen KM, Ling HW, Sun F, Li X, Wan T, et al. Correlation of iron in the hippocampus with MMSE in patients with Alzheimer's disease. Journal of magnetic resonance imaging : JMRI. 2009 Apr;29(4):793-8.
97.Barlow BK, Cory-Slechta DA, Richfield EK, Thiruchelvam M. The gestational environment and Parkinson's disease: evidence for neurodevelopmental origins of a neurodegenerative disorder. Reproductive toxicology. 2007 Apr-May;23(3):457-70.
98.Kaur D, Andersen J. Does cellular iron dysregulation play a causative role in Parkinson's disease? Ageing research reviews. 2004 Jul;3(3):327-43.
99.Doorn KJ, Lucassen PJ, Boddeke HW, Prins M, Berendse HW, Drukarch B, et al. Emerging roles of microglial activation and non-motor symptoms in Parkinson's disease. Progress in neurobiology. 2012 Aug;98(2):222-38.
100.Parkinson's Disease Foundation; Statistics on Parkinson's disease. [cited 2012 21 October]; Available from: www.pdf.org.
101.Toulouse A, Sullivan AM. Progress in Parkinson's disease-where do we stand? Progress in neurobiology. 2008 Aug;85(4):376-92.
102.Jankovic J. Searching for a relationship between manganese and welding and Parkinson's disease. Neurology. 2005 Jun 28;64(12):2021-8.
103.Gorell JM, Peterson EL, Rybicki BA, Johnson CC. Multiple risk factors for Parkinson's disease. J Neurol Sci. 2004 Feb 15;217(2):169-74.
104.Gibb WR, Lees AJ. Anatomy, pigmentation, ventral and dorsal subpopulations of the substantia nigra, and differential cell death in Parkinson's disease. Journal of neurology, neurosurgery, and psychiatry. 1991 May;54(5):388-96.
105.Barapatre N, Morawski M, Butz T, Reinert T. Trace element mapping in Parkinsonian brain by quantitative ion beam microscopy. Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms. 2010;268(11–12):2156-9.
106.Dinis-Oliveira RJ, Remiao F, Carmo H, Duarte JA, Navarro AS, Bastos ML, et al. Paraquat exposure as an etiological factor of Parkinson's disease. Neurotoxicology. 2006 Dec;27(6):1110-22.
107.Cole NB, Murphy DD, Lebowitz J, Di Noto L, Levine RL, Nussbaum RL. Metal-catalyzed oxidation of alpha-synuclein: helping to define the relationship between oligomers, protofibrils, and filaments. The Journal of biological chemistry. 2005 Mar 11;280(10):9678-90.
108.Alimonti A, Bocca B, Pino A, Ruggieri F, Forte G, Sancesario G. Elemental profile of cerebrospinal fluid in patients with Parkinson's disease. Journal of Trace Elements in Medicine and Biology. 2007;21(4):234-41.
109.Behnke S, Berg D, Becker G. Does ultrasound disclose a vulnerability factor for Parkinson's disease? Journal of neurology. 2003 Feb;250 Suppl 1:I24-7.
110.Cass WA, Grondin R, Andersen AH, Zhang Z, Hardy PA, Hussey-Andersen LK, et al. Iron accumulation in the striatum predicts aging-related decline in motor function in rhesus monkeys. Neurobiology of aging. 2007 Feb;28(2):258-71.
111.Fredriksson A, Archer T. Effect of postnatal iron administration on MPTP-induced behavioral deficits and neurotoxicity: behavioral enhancement by L-Dopa-MK-801 co-administration. Behavioural brain research. 2003 Feb 17;139(1-2):31-46.
112.Cheah JH, Kim SF, Hester LD, Clancy KW, Patterson SE, 3rd, Papadopoulos V, et al. NMDA receptor-nitric oxide transmission mediates neuronal iron homeostasis via the GTPase Dexras1. Neuron. 2006 Aug 17;51(4):431-40.
113.Maharaj DS, Maharaj H, Daya S, Glass BD. Melatonin and 6-hydroxymelatonin protect against iron-induced neurotoxicity. Journal of neurochemistry. 2006 Jan;96(1):78-81.
114.Mendez-Alvarez E, Soto-Otero R, Hermida-Ameijeiras A, Lopez-Martin ME, Labandeira-Garcia JL. Effect of iron and manganese on hydroxyl radical production by 6-hydroxydopamine: mediation of antioxidants. Free radical biology & medicine. 2001 Oct 15;31(8):986-98.
115.Gupta A, Dawson VL, Dawson TM. What causes cell death in Parkinson's disease? Annals of neurology. 2008 Dec;64 Suppl 2:S3-15.
116.Levenson CW, Tassabehji NM. Iron and ageing: an introduction to iron regulatory mechanisms. Ageing research reviews. 2004 Jul;3(3):251-63.
117.Popescu BF, George MJ, Bergmann U, Garachtchenko AV, Kelly ME, McCrea RP, et al. Mapping metals in Parkinson's and normal brain using rapid-scanning x-ray fluorescence. Physics in medicine and biology. 2009 Feb 7;54(3):651-63.
118.Beshgetoor D, Hambidge M. Clinical conditions altering copper metabolism in humans. The American journal of clinical nutrition. 1998 May;67(5 Suppl):1017S-21S.

119.Riederer P, Sofic E, Rausch WD, Schmidt B, Reynolds GP, Jellinger K, et al. Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. Journal of neurochemistry. 1989 Feb;52(2):515-20.
120.Boll MC, Sotelo J, Otero E, Alcaraz-Zubeldia M, Rios C. Reduced ferroxidase activity in the cerebrospinal fluid from patients with Parkinson's disease. Neuroscience letters. 1999 Apr 23;265(3):155-8.
121.Arredondo M, Nunez MT. Iron and copper metabolism. Molecular aspects of medicine. 2005 Aug-Oct;26(4-5):313-27.
122.Aguirre P, Mena N, Tapia V, Arredondo M, Nunez MT. Iron homeostasis in neuronal cells: a role for IREG1. BMC neuroscience. 2005;6:3.
123.Aigner E, Theurl I, Haufe H, Seifert M, Hohla F, Scharinger L, et al. Copper availability contributes to iron perturbations in human nonalcoholic fatty liver disease. Gastroenterology. 2008 Aug;135(2):680-8.
124.Leigh PN, Abrahams S, Al-Chalabi A, Ampong MA, Goldstein LH, Johnson J, et al. The management of motor neurone disease. Journal of neurology, neurosurgery, and psychiatry. 2003 Dec;74 Suppl 4:iv32-iv47.
125.Ostachowicz B, Lankosz M, Tomik B, Adamek D, Wobrauschek P, Streli C, et al. Analysis of some chosen elements of cerebrospinal fluid and serum in amyotrophic lateral sclerosis patients by total reflection X-ray fluorescence. Spectrochimica Acta Part B: Atomic Spectroscopy. 2006;61(10–11):1210-3.
126.Wijesekera LC, Leigh PN. Amyotrophic lateral sclerosis. Orphanet journal of rare diseases. 2009;4:3.
127.Gurney ME, Pu H, Chiu AY, Dal Canto MC, Polchow CY, Alexander DD, et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science. 1994 Jun 17;264(5166):1772-5.
128.Ince PG, Shaw PJ, Candy JM, Mantle D, Tandon L, Ehmann WD, et al. Iron, selenium and glutathione peroxidase activity are elevated in sporadic motor neuron disease. Neuroscience letters. 1994 Nov 21;182(1):87-90.
129.Wang XS, Lee S, Simmons Z, Boyer P, Scott K, Liu W, et al. Increased incidence of the Hfe mutation in amyotrophic lateral sclerosis and related cellular consequences. J Neurol Sci. 2004 Dec 15;227(1):27-33.
130.Thompson KJ, Shoham S, Connor JR. Iron and neurodegenerative disorders. Brain research bulletin. 2001 May 15;55(2):155-64.
131.Hayward LJ, Rodriguez JA, Kim JW, Tiwari A, Goto JJ, Cabelli DE, et al. Decreased metallation and activity in subsets of mutant superoxide dismutases associated with familial amyotrophic lateral sclerosis. The Journal of biological chemistry. 2002 May 3;277(18):15923-31.
132.Valentine JS, Hart PJ. Misfolded CuZnSOD and amyotrophic lateral sclerosis. Proceedings of the National Academy of Sciences of the United States of America. 2003 Apr 1;100(7):3617-22.
133.Hart PJ. Pathogenic superoxide dismutase structure, folding, aggregation and turnover. Current opinion in chemical biology. 2006 Apr;10(2):131-8.
134.Estevez AG, Crow JP, Sampson JB, Reiter C, Zhuang Y, Richardson GJ, et al. Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. Science. 1999 Dec 24;286(5449):2498-500.
135.Willis JB. The early days of atomic absorption spectrometry in clinical chemistry. Spectrochimica Acta Part B: Atomic Spectroscopy. 1999;54(14):1971-5.
136.Parsons PJ, Barbosa Jr F. Atomic spectrometry and trends in clinical laboratory medicine. Spectrochimica Acta Part B: Atomic Spectroscopy. 2007;62(9):992-1003.
137.Todd AC, Parsons PJ, Carroll S, Geraghty C, Khan FA, Tang S, et al. Measurements of lead in human tibiae. A comparison between K-shell x-ray fluorescence and electrothermal atomic absorption spectrometry. Physics in medicine and biology. 2002 Feb 21;47(4):673-87.
138.Thermo Elemental; AAS, GFAAS, ICP or ICP-MS? Which technique should I use? 2001 [cited 2012 21 October]; Available from: http://www.thermo.com/eThermo/CMA/PDFs/Articles/articlesFile_18407.pdf.
139.Concordia College. [cited 2012 21 October]; Available from: http://www.cord.edu/dept/chemistry/analyticallabmanual/experiments/aa/intro.html.
140.Bettmer J, Montes Bayon M, Encinar JR, Fernandez Sanchez ML, Fernandez de la Campa Mdel R, Sanz Medel A. The emerging role of ICP-MS in proteomic analysis. Journal of proteomics. 2009 Aug 20;72(6):989-1005.
141.Piguet O, Double KL, Kril JJ, Harasty J, Macdonald V, McRitchie DA, et al. White matter loss in healthy ageing: a post-mortem analysis. Neurobiology of aging. 2009 Aug;30(8):1288-95.

142.Bilgic B, Pfefferbaum A, Rohlfing T, Sullivan EV, Adalsteinsson E. MRI estimates of brain iron concentration in normal aging using quantitative susceptibility mapping. NeuroImage. 2012 Feb 1;59(3):2625-35.
143.Xu X, Wang Q, Zhang M. Age, gender, and hemispheric differences in iron deposition in the human brain: an in vivo MRI study. NeuroImage. 2008 Mar 1;40(1):35-42.
144.Andrási E, Igaz S, Szoboszlai N, Farkas É, Ajtony Z. Several methods to determine heavy metals in the human brain. Spectrochimica Acta Part B: Atomic Spectroscopy. 1999;54(5):819-25.
145.Rajan MT, Jagannatha Rao KS, Mamatha BM, Rao RV, Shanmugavelu P, Menon RB, et al. Quantification of trace elements in normal human brain by inductively coupled plasma atomic emission spectrometry. Journal of the Neurological Sciences. 1997;146(2):153-66.
146.Andrasi E, Farkas E, Gawlik D, Rosick U, Bratter P. Brain Iron and Zinc Contents of German Patients with Alzheimer Disease. Journal of Alzheimer's disease : JAD. 2000 Mar;2(1):17-26.
147.Lutsenko S, Bhattacharjee A, Hubbard AL. Copper handling machinery of the brain. Metallomics : integrated biometal science. 2010 Sep;2(9):596-608.
148.Takeda A. Manganese action in brain function. Brain research Brain research reviews. 2003 Jan;41(1):79-87.
149.Tracqui A, Tayot J, Kintz P, Alves G, Bosque MA, Mangin P. Determination of manganese in human brain samples. Forensic science international. 1995 Dec 29;76(3):199-203.
150.Gotz ME, Double K, Gerlach M, Youdim MB, Riederer P. The relevance of iron in the pathogenesis of Parkinson's disease. Annals of the New York Academy of Sciences. 2004 Mar;1012:193-208.
151.Rhodes SL, Ritz B. Genetics of iron regulation and the possible role of iron in Parkinson's disease. Neurobiology of disease. 2008 Nov;32(2):183-95.
152.Deibel MA, Ehmann WD, Markesbery WR. Copper, iron, and zinc imbalances in severely degenerated brain regions in Alzheimer's disease: possible relation to oxidative stress. J Neurol Sci. 1996 Nov;143(1-2):137-42.
153.Panayi AE, Spyrou NM, Iversen BS, White MA, Part P. Determination of cadmium and zinc in Alzheimer's brain tissue using inductively coupled plasma mass spectrometry. J Neurol Sci. 2002 Mar 15;195(1):1-10.
154.Plantin L, Lying-Tunell U, Kristensson K. Trace elements in the human central nervous system studied with neutron activation analysis. Biological trace element research. 1987;13(1):69-75.
155.Hung YH, Bush AI, Cherny RA. Copper in the brain and Alzheimer's disease. Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry. 2010 Jan;15(1):61-76.
156.Guilarte TR. APLP1, Alzheimer's-like pathology and neurodegeneration in the frontal cortex of manganese-exposed non-human primates. Neurotoxicology. 2010 Sep;31(5):572-4.
157.Bowler RM, Gysens S, Diamond E, Nakagawa S, Drezgic M, Roels HA. Manganese exposure: Neuropsychological and neurological symptoms and effects in welders. Neurotoxicology. 2006;27(3):315-26.
158.Bouchard M, Mergler D, Baldwin M, Panisset M, Bowler R, Roels HA. Neurobehavioral functioning after cessation of manganese exposure: A follow-up after 14 years. American Journal of Industrial Medicine. 2007;50(11):831-40.
159.Bowler RM, Koller W, Schulz PE. Parkinsonism due to manganism in a welder: Neurological and neuropsychological sequelae. Neurotoxicology. 2006;27(3):327-32.
160.Barnham KJ, Bush AI. Metals in Alzheimer's and Parkinson's diseases. Current opinion in chemical biology. 2008 Apr;12(2):222-8.
161.von Bohlen und Halbach O, Dermietzel R. Neurotransmitters (chapter 3). Neurotransmitters and Neuromodulators: Wiley-VCH Verlag GmbH & Co. KGaA; 2003.
162.Cersosimo MG, Koller WC. The diagnosis of manganese-induced parkinsonism. Neurotoxicology. 2006 May;27(3):340-6.
163.Covy JP, Giasson BI. α-Synuclein, leucine-rich repeat kinase-2, and manganese in the pathogenesis of parkinson disease. Neurotoxicology. 2011;32(5):622-9.
164.Pifl C, Khorchide M, Kattinger A, Reither H, Hardy J, Hornykiewicz O. α-Synuclein selectively increases manganese-induced viability loss in SK-N-MC neuroblastoma cells expressing the human dopamine transporter. Neuroscience letters. 2004;354(1):34-7.
165.Prabhakaran K, Chapman GD, Gunasekar PG. α-Synuclein overexpression enhances manganese-induced neurotoxicity through the NF-κB-mediated pathway. Toxicology Mechanisms and Methods. 2011;21(6):435-43.

166.Kasarskis EJ, Tandon L, Lovell MA, Ehmann WD. Aluminum, calcium, and iron in the spinal cord of patients with sporadic amyotrophic lateral sclerosis using laser microprobe mass spectroscopy: a preliminary study. J Neurol Sci. 1995 Jun;130(2):203-8.
167.da Rocha AJ, Oliveira AS, Fonseca RB, Maia AC, Jr., Buainain RP, Lederman HM. Detection of corticospinal tract compromise in amyotrophic lateral sclerosis with brain MR imaging: relevance of the T1-weighted spin-echo magnetization transfer contrast sequence. AJNR American journal of neuroradiology. 2004 Oct;25(9):1509-15.

VI. ATTACHMENTS

Attachment 1 – Description of analyzed subjects with no documented neurodegenerative diseases.

Age Range	Cause of Death	Gender	Age	Brain Hemisphere	*Post-mortem* Interval (hours)
20-30	Chest trauma by firearm	M	20	R	24
	Asphyxia from hanging	M	24	R	24
50-60	Asphyxia from hanging	M	57	R	24
	Burning lesions	M	53	R	24
	AMI	M	58	R	24
	ANHP	M	56	R	24
	AMI	M	54	R	
	?	M	57	R	24
	?	M	54	R	
	AMI	M	55	R	72
	AAA rupture	F	54	R	24
	Asphyxia from hanging	M	56	R	24
60-70	AMI	M	68	R	48
	AMI	M	60	R	24
	AMI	M	60	L[1]	14
	AMI	M	66	R	48
	AMI	M	63	R	24
	AMI	M	68	R	24
	Multiorgan failure	F	69	L[1]	24
	AMI	M	63	R	72
	?	F	60	R	
	asphyxia from drowning	f	68	L[1]	12
70-80	Acute pancreatitis	F	76	R	24
	Cardiorespiratory insufficiency	M	75	R	24
	AAA rupture	M	71	R	24
	Cardiomyopathy ischemic	F	71	R	
	PA rupture	M	72	R	48
	Asphyxia from hanging	M	72	R	36
	?	F	71	R	48
	?	F	70	R	20
	?	F	73	R	24
	Acute pancreatitis	M	78	R	14

Age Range	Cause of Death	Gender	Age	Brain Hemisphere	*Post-mortem* Interval (hours)
80-90	Digestive hemorrhage	M	81	R	72
	Pulmonary infection	F	84	R	18
	AAA rupture	M	84	R	48
	?	F	82	R	
	?	M	80	R	12
	Chest trauma by firearm	M	81	R	24
	Asphyxia from hanging	M	88	R	24
		M	84	R	
	Asphyxia from hanging	F	88	R	24
≥90	Pneumonia	F	92	R	
	Cardiac rupture	F	90	L[1]	
	Respiratory infection	F	102	R	

ANHP: Acute Necrohemorrhagic Pancreatitis; AMI: Acute Myocardial Infarction*;* AAA: aortic abdominal aneurysm*;* PA: Pulmonary artery; *?: undetermined cause of death;* M: Male; F: Female; R: Right; L: Left.

Attachment 2 – Description of analyzed subjects with documented neurodegenerative diseases.

ND	Cause of Death	Gender	Age	Brain Hemisphere	*Post-mortem* Interval (hours)
AD	Pulmonary thromboembolism	F	73	R	
AD	Consumption coagulopathy	F	85	R	
PD		F	91	R	
ALS	Vomit aspiration	M	84	R	24

[1] It was sampled the left hemisphere due to non-recent stroke areas on right hemisphere.

Attachment 3 – Distribution of Cu (µg/g dry tissue) in different regions of healthy human brain.

Age range	1	2A	2B	3A	3B	3C	4	5	6	7	8	9	10	11
20-30	21	32	15	5	119	14	52	10	24	21	21	6	6	32
	19	19	16	18	33	21	17	20	20	18	18	24	5	10
50-60	37	28	34	30	35	27	27	34	37	32	39	31	21	29
	40	38	37	52	47	29	46	27	43	40	38	36	9	31
	22	39	16	14	34	15	13	11	40	20	19	4	11	19
	22	23	20	30	30	16	24	16	21	23	20	6	10	23
	15	12	20	11	36	11	19	16	16	16	19	6	4	23
	20	21	35	29	22	36	32	33	13	24	15	30	10	27
	21	21	19	2	27	17	14	18	26	23	31	7	15	27
	31	40	28	71	55	2	1	30	23	27	25	11	15	27
	20	39	17	3	70	45	30	17	30	22	18	11	7	32
	18	17	23	16	42	15	8	17	25	16	22	6	13	18
60-70	18	18	18	23	23	18	16	15	18	17	24	23	30	4
	13	14	9	15	65	16	18	9	13	17	18	9	3	23
	20	20	20	21	29	21	18	16	15	22	19	19	6	23
	18	14	16	28	31	17	17	16	25	25	26	29	7	26
	14	14	14	13	21	15	13	12	16	19	24	23	9	23
	20	19	22	15	31	31	22	16	17	17	15	13	7	23
	19	18	23	31	38	24	18	16	18	23	25	17	16	28
	25	21	24	29	34	22	24	17	22	23	22	51	28	6
	27	31	29	33	42	59	13	21	26	29	21	22	10	26
	16	13	30	5	76	14	12	15	26	20	19	6	9	16
70-80	7	26	28	34	42	19	26	45	18	29	37	29	48	18
	31	37	38	22	30	26	30	26	31	32	25	19	14	29
	23	15	21	31	33	19	14	12	15	17	14	7	7	12
	19	27	23	26	32	19	27	25	27	26	33	22	20	31
	16	16	18	15	23	18	13	10	22	14	20	12	8	17
	17	18	20	9	22	31	23	10	32	22	24	5	13	21
	17	17	20	4	91	12	52	17	23	19	9	7	8	22
	24	18	15	20	33	41	8	53	8	27	24	8	14	24
	26	16	27	48	31	21	33	18	35	33	24	10	15	33
	17	16	17	22	27	19	2	9	13	15	16	12	21	4
80-90	23	22	22	27	47	28	21	16	19	17	26	16	5	64
	19	19	19	21	29	25	18	15	20	22	22	19	15	31
	14	15	18	14	26	14	9	10	10	11	11	4	7	16
	22	21	X	38	34	19	15	15	18	21	27	11	12	23
	19	31	17	8	61	30	19	9	90	23	21	8	10	24
	18	16	28	41	36	22	21	20	79	27	31	7	6	16
	25	25	24	26	33	22	23	18	23	23	23	15	11	28
	9	12	8	8	23	19	7	6	8	9	11	X	2	11
	14	26	18	24	26	14	14	15	17	20	21	7	5	19
> 90	21	19	20	32	39	27	20	18	20	23	26	27	6	20
	32	25	29	17	38	17	30	21	29	23	28	20	11	21
	13	17	42	22	37	29	23	18	19	20	19	20	10	15

Attachment 4 – Distribution of Fe (µg/g dry tissue) in different regions of healthy human brain.

Age range	1	2A	2B	3A	3B	3C	4	5	6	7	8	9	10	11
20-30	318	277	231	402	414	884	169	211	251	290	222	80	86	182
	246	188	171	195	404	498	142	184	184	237	259	47	23	114
50-60	463	497	478	574	701	1083	329	353	412	431	273	125	68	206
	232	262	316	872	1337	703	1572	252	246	270	312	100	23	155
	336	393	328	242	624	430	264	282	336	391	349	84	76	131
	254	267	247	581	706	419	198	191	261	285	251	75	38	256
	185	217	187	380	447	251	117	154	189	181	169	29	35	156
	207	264	219	609	934	924	185	234	281	220	107	418	49	164
	237	282	295	27	908	460	85	248	271	378	206	83	53	422
	232	293	346	926	835	401	170	261	342	394	290	131	63	306
	248	210	225	621	861	697	180	204	211	288	203	80	20	520
	234	231	257	500	415	402	215	207	242	282	409	74	52	120
60-70	330	347	244	908	488	343	187	230	263	300	437	88	43	124
	255	259	246	557	731	1004	206	136	444	340	212	146	13	238
	329	315	367	486	830	1040	208	281	212	322	351	106	59	385
	282	337	232	532	855	440	184	232	304	395	314	95	37	309
	304	211	285	306	839	646	235	277	251	228	278	142	28	450
	315	343	345	278	691	1194	360	368	296	317	360	79	101	457
	279	289	325	962	1973	1350	257	284	372	436	572	151	79	358
	216	273	247	248	858	799	229	213	231	290	311	51	64	150
	211	207	205	396	909	395	262	170	183	152	206	29	60	109
	282	310	267	648	659	382	186	205	270	299	173	89	77	287
70-80	245	291	429	763	1042	501	215	196	219	327	367	123	64	188
	314	326	320	199	504	225	312	283	287	279	271	110	53	244
	375	451	389	672	958	724	242	274	395	355	342	112	69	416
	272	295	299	528	845	428	257	292	345	475	326	101	179	425
	308	292	337	334	500	572	291	156	345	604	420	121	89	239
	427	297	235	502	627	281	191	240	287	365	214	70	76	156
	180	193	176	579	1132	789	145	149	198	206	54	11	50	315
	306	263	295	859	328	459	336	250	302	758	293	148	115	345
	333	311	234	537	1108	867	258	259	365	274	219	69	68	409
	389	397	364	1103	1439	1078	218	271	311	314	260	130	55	249
80-90	290	257	248	902	2742	1539	224	281	265	289	247	71	18	468
	296	364	316	313	1628	1870	325	332	376	487	481	145	95	410
	293	178	588	825	1460	428	208	337	215	180	237	35	72	406
	192	223	X	787	782	1061	162	222	232	293	492	116	71	192
	209	270	240	1477	1563	745	167	206	230	335	470	94	28	218
	582	165	269	877	1037	1510	384	232	457	443	308	253	17	225
	174	291	202	528	973	818	156	156	189	261	224	89	31	349
	282	327	278	284	1149	1255	231	178	291	312	230	X	23	241
	213	452	235	691	651	331	172	272	339	162	252	129	275	237
> 90	428	343	378	646	677	735	221	227	256	328	246	121	63	200
	330	366	303	268	1246	405	292	226	296	423	415	35	0	447
	161	208	465	279	1007	1066	214	228	265	289	152	98	82	215

Attachment 5 – Distribution of Mn (µg/g dry tissue) in different regions of healthy human brain.

Age range	1	2A	2B	3A	3B	3C	4	5	6	7	8	9	10	11
20-30	1,06	0,95	0,92	2,22	1,98	1,62	0,85	1,21	0,87	1,13	1,06	0,67	0,64	0,81
	0,86	0,94	0,96	1,84	2,35	1,57	0,92	1,14	1,02	1,01	1,14	0,85	0,83	0,77
50-60	0,99	0,59	1,02	0,92	1,63	1,36	0,76	0,77	0,98	1,02	1,06	0,81	0,72	0,49
	1,15	1,23	0,95	2,63	3,33	3,02	1,55	1,82	1,60	1,53	1,67	1,18	0,32	0,96
	1,12	1,07	1,09	1,71	2,50	1,63	1,04	1,55	1,03	1,29	1,37	0,76	0,98	0,90
	0,99	0,91	0,88	2,28	2,28	1,32	1,00	1,12	0,90	0,91	0,92	0,63	0,68	0,68
	0,96	1,00	0,99	2,39	2,04	1,19	0,91	1,22	0,89	0,93	1,15	0,80	0,57	0,76
	0,81	0,94	0,98	2,10	2,61	1,88	0,92	1,13	0,96	1,04	0,63	1,73	0,80	0,75
	0,67	0,64	0,65	0,04	1,75	0,81	0,19	1,34	1,06	0,84	1,30	0,81	0,76	1,08
	1,07	1,14	1,08	4,04	2,75	1,97	1,07	1,13	1,18	1,04	1,18	1,00	0,83	1,02
	1,04	0,98	1,02	2,49	2,53	1,88	0,99	1,22	0,98	1,38	1,14	0,93	0,98	0,93
	0,94	1,09	0,98	2,10	1,64	1,18	0,92	1,21	0,88	1,02	1,13	0,52	0,65	0,68
60-70	0,97	0,92	0,99	2,35	1,83	1,37	1,00	1,53	1,01	1,04	1,27	0,99	1,24	0,45
	0,88	0,88	0,85	1,94	1,81	1,54	0,95	0,53	2,12	0,92	0,85	0,50	0,24	2,07
	0,99	0,97	1,04	1,59	2,27	1,89	0,99	1,24	0,75	1,59	1,08	0,81	0,65	0,79
	0,89	0,54	0,73	8,97	3,24	1,67	3,28	1,17	1,44	1,91	1,27	1,13	0,99	0,78
	0,76	0,73	0,79	0,92	1,40	0,88	0,85	0,75	0,80	1,14	0,97	1,16	0,82	0,57
	0,40	0,37	0,41	0,43	0,68	0,63	0,40	0,40	0,64	0,54	0,51	0,46	0,27	0,49
	1,03	0,91	1,10	2,75	3,20	2,30	1,04	1,20	1,06	1,51	1,41	0,93	0,97	0,99
	1,16	1,02	1,02	2,39	2,69	2,66	1,02	1,36	1,06	1,21	1,19	1,45	1,21	0,52
	1,35	1,79	1,77	3,88	4,53	3,53	3,76	2,23	1,84	2,58	2,69	1,97	1,81	1,80
	0,92	0,94	0,90	2,00	1,84	1,17	0,85	0,94	0,86	1,03	0,98	0,59	0,74	0,84
70-80	0,60	1,12	1,16	2,63	3,03	1,47	1,08	2,06	0,74	1,67	1,64	1,42	2,40	0,87
	1,00	1,05	0,99	2,31	0,72	1,46	1,05	1,10	1,03	1,23	0,76	0,72	0,72	0,78
	1,63	0,78	1,60	3,94	3,26	2,27	1,16	1,59	1,24	1,39	0,92	0,68	0,76	0,83
	1,38	1,57	1,44	2,48	2,80	1,30	1,05	1,16	1,15	1,38	1,16	0,85	1,11	0,96
	0,91	0,93	1,02	1,50	1,71	1,36	0,83	0,67	1,24	0,85	0,97	0,70	0,74	0,75
	0,98	0,95	0,91	2,67	2,20	1,64	1,03	1,50	1,23	1,29	1,22	0,66	1,07	0,85
	0,65	1,05	0,69	2,46	2,97	2,55	1,02	1,23	1,00	1,18	0,70	0,70	0,68	1,03
	1,23	1,05	1,14	2,34	1,26	1,58	1,21	1,26	1,10	2,21	1,19	0,80	0,97	0,83
	0,92	0,87	1,05	1,68	2,53	1,42	2,33	1,17	1,02	1,00	0,85	0,59	0,75	0,83
	1,12	1,03	1,15	2,68	3,15	2,87	1,01	1,19	0,95	1,01	0,92	0,83	1,22	0,77
80-90	1,08	1,18	1,05	2,38	3,64	3,27	4,04	1,14	0,96	1,06	0,83	0,61	0,38	1,83
	0,94	1,09	0,86	2,61	2,23	2,07	0,69	0,98	1,09	1,23	0,99	0,59	0,81	0,72
	0,91	0,84	0,95	1,76	2,08	0,96	0,86	1,10	0,79	1,00	0,81	0,53	0,65	0,64
	1,12	0,91	X	2,60	2,56	7,67	0,79	1,29	0,82	0,95	0,98	0,73	0,82	0,83
	0,90	0,85	0,95	2,66	2,94	1,40	0,83	1,10	0,89	0,88	0,89	0,57	0,79	0,77
	0,80	0,80	1,61	3,14	4,46	5,17	2,61	1,84	6,93	2,72	2,13	0,69	0,98	1,00
	0,88	1,08	0,90	1,56	2,12	1,29	0,68	0,89	0,67	0,76	0,78	0,62	0,62	0,83
	1,15	1,36	1,12	1,92	3,09	2,79	1,13	1,17	1,12	1,17	1,10	X	0,88	1,18
	0,75	1,41	1,02	2,60	2,31	1,26	1,02	1,21	0,94	1,04	0,99	0,56	0,81	0,82
> 90	1,13	0,93	1,26	2,08	2,24	2,79	1,07	1,25	1,03	1,18	1,02	0,97	0,59	0,80
	1,50	1,49	1,36	1,52	3,25	1,97	1,54	1,40	1,22	1,25	1,29	0,93	0,89	1,03
	0,69	0,94	1,98	1,71	2,72	2,23	1,18	1,44	1,08	1,02	1,05	1,12	1,37	1,18

Attachment 6 – Distribution of Zn (µg/g dry tissue) in different regions of healthy human brain.

Age range	1	2A	2B	3A	3B	3C	4	5	6	7	8	9	10	11
20-30	62	60	59	83	62	48	43	69	55	54	38	28	26	39
	53	60	64	39	72	37	43	66	48	43	32	30	34	30
50-60	72	57	76	67	63	47	53	81	72	63	44	42	43	32
	51	49	52	72	53	37	69	57	60	54	37	30	11	26
	56	23	63	45	48	35	44	71	43	46	33	26	31	33
	54	61	54	44	49	27	72	78	58	57	34	26	31	25
	53	45	57	53	42	27	53	69	45	49	34	40	23	33
	53	65	75	63	70	53	60	69	58	56	23	51	41	34
	61	56	61	7	54	31	33	86	58	50	51	38	41	46
	72	61	72	73	68	34	59	50	61	65	41	33	34	41
	67	70	65	71	65	54	59	65	56	52	38	35	24	38
	60	61	59	52	47	33	52	79	55	54	34	24	32	32
60-70	65	57	67	86	53	38	57	79	60	49	41	38	39	18
	50	59	62	56	47	47	57	26	142	45	26	19	9	87
	63	63	69	46	62	51	55	70	45	47	32	30	59	43
	50	33	49	70	61	43	49	59	82	64	47	40	34	36
	61	53	63	43	55	41	59	57	63	68	56	50	43	47
	67	67	81	46	68	66	63	71	69	55	49	39	41	54
	62	54	75	73	85	59	59	72	68	65	48	42	47	47
	71	55	66	60	61	44	58	65	63	54	40	49	36	20
	60	66	65	53	60	124	20	64	53	59	35	26	24	41
	51	59	59	46	50	37	40	56	51	55	37	26	32	32
70-80	29	56	68	72	68	35	67	95	41	96	88	66	69	32
	65	79	78	55	57	58	59	66	61	59	37	33	36	39
	92	51	78	81	68	42	58	64	52	55	27	24	23	27
	55	75	65	59	51	28	70	81	60	57	46	42	56	45
	68	58	77	48	65	41	50	49	77	46	39	32	33	36
	45	44	43	58	44	35	41	69	49	53	33	28	33	32
	29	70	46	56	59	63	56	64	59	54	28	28	30	44
	68	61	69	56	51	41	67	71	60	65	38	31	35	34
	59	51	79	57	75	47	67	87	71	73	34	26	38	41
	72	57	77	71	73	52	54	63	56	43	32	29	43	18
80-90	67	66	78	62	94	63	60	65	61	48	36	28	14	76
	69	49	65	77	66	61	53	69	75	55	42	27	36	44
	72	65	86	46	86	47	37	51	38	45	32	21	41	39
	78	67	X	88	77	52	50	74	50	49	42	34	47	36
	67	57	73	70	78	42	55	72	60	58	44	33	37	37
	71	66	94	74	73	55	70	78	178	69	60	22	26	31
	63	67	67	54	65	46	62	78	73	62	42	35	39	44
	75	88	62	32	63	48	53	57	56	44	31	X	22	35
	43	85	69	69	55	29	46	64	55	52	34	23	23	32
> 90	68	58	71	79	69	64	71	84	71	60	52	47	31	36
	80	56	78	61	62	39	72	79	69	54	45	26	31	41
	51	56	162	59	82	70	78	80	69	60	42	39	69	32

Attachment 7 – Distribution of Cu, Fe, Mn and Zn (µg/g dry tissue) in different regions of ND patients.

Brain	PD1				AD1				AD2				ALS			
region	Fe	Mn	Cu	Zn	Fe	Mn	Cu	Zn	Fe	Mn	Cu	Zn	Fe	Mn	Cu	Zn
1	247	1,1	17,0	38	198	0,8	12	67	280	1,2	36	89	190	0,9	17	64
2a	348	0,7	15,5	54	356	0,8	13	64	352	1,2	33	87	166	0,6	17	45
2b	294	0,9	14,0	70	323	1,0	12	58	267	1,1	27	81	198	0,8	17	56
3a	932	2,0	18,0	54	1170	1,9	18	64	1268	3,7	45	88	310	1,7	24	49
3b	1453	9,1	22,5	58	856	2,2	20	72	1539	4,4	52	95	1186	2,5	29	64
3c	1660	1,5	20,8	71	1433	2,1	26	129	1410	4,0	31	66	619	1,3	23	40
4	356	2,0	13,3	68	252	0,6	7	41	267	1,0	24	66	174	0,8	18	48
5	295	1,8	13,4	93	505	0,8	12	72	327	1,6	27	87	256	1,4	14	68
6	147	1,6	16,6	68	340	0,9	15	71	323	1,5	35	80	196	0,7	16	40
7	433	1,6	17,6	54	312	1,1	16	63	506	1,6	34	67	284	1,0	21	58
8	327	1,6	25,2	38	275	0,8	14	40	589	1,8	39	66	285	1,0	22	50
9	111	1,0	5,5	30	102	0,6	9	29	145	1,0	21	44	50	0,6	10	32
10	61	2,0	11,5	47	55	0,7	8	45	123	1,4	20	56	36	0,9	8	31
11	471	0,9	16,4	37	351	0,6	24	47	360	0,9	27	41	339	1,0	29	45