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PhD THESIS WORK:

RELATIONSHIP BETWEEN EXPOSURE TO THE ENVIRONMENTAL TOXICANT METHYLMERCURY AND THE TRANSCRIPTIONAL REPRESSOR REST IN VITRO MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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INTRODUCTION

1.1 Introduction to the topic

The human being is a complex biological system of a dynamic type continually reacting to both internal and external stimulus and trying to adapt to the witnesses' changes. Particularly, the interaction with the external environment puts the biological systems to continuous changes not only of macroscopic type but also of molecular and cellular order which must contain a possible and probable damage that the change itself might produce. If we consider only the respiratory and nutritional activities we realize that there are thousands of molecules that we continually introduce into our body. If we add to these two activities all the problems connected to air, water and soil pollution, the number of xenobiotics our organism is subjected to is almost endless. The human body has developed many systems of biotransformation of exogenous substances with the aim of canceling or simply reducing their toxicity. However, in some cases some substances are recognized as foreign by our organism and when they are subjected to these biotransformation systems they are activated and they produce metabolic intermediates or even final products being inert substances that exert a powerful toxic action even more powerful than the substance of origin on the various organs and systems. Moreover, since the different substances are never absorbed individually but in different combinations for typology and concentrations, extremely variable and unpredictable toxicity dynamics are established at times. In recent years, environmental medicine has been developing more and more; this is a discipline studying the biological and

etiopathogenic implications produced by the interaction of biological systems and the main families of environmental contaminants. The combined effect of several substances can be additive or multiplicative and it greatly amplifies the damage produced individually by them and the mechanisms through which these processes are established can be much more complex and regulated by a plethora of biological substances and molecules responsible for the harmful final effect. Every day hundreds of new chemical substances are placed on the market whose toxic effects can occur even after years or even decades from exposure. To produce damage, exposure does not have to be massive or of considerable entity: in fact, all the problem linked to microdoses of xenobiotics must not be underestimated also because there is no dream dose for some molecules capable of influencing biological systems; for this reason the problem is even more complex and difficult to approach. Furthermore, numerous non-modifiable parameters, such as sex, age, genetic pattern and others that are considered modifiable such as eating habits, lifestyles, physical activity and the consumption of substances for pleasure purposes, greatly affect the toxic activity exerted by the xenobiotics. The central and peripheral nervous system, in consideration of its morpho-functional complexity, turns out to be a particularly vulnerable biological target despite the fact that the blood brain barrier protects it from the action of some hydrophilic toxic substances. The major problem arises for lipophilic substances which, being able to cross the cell membrane thanks to their chemical-physical characteristics, are able to reach the nervous tissue causing serious damage: in particular, persistent, bioaccumulative and toxic substances exert a particularly negative influence sometimes leading to the death of cells and to impoverish the nervous system of cellular elements even with different cytotypic specificity with production of damage that may also be seen clinically and giving rise to important pathologies compromising the quality and duration of life. In particular, mercury in its organic form is fully reflected in this type of substances and its effects on the nervous system seem to be particularly dangerous. In this work we will evaluate the

exposure of neurons to mercury and the early onset of cell damage linked to amyotrophic lateral sclerosis which is a serious degenerative pathology of motor neurons.

1.2 Chemistry of mercury in the environment

Mercury is a powerful and dangerous environmental pollutant. No longer it is considered a local and regional problem but a global pollutant (Schroeder and Munthe, 1998). Its symbol, Hg, derives from the Greek term *Hydrargyros*, this means it is composed of hydros (water) to argyrion (silver) from which liquid silver; in fact this metal is sometimes called "quicksilver". Some of mercury properties are listed in Table 1.1. Mercury occurs rarely in nature as a native metal and most often in cinnabar (or mercuric sulfide, HgS), living stonite ($HgSb_4S_8$) and other minerals. It is third series element of transition metals belonging to group IIB. Its vapor pressure is lower than liquids ones but is considerably higher than any other metal. Because of the value of its reduction potential, mercury at room temperature behaves almost like a noble metal, being unassailable by non-oxidizing acids, oxygen, nitrogen, hydrogen, phosphorus, carbon, ammonia, hydrofluoric acid and hydrochloric acid. It is sensitive to anhydrous nitric, hydrobromic and hydrogen iodide acids, halogens and ozone. Mercury combines with many other metals, particularly with gold, silver, zinc, tin and alkali metals and leading to the formation of alloys called amalgams which are liquid only if mercury is present in big quantities compared to the other elements. There is no amalgam formation with iron, nickel and cobalt, and for this reason mercury is transported and stored in iron containers. Beyond its elemental form that’s a highly volatile chemical species, mercury can form both inorganic and organic compounds. As for inorganic compounds, unlike

the other elements of the chemical group to which it belongs, another peculiarity of is that it appears in the +1 oxidation state (mercury compounds) as well as in the +2 state (mercury compounds). Furthermore, in all mercury compounds there is the dimer ion Hg2+ in which two mercury atoms are bound by a metal-metal covalent bond. The low presence of Hg2 + compounds is due to the strong tendency to disproportionate:

$$Hg2 + \rightarrow Hg0 + Hg2 + \text{(I.1)}$$

The most stable and most abundant inorganic compounds of mercury in nature are mercury oxide, HgO, halides, $HgCl_2$ and HgI_2, mercury sulphides, HgS, mercury cyanide, Hg $(CN)_2$, mercury sulphate, $HgSO_4$, and nitrate Hg $(NO_3)_2$. Because of the diffusion in the environment it is useful to pay particular attention to the characteristics of chloride and mercury oxide. The first is a white solid easily sublimated and very toxic (it is also known as a corrosive sublimate). Oxide is a highly reactive yellow or red solid and decomposes at 500 °C into the elements mercury and oxygen. It is able to oxidize numerous reducing substances including hydrogen, sulfur dioxide and formaldehyde, with the formation of metallic mercury.

Atomic number	80.0	Melting point [° C]	-38.9
Atomic weight [g mol^{-1}]	200.6	Boiling point [° C]	356.6
Atomic radius [nm]	0.15	Thermal conductivity [Wcm-1K-1]	$8.34 \cdot 0^{-2}$
Atomic volume [$cm^3 \cdot mol^{-1}$]	14.8	Heat of fusion [kJ · mol^{-1}]	2.29
State of oxidation	0;+1;+2	Heat of vaporization [kJ · mol^{-1}]	59.23

Electronegativity	2	Specific heat [J g^{-1} K^{-1}]	0.14
Density at 20 ° C [gcm^{-3}]	13.35	Electrical conductivity [Ω]	$1.04 \cdot 10^4$

Table 1.1 - Chemical-physical properties of mercury

The organometallic compounds of mercury are grouped into two categories: alkylmercury compounds (containing methylmercury CH_3Hg^+, ethylmercury $C_2H_5Hg^+$) and arylmercury compounds (containing phenylmercury $C_6H_5Hg_+$). No doubt the cation of big interest is the monomethylmercury cation coming largely from the methylation of inorganic mercury in the sediments of the bottom of lake, marshy and marine environments (and possibly also in the aqueous phase of soils). The monomethylmercury cation gives rise to a wide range of compounds, including dimethylmercury, $(CH_3)_2Hg$, and diethylmercury, $(C_2H_5)_2Hg$ which are by far the most toxic forms for organisms.

1.3 Natural sources and transport mechanisms in the environment

Mercury is a metal naturally occurring in ecosystems and showing a background level in the environment independently by man-made emissions. This element is naturally released into the atmosphere through erosion and dissolution of the mercury minerals in the rocks and soil or as a result of volcanic eruptions. According to the latest estimates total natural emissions are around 1600 tons per year which is equivalent to about 10% of the total. The release of mercury from natural sources has remained more or less the

same over the centuries. The transport mechanisms in the environment are related to atmospheric precipitation, the phenomena of dissolution of mineral deposits and those of sedimentation. Total mercury levels are generally below 10 $ng \cdot g^{-1}$ in non-mercuriferous rocks and minerals, such as granite or feldspar, while levels in non-mercury soils and sediments in areas not directly subject to anthropogenic impacts or volcanic emissions they oscillate between 50 and 200 $ng \cdot g^{-1}$with an increase in the vicinity of urbanized areas. Natural emissions of gaseous mercury entirely consist of elemental mercury Hg^0 generally in a percentage higher than 90% compared to other forms of volatile or particulate mercury (Zhang et al., 2009). This is due both to its longer residence time and to the fact that it is the main form of mercury emitted from sources of air pollution. The average residence time in the troposphere of elemental mercury is about one year. This relatively long residence time allows the mercury in the atmosphere to travel long distances by exploiting the dominant currents. Mercury is transported directly in its gaseous form or by attaching itself to atmospheric dust. It has been shown that mercury in the atmosphere can travel up to 2500 km in 72 hours. The possibility of staying in the atmosphere for a long time in addition to the aforementioned diffusion capacity in all sectors it is useful to explain the cross-border nature of mercury pollution in the world. The residence time of the oxidized compounds of mercury is much shorter than the elemental mercury one and therefore undergoes both dry and wet deposition phenomena in these forms mercury. Combined with data collected on this topic, the atmospheric deposition models of mercury show that in humid climate zones there is higher deposition rate than in arid climate ones. (US EPA Vol. III, 1997).The mercury diffusion is managed by a complex global cycle involving all natural compartments that are the atmosphere, the hydrosphere and the geosphere (figure 1.1).

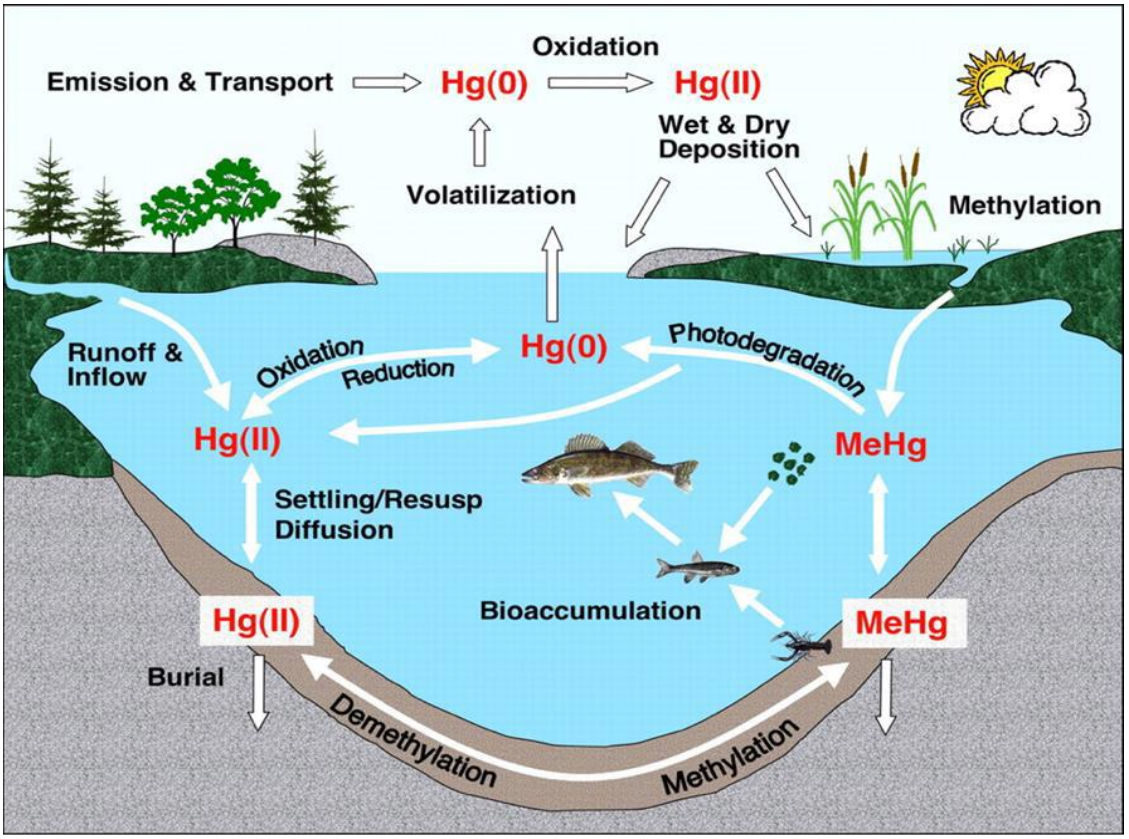

Figure 1.1 - Diagram of the biogeochemical cycle of mercury

Despite the long residence time, elemental mercury in the atmosphere still has a certain reactivity (Driscoll et al., 2013). The mercury cycle is based on the conversion in soil and water of divalent inorganic mercury ($Hg2^{+}$) to elemental mercury (Hg^{0}) and dimethylmercury ($(CH_3)_2Hg$) volatile forms that can therefore pass into the atmosphere; this is followed by the atmospheric reoxidation of elemental mercury which falls on the aquatic and terrestrial surfaces through dry and humid depositions. If mercury is deposited or transported on aquatic surfaces it can undergo a variety of transformations. In natural waters, much of Hg^{2+} is combined with suspended particulate matter and then

deposited in sediments. If together with anions creating covalent bonds, the mercury ion forms covalent molecules rather than a solid ion; and a volatile molecular liquid is formed with the methyl anion, CH3-, dimethylmercury, $Hg(CH_3)_2$. The process of formation of dimethylmercury takes place in the muddy sediments of rivers and lakes, especially with anaerobic conditions when bacteria and anaerobic microorganisms convert Hg^{2+} into $Hg(CH_3)_2$. In this process the methylating agent is a common constituent of the microorganisms called methylcobalamine that is a derivative of vitamin B12 in which a CH3-anion is bound to the cobalt. Due to its volatility dimethylmercury leaves water relatively quickly unless it is transformed into monomethyl by the acidic conditions. Other compounds formed have the formula CH_3Hg_X (for example CH_3HgCl and CH_3HgOH) and therefore they are made up of the monomethylmercury ion CH_3Hg^+ and this is very toxic because of its solubility in the adipose tissues of animals where it undergoes bioaccumulation and biomagnification. Once ingested the CH^3Hg^X compound converts to other compounds where X is a sulfur-containing amino acid; in some of these forms it is soluble in biological tissue and can thus cross both the human blood-brain and placental barrier, presenting a double risk. In fact, methylmercury represents the riskiest form of mercury and it is followed only by the vapor of the element itself. The mercuric ion Hg^{2+} is not very toxic in the stomach as it combines with the chlorine ion producing Hg_2Cl_2. Mercury is present in humans largely in the form of methylmercury taken almost entirely through a fish-based diet in which at least 80% of mercury is found in the form of methylmercury. Fish introduce this substance into their bodies through the gills and food. Methylmercury is able to bind to the sulfhydryl group of proteins so as to be distributed throughout the muscular system of fish. The fish mercury is generally higher in acidic waters probably because mercury methylation is faster in the presence of low pH values. Therefore, the acidification of natural waters that are also caused by the increase in the concentration of

CO_2 in the atmosphere as a result of anthropogenic pollution increases exposure to methylmercury in no direct way by the ones eating plenty fish.

1.4 Anthropogenic emission of mercury

Anthropogenic activity has introduced a large element of imbalance in the global mercury cycle. It is estimated that about two thirds of the mercury we have in the environment was produced during the twentieth century and that the today load of mercury introduced has increased by about 3 times compared to the early twentieth century. Surely the most involved environmental sector in mercury emissions is the atmospheric one from which the element spreads to other sectors as well. Figure 1.2 clearly shows how much human activities starting from 800 with the industrial revolution, have increased the amount of mercury in the atmosphere.

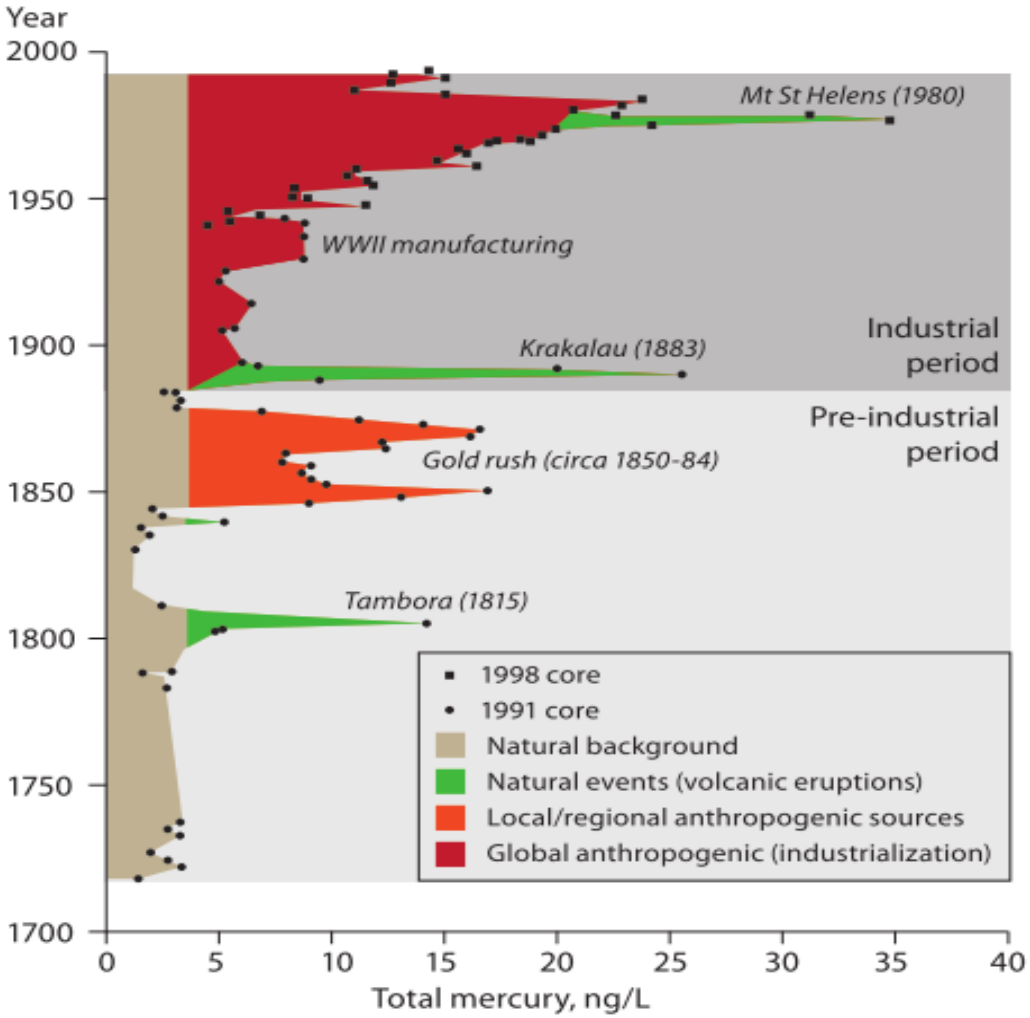

Figure 1.2 - Trend of the environmental level of mercury with industrialization

Gaseous anthropogenic emissions have both an elemental mercury component and an additional component consisting of mercury adsorbed on particulate matter (TPM, *Total Particulate Mercury*) or forms of mercury with different volatility and reactivity characteristics but mainly bivalent. The latter component is called reactive gaseous mercury (RGM, *Reactive Gaseous Mercury*) and is mainly made up of $HgCl_2$. elemental mercury enters the cycle in a very easy way instead RGM and TPM are more prone to

local or regional deposition causing mercury levels to rise at sites close to major emission sources. It is estimated that the emitted mercury fluxes are in the order of 2000 tons per year (Pacyna et al., 2010). Anthropogenic sources of mercury can be divided into primary and secondary sources. The primary sources are the ones with geological mercury mobilized and released into the environment. The two main primary sources are mining and the combustion of fossil fuels which contain trace contaminant mercury. Secondary sources are the ones in which the emission is caused by the intentional use of mercury as in the case of certain products, industrial processes, dental applications and artisanal and small-scale gold mines. apart mining, in primary sources the emission of mercury always occurs as an intermediate process where mercury is present as an impurity. The most relevant mercury source in the atmosphere is constituted by coal-fired thermoelectric plants or with oil in a very smaller part followed by solid waste incineration plants, chlorine-soda plants, smelting processes for the production of copper, lead and zinc and cement factories. Among the secondary sources, small-scale gold mining remains the main sector of use for mercury; it contributes to the increase in the quantities of mercury dispersed in the environment, although in this case the emission does not occur in the atmospheric compartment but in the water with soil pollution almost always localized in far sites from the most densely populated areas. In these, the mercury allows the separation of small quantities of gold flakes from large volumes of soils or sediments. Other secondary sources of pollution include (Pacyna et al., 2010):

➢ the use of mercury and its compounds in industrial (e.g. vinyl chloride production, wood processing, paint production, etc.), agricultural and pharmaceutical sectors;

➢ cremation;

➢ the inadequate elimination of mercury-containing products especially in the medical, scientific (thermometers, sphygmomanometers, etc.) and household (thermostats, batteries, fluorescent lamps) fields.

United Nations Environment Program Agency (UNEP) in 2013 published a study ("*Global Mercury Assessment 2013*") reporting the anthropogenic emissions of mercury from the various sources. Figure I.3 shows the graph with the results. Overall, according to UNEP, 37% of emissions into the environment are attributed to gold mines and 24% to sources that use combustion sources. However, the UNEP study specifies that the determination of mercury emissions still retains many uncertainties and admits that other researchers have provided different estimates of emissions even using similar methods. Figure I.4 shows the anthropogenic emissions of mercury of each nation. According to the UNEP inventory, only China contributes 40% of total emissions (Pacyna et al., 2010). There is another study estimating that China is responsible for as much as 54-56% of world mercury emissions (Li et al., 2009). In any case, this is a very high percentage, which moreover is destined to increase given the strong momentum of the Chinese economy (for example, in 1990 the estimate of Chinese emissions compared to the world total was 30%). In particular, the emission source destined to grow dramatically will be that of coal plants, since the economic development of China is causing a rise in energy needs (Pirrone et al., 2013).

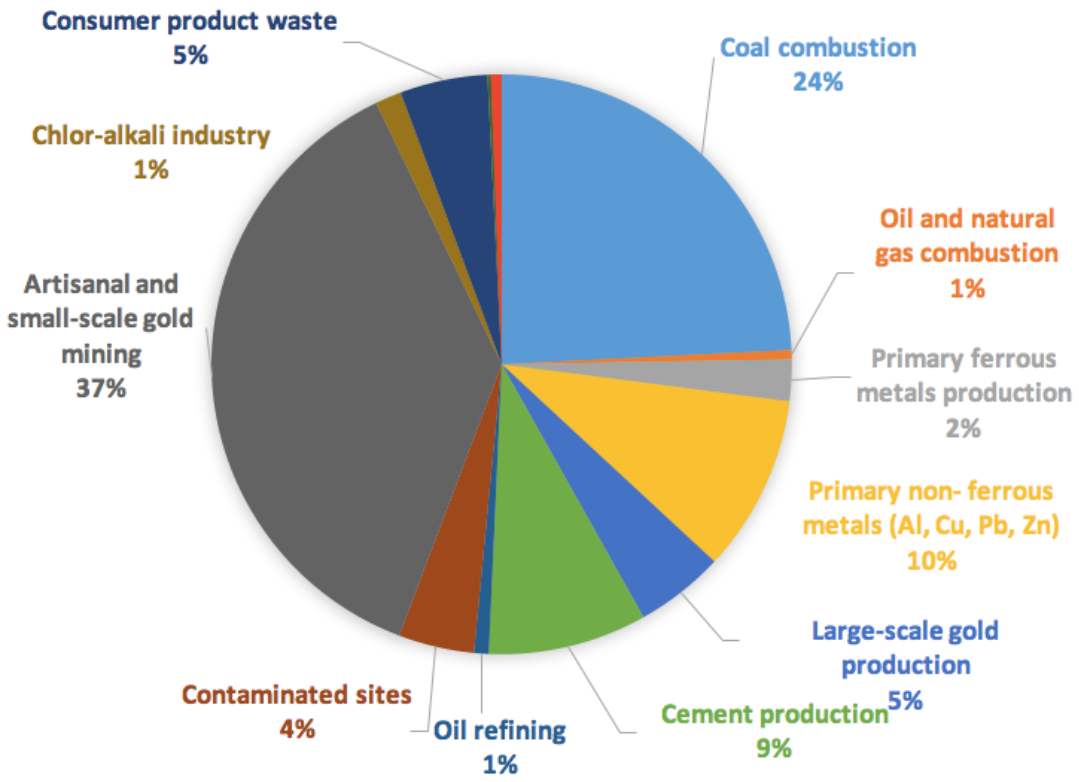

Figure 1.3 - Estimated atmospheric emissions of each sector reported in the UNEP Global Mercury Assessment 2013 study and expressed as a percentage of tons of Hg0 emitted per year with respect to total anthropogenic emissions.

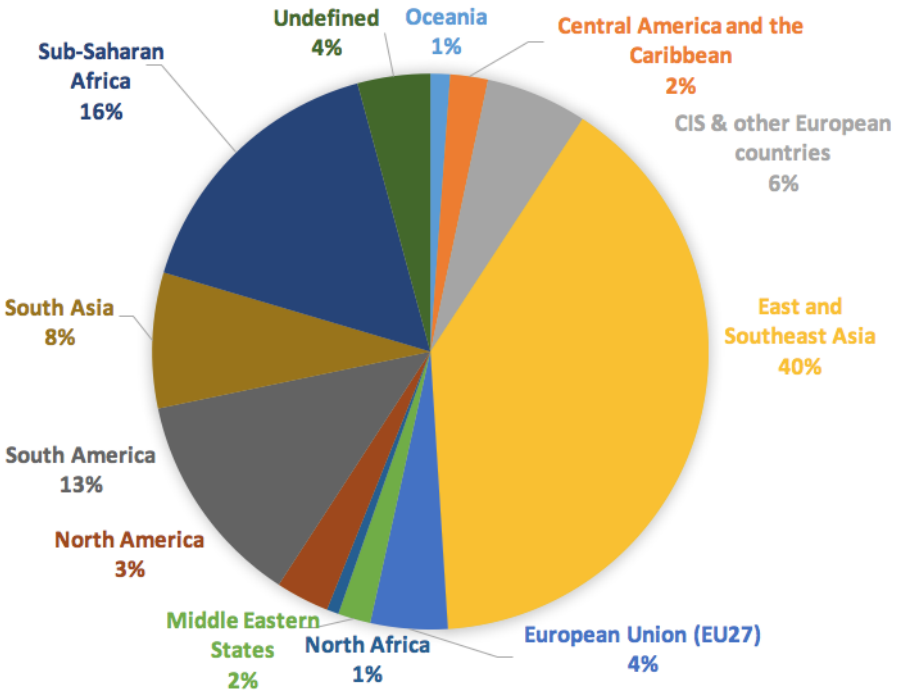

Figure 1.4 - Estimation of atmospheric mercury emissions for each geographical area reported in the UNEP Global Mercury Assessment 2013 study and expressed as a percentage of tons of Hg0 emitted per year with respect to total anthropogenic emissions.

This problem for Western economies is of lesser importance because of the fact that more oil or gas-fired thermoelectric plants (emitting very little mercury) are used rather than coal-fired plants. The case of China is the most evident one but mercury pollution is a problem of great concern in all Asian developing economies: cases of sites contaminated by high mercury pollution are also documented in Japan, India, Korea from the South, Kazakhstan and the Philippines (Li et al., 2009). Globally, mercury emissions from human activities are estimated to increase by 5% every year.

1.5 Normative references

Mercury pollution released from coal-fired plants is an environmental problem on a global scale requiring immediate action at both political and technical-scientific level. There is total consensus on this in the international scientific community. The Minamata convention (named after a Japanese location where tragic clinical episodes occurred following copious emissions of mercury by chemical companies) signed in Geneva in February 2013 as part of UNEP was one of the most important international initiatives on the reduction of mercury emissions globally. This regulatory instrument, which is constantly evolving, has provided suitable modification procedures that will allow the 139 signatory countries to introduce future and further measures aimed at reducing the rate of mercury pollution (UNEP Minamata, 2013). The convention is the first global agreement on the environment and health in nearly a decade, and may bode well for renewed intergovernmental cooperation on environmental protection; moreover, its subscription is significant as many countries have nevertheless committed themselves to allocating resources for the fight against the harmful effects of mercury despite the prolongation of the economic crisis. No doubt Japan was among the first signatories of the Minamata Convention providing for controls and reductions on the whole range of products, processes and industries in which mercury is used, released or emitted; the treaty also addresses issues such as direct extraction, export and import, safe storage of mercury waste and also aims to identify populations at risk, increase medical care and better training of doctors in identifying and treating the effects of mercury poisoning. Under the provisions of the Minamata Convention, the production, import and export of a range of mercury-containing products will be prohibited from 2020, including: batteries, switches and relays, cold cathode fluorescent lamps and external electrode fluorescent lamps, soaps and cosmetics. Furthermore, reduction measures have been

determined for the main sources of mercury emissions from large industrial plants such as coal power plants, industrial boilers, incinerators. Canada and the United States were the first countries to adopt stringent limits on mercury emissions from coal-fired power plants. Canada has the aim of eliminating mercury emissions by closing off non-standard coal plants and replacing fuel; on the other hand, by equipping existing plants with efficient Hg0 capture systems. The United States intends to permanently reduce mercury emissions from power plants establishing a maximum emission limit of 15 tons per year starting from 2018, a limit less than 30% compared to the 1999 emission level. With the close collaboration of the Member States, the European Union is committed to promoting the production of electricity from renewable sources to exclude the use of coal as a fuel from the energy mix. A fundamental EU instrument obliging industrial plants to acquire integrated environmental authorizations based on the concept of "best available techniques" (BAT - *Best Available Techniques*), the IPPC (*Integrated Pollution Prevent and Control*) Directive is part of this. The adoption by the European Parliament of the IED Directive (*Industrial Emissions Directive*) represented a particularly important step in the evolution of the legislation on pollutant emissions from industrial plants. According to the new environmental directive working since 2016, it is necessary to demonstrate that the plant complies with the new parameters for the four pollutants (SO_X, NO_X, PM_X powder and Hg^0) and which is also characterized by BAT for the reduction of emissions of pollutants to obtain permission to use and/or build a combustion plant from the competent authorities, IED does not have a limit for mercury emissions.

1.6 Mercury in flue gas

In recent decades research has invested part of its resources in the development of new technologies for the abatement and capture of this element to be applied to coal-fired power plants as being the primary source of the anthropogenic emission of mercury into the atmosphere. Emissions from individual boilers vary widely depending on the type of coal and the pollution control systems available. Although the mercury content in coal is relatively low (~ 0.1 mg kg-1) the amount of mercury released by coal-fired boilers is very high due to the high amounts of coal burned. It is essential to know the chemical and physical transformations of this element into flue gas summarized in figure 1.5 (Galbreath et al., 2000) in order to understand the mechanisms that regulate the transport and abatement of mercury released by coal-fired boilers into the atmosphere.

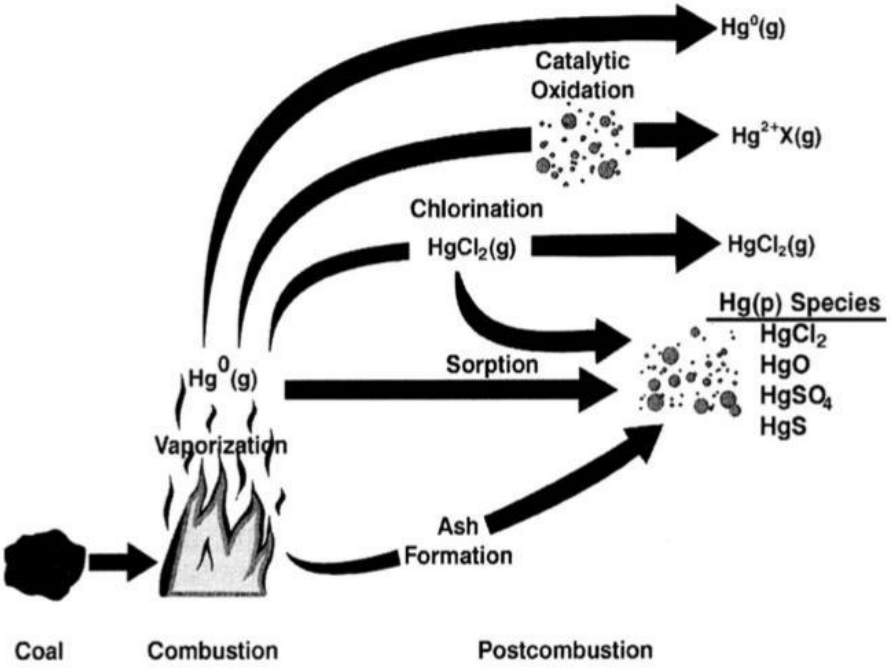

Figure 1.5 - Transformation of mercury during the combustion process and subsequently in the exhaust fumes

During the combustion of coal, mercury begins to volatilize already at temperatures below 200 °C regardless of its form and in flue gas it is mainly found in the elemental form, Hg^0 (at 1000 °C). In the complex post-combustion environment, as the current temperature decreases, elemental mercury can remain as a monatomic species or react to form oxidized mercury, $Hg2^+$, or mercury, $Hg2^+$ species (Senior et al., 2000). Due to the instability of mercury compounds at low concentrations, it is assumed that part of Hg^0 is oxidized, homogeneously or heterogeneously, only to Hg^{2+}. Several researches have also led to the conclusion that, leaving the high combustion temperatures, the mercury at 600 ° C is found in the form of inorganic materials while at 400 °C of organic materials. At higher temperatures, mercury reacts with other components, initially producing HgO and $HgCl_2$ at temperatures below 430 °C. According to this, the following reactions have been proposed in the Frandsen model:

$$HgO(g) \leftrightarrow Hg(g)0 + 0.5O2\,(g) \quad (1.2)$$

$$HgCl2\,(g) + H2O(g) \leftrightarrow HgO(g) + 2HCl(g) \quad (1.3)$$

$$HgSO4\,(g) \leftrightarrow HgO(g) + SO2\,(g) + 0.5O2\,(g) \quad (1.4)$$

$$HgO(s) \leftrightarrow HgO(g) \quad (1.5)$$

$HgSO4\,(s) + Cl2\,(g) \leftrightarrow HgCl2\,(g) + SO2\,(g) + O2\,(g)$ (1.6)

Reaction 1.2 takes place at 320°C in the absence of chlorine and 680°C in the presence of chlorine, 1.3 takes place at 430°C in the presence of chlorine, 1.4 takes place at 320 ° C, 1.5 at 170 ° C and 1.6 at 110 °C in the presence of chlorine (Pavlish et al., 2003). At about 400°C the elemental mercury can then be completely converted into the forms Hgp (mercury bound to the particulate) and $Hg^{2+}X$. It is also possible to reduce the $Hg^{2+}X$ species in the combustion fumes:

$HgO(s) + SO_2(g) \rightarrow Hg(g)^0 + SO_3\,(g)$ (1.7)

$HgO(s) + CO\,(g) \rightarrow Hg(g)^0 + CO_2\,(g)$ (1.8)

Many compounds such as Fe, S, Ca and Cl, contained in coal influence the presence of the different forms of mercury in the combustion fumes: Fe catalyzes the mercury oxidation reaction; Ca reacts with chlorine during the combustion process, inhibiting its activity to promote the oxidation of mercury. Experimental evidence has also shown the reduction of HgCl2 on heated steel, according to the following reaction:

$3HgCl_2\,(g) + 2Fe(s) \rightarrow 3Hg(g)^0 + 2FeCl_3\,(s)$ (1.9)

The dominant mechanism of mercury transformation in the coal post-combustion zone is chlorination that is the reaction of Hg^0 with HCl or Cl_2 to form $HgCl_2$, although the latter has never been measured. Chlorine speciation is a very important factor to consider for understanding the reaction mechanism between mercury and the chlorinating agent. Many theoretical and experimental analyzes have indicated that, at low temperatures (<400°C), Cl_2 is the most active chlorinating agent. During combustion, HCl is developed, downstream of the combustion process Cl_2 is formed by the Deacon reaction:

$$2HCl(g) + 0.5O_2(g) \rightarrow Cl_2(g) + H2^O(g) \quad (1.10)$$

However, it is estimated that only 1% of HCl contained in the combustion fumes is transformed into Cl_2 as this transformation is not governed by a thermodynamic equilibrium, in fact it proceeds only in the presence of a metal catalyst. Furthermore, the presence of SO_2 in the combustion fumes can inhibit the formation of chlorinated compounds as it reduces the concentration of Cl2 according to the following reaction:

$$Cl2(g) + SO2(g) + H2O(g) \leftrightarrow 2HCl(g) + SO3(g) \quad (1.11)$$

Consequently, the presence of a high sulfur/chlorine ratio inhibits the formation of Cl2 and therefore also of $HgCl_2$. Following chlorination, $HgCl_2$ can remain in the exhaust gas or be adsorbed on the ash particles carried by the gas flow. The morphology and the porous surface of the particles are generally dominant factors in controlling the adsorption of $HgCl_2$. In addition, O_2 and NO_2 can also react with mercury in the

combustion fumes: however, kinetic limitations and relatively low residence time in the ducts exclude the occurrence of any homogeneous reactions involving Hg^0 and O_2 or NO_2. Instead, in the presence of inorganic and carbonaceous ash particles, these gases show interesting phenomena of adsorption and oxidation of elemental mercury. For example, there is a significant adsorption of elemental mercury on fly ashes in the presence of oxygen especially in the temperature range between 100-300 °C. Sulfur rich ashes entrained in the combustion fumes are potential sorbents for elemental mercury; the chemiadsorption of sulfur compounds on the surface of the particles can create active sites for the adsorption of mercury. In summary, in the combustion environment, mercury is initially present in the form of Hg^0 but downstream of the combustion process, various mechanisms control its speciation. It is hypothesized that chlorination is the dominant mechanism of transformation of mercury; the other mechanisms reported in figure I.5 may involve interactions between mercury and the surface of the ash particles where reactive chemical species, catalysts, and active sites are available for the conversion of Hg^0 into $Hg^{2+}X$ or Hg_P.

1.7 Techniques for abatement of mercury from flue gas

The abatement of mercury in coal boilers can occur before, during or after combustion. The most effective control technique is the treatment of the effluent streams from the plant, thus removing the pollutant from flue gas. Clearly, based on the form of mercury taken into consideration, a different removal strategy is carried out:

➢ Mercury bound to particulate matter, Hg_P, is removed directly with a dedusting device such as ESP electrostatic precipitators or FF bag filters;

➢ The oxidized mercury Hg^{2+} is removed by wet desulfurization, Wet Flue Gas Desulfurization (WFGD) or by wet scrubbers. The wet WFGD system consists of a scrubber with limestone as it is and one with limestone and magnesium;

➢Elemental mercury is difficult to remove due to its thermodynamic stability at high temperatures, inertia towards oxidation reactions at low temperatures, low melting point (-38.9 ° C), high vapor pressure (0.25 Pa at 25°C) and low solubility in water.

A promising method for this form could be the combination of catalytic oxidation of Hg^0 to Hg^{2+} and wet scrubber or WFGD or Selective Catalytic Reduction (SCR), which consists in the removal of NOX with the use of NH_3.This last control technique is becoming increasingly important in scientific research as oxidized species are easily removable (Brown et al., 1999). So far there are three groups of main catalysts for oxidation: noble metals; SCR catalysts, transition metals. Among the noble metals

studied, Pd is considered one of the most efficient catalysts, supported in particular by TiO_2 and γ-Al_2O_3 which improve its activity, stability and regenerability (Presto and Granite, 2008). The results showed that Pd can oxidize more than 95% of elemental mercury in the initial stage and that it can be effectively regenerated by heating at about 315°C for several hours; in fact, after 3 months the degree of conversion of Hg_0 remains at 85% and after 20 months at 65%. Au also proved to be a good catalyst due to its ability to absorb mercury and chlorine excluding the other species (HNO3, SO2 and H2O) (Zhao et al., 2006). The activity of Pd and Au decreases as the concentration of HCl decreases; Pt has instead a contrary trend (Presto and Granite, 2008). Although that of noble metals is the most promising category, the high costs of these compounds have led to further research and a greater development of the other two categories on the process of reducing mercury by catalytic oxidation.SCR catalysts are classified, based on operating temperature, into high temperature (High Temperature Selective Catalytic Reduction, HTSCR) and low temperature (Low Temperature Selective Catalytic Reduction, LTSCR) SCR catalysts. The first category consists of compounds based on V_2O_5, such as V_2O_5-WO_3/TiO_2, VOX/TiO_2, SiO_2-TiO_2-V_2O_5. LTSCR include MnOx-based compounds such as MnOX/Al_2O_3, Mo-MnOX/Al_2O_3 and MnOX-CeO_2/TiO_2. The HTSCR systems guarantee excellent oxidation of elemental mercury in a temperature range between 300 and 400°C, the degree of which depends directly proportional to the concentration of HCl in flue gas and inversely proportional to the quantity of NH_3 used (Lee et al., 2012; He at al., 2009).Among the most effective and widely used combinations is V_2O_5/TiO_2 whose oxidation activity improves in the presence of HCl (Arena et al., 1999). A thermodynamic evaluation of this oxidation confirms the high reactivity of the catalyst under standard conditions: the conversion of Hg_0 is maintained at 90% in a temperature range between 250 and 350°C and decreases with increasing temperature. The exact mechanism of Hg0 oxidation by SCR catalysts is still not very clear, although various models have been presented in the literature. Niksa and Fujivara

have assumed that in this process HCl is first adsorbed on the active sites of V_2O_5 after which the reaction between adsorbed HCl and gaseous or weakly bound Hg_0 can occur. In the model that assumes the Eley-Rideal mechanism, chlorine is produced by the Deacon reaction (reaction I.10), Cl_2 then reacts with Hg_0 forming $HgCl_2$ (Senior, 2006). The Langmuir-Hinshelwood mechanism is based on the initial adsorption of both HCl and Hg_0 on the active sites to form $HgCl_2$ and VOH followed by the reoxidation of VOH by O_2 with the formation of VO and H_2O (He at al., 2009).LTSCR systems have also aroused great interest in the scientific community. Qiao et al. studied the adsorption and catalytic oxidation of gaseous elemental mercury on MnOX/α-Al_2O_3. The active phase tends to form a dense shell on α-Al_2O_3 and in this layer MnOX is inaccessible in depth and cannot adsorb Hg_0 due to the high resistance to diffusion of the gas. The superficially adsorbed mercury is found mainly in the form Hg^0 and Hg-O-$MnOX^{-1}$; their ratio varies with the amount of adsorbed species and the MnOX loading. To improve the catalytic activity and resistance to acid gases, various metal elements were used as promoters. Among all those tested, the best performance was obtained with molybdenum. Then Li et al. observed a significant synergy for mercury oxidation between MnOX and CeO_2:using titania as a support, a degree of oxidation of 90% was obtained (Li et al., 2012). The advantage of using these compounds is certainly the simultaneous removal of two pollutants Hg_0 and NO but however their use is limited for three reasons: the concentration of HCl in flue gas, the interference with NH3 and the removal of toxins from industrial waste. Among the most captivating properties of transition metal oxides there is excellent catalytic activity, easy preparation and lower costs compared to noble metals. The abatement of mercury from flue gas by catalytic oxidation could be the most effective solution, having as an advantage also the removal of more pollutants, but the catalysts analyzed up to now still have insurmountable disadvantages: the high costs of noble metals; the interference of the smoke components with regard to the use of SCR catalysts and the poisoning of SO_2 for transition metals.

1.8 Adsorption of mercury from combustion fumes

The focus is now on the problem of the abatement of elemental mercury vapors by adsorption with suitable materials. More effective strategies for the control of mercury emissions must in fact be based on the formulation of new materials with advanced adsorption properties of metallic mercury. It has been highlighted in the literature that many materials (in particular transition metal oxides and noble metals) possess promising properties of mercury capture, as well as oxidation.So far the most effective technique for this abatement uses activated carbon as adsorbent materials. Interesting properties for this purpose can also be found in the oxides of noble metals and transition metals: possibility of regeneration in order to reduce operating costs; possibility of capturing mercury at relatively high temperatures (200-400 °C). In the next paragraphs we will analyze the fundamental properties of adsorption and the capture performances of the main adsorbent materials investigated in the capture of mercury.

1.9 The adsorption process

Adsorption is a surface phenomenon of molecular attraction that occurs at the interface between two phases: a solid phase, the adsorbent, and a liquid or gaseous phase, called adsorbate. From a thermodynamic point of view it is possible to observe that, being a spontaneous process ($\Delta G < 0$) and being characterized by a decrease in the entropy of the adsorbed substance ($\Delta S < 0$), adsorption is an exothermic phenomenon ($\Delta H < 0$) and as such it is favored by low temperature values. Between the adsorbate and the solid, two different types of forces of attraction must be considered: van der Waals forces and forces of a chemical nature (Perry and Green, 2008). The first type of adsorption is generally called "physical adsorption". It occurs at low temperatures (with values almost close to environmental ones) and is non-specific: the adsorbate molecules do not bind to a specific site but are rather free to undergo translational movements on the interface. Van der Waals forces are weak intermolecular forces and for this reason the process is considered reversible. If the adsorbate undergoes chemical interactions with the adsorbent then we speak of "chemical adsorption" (chemiadsorption). The chemically adsorbed molecules, unlike the previous case, are not free to move on the surface since the adsorbate forms very strong localized bonds with the adsorbent sites. The chemical interaction between adsorbent and adsorbate is favored by high temperatures: chemical reactions, in fact, proceed faster at high temperatures than at low temperatures. Furthermore, the chemical adsorption involves the formation of a monolayer given the high degree of specificity and the relevant bond strengths. Table 1.2 summarizes the main characteristics of the two types of adsorption (Ruthven, 1984).

	Physical adsorption	Chemical adsorption
Heat of adsorption	low (≤5 kcal mol^{-1})	high (10100 kcal mol^{-1})
Adsorbent-adsorbate interactions	Not specific	Specific
Type of adsorption	mono or multilayer	single layer only
Temperature range	significant only at low	wide range
Other characteristics	quick, not activated, reversible	activated, it can be slow and reversible

Table 1.2 - Main distinctive features of physical and chemical adsorption

In order for an adsorption process in a liquid/solid system to be successful, the solute must be removed from the solution and its consequent adsorption on the surface of the adsorbent material. In other words, when the adsorption rate is equal to the desorption rate, then the thermodynamic equilibrium condition has been reached, which means that the adsorbing capacity of the material is exhausted. The adsorption phenomenon can be characterized from a strictly kinetic point of view through the study of the fluid-dynamic parameters of the liquid stream to be treated, including flow rate, speed and therefore solid-liquid contact time, in the plant configuration chosen for the conduction of the process (McCabe et al., 1985). The overall kinetics of the adsorption process can be broken down into three stages:

➢external diffusion: transport of the species to be adsorbed from the fluid stream to the surface of the solid;

➢ internal diffusion in intraparticle cavities;

➢ adsorption: the species is adsorbed on the active sites.

The inverse desorption process involving the species adsorbed on the solid is characterized by stages which are mirrored to the first two stages. Figure 1.6 schematically represents the porous surface of an adsorbent solid, the boundary layer that surrounds it and the adsorption-desorption path taken by the solute. The overall kinetics of the process depends on that of the individual stages. In any case, the slowest of all will constitute the limiting step and consequently control the adsorption rate; generally in a turbulent flow regime the internal diffusion offers the greatest resistance. In general, the speed of the process decreases as the molecule size increases because the diffusion coefficients decrease and, where the control mechanism is internal diffusion, it increases as the pore size decreases. It can therefore be said that at low temperatures the system is in a purely kinetic regime, but by increasing the temperature it passes to a mixed regime in which diffusion in the pores becomes limiting, finally at high temperatures it passes to the external diffusion regime, where the extraparticle diffusion is dominant. In the Arrhenius diagram, where there is the adsorption rate as a function of the reciprocal of the temperature, the transitions from one regime to another are clearly identified by the slope changes.

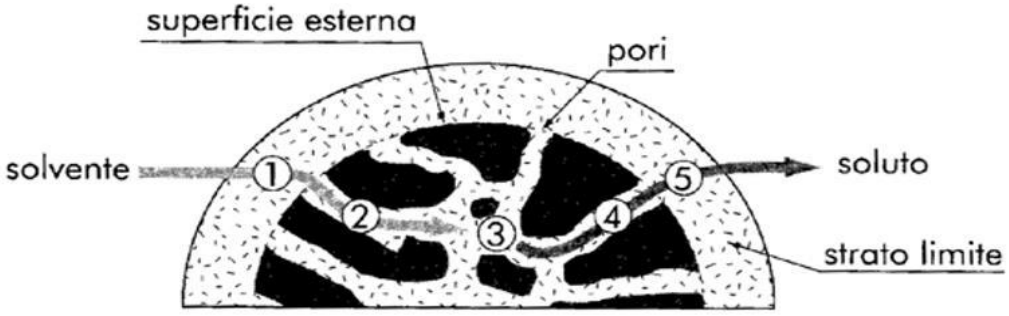

Figure 1.6 - Representation of the porous surface of an adsorbent solid and boundary layer that surrounds it; the phases of the adsorption-desorption process are also highlighted.

Most of the adsorption processes are carried out in fixed bed columns; these are tubular reactors inside which a granular adsorbent material is placed through which, subsequently, the current containing the pollutant to be removed is passed. The treated current, gradually depleting of the solute, reduces the pushing force that regulates the exchange of matter, thus slowing down the adsorption kinetics. The kinetic study of the adsorption phenomena and of the parameters that influence their behavior is carried out using a process configuration that re-proposes, on a pilot scale, the real one or in a fixed bed column and through the creation of breakthrough curves, in which the concentration dimensionless of the pollutant in the stream leaving the treatment column is related to the time (Figure 1.7). In general, the breakthrough curve, with a sigmoidal trend, is characterized by two times referred to two reference concentration levels (C = 5 and 95% of the input concentration C_0); in particular the breakpoint time, tb, represents the time at which C/C0 = 0.05, while the saturation time, ts, is the time at which C/C_0=0.95. Initially the output concentration C is zero as the solid is able to adsorb all the substance introduced into the reactor; as the initial areas of the column become saturated, the

material transfer area moves towards the reactor outlet and ever increasing outgoing concentration values are observed until, once the last stretch of the adsorbent bed is saturated, the outlet is finds the same concentration of the solution at the reactor inlet ($C = C_0$).

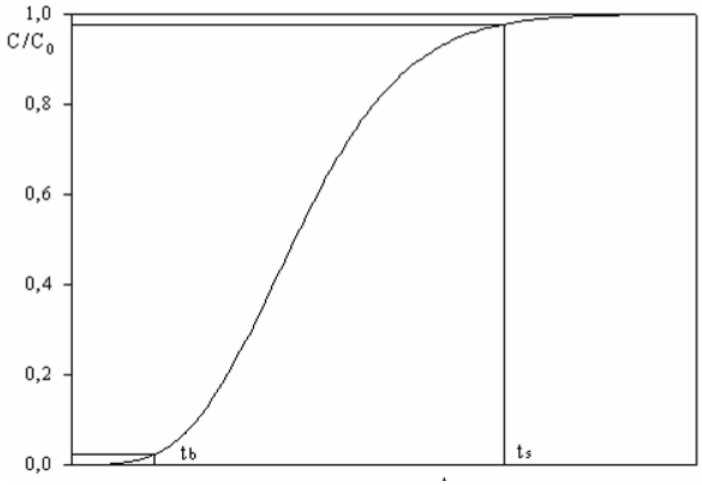

Figure 1.7 - Characteristic trend of the breakthrough curve of the adsorbent bed.

1.10 Characteristics of adsorbent materials

The choice of an appropriate sorbent for capturing Hg^0 represents a rather complex problem because of the numerous requirement it must possess in order to allow its application in separation processes (Carey et al., 1998):

➢ Adsorption capacity: the quantity of adsorbent required for the capture process and therefore the dimensions of the adsorption equipment are directly related to the Hg^0 capture capacity of the sorbent in saturation conditions;

➢ Selectivity towards Hg^0: the ratio between the capture capacity of the sorbent towards mercury obtainable towards other species present in flue gas has a direct impact on the purity of mercury recovered downstream of the regeneration process;

➢ Adsorption/desorption kinetics: in the process operating conditions, it is essential the sorbent shows rapid adsorption/desorption kinetics directly linked to the diffusion phenomena concerning the transport of mercury from the gaseous bulk to the active sites of the sorbent, as well as to the intrinsic kinetics of the reactive capture process;

➢ Thermal regeneration of the sorbent: for the capture process to be efficient and economically advantageous it must be possible regenerate the sorbent, in such a way as to recover most of the original capture capacity, in a step that is not very expensive, especially in terms of energy required for recovery of the adsorbate;

➢ Chemical and thermo-mechanical stability: the sorbent must show good structural stability retaining its morphological characteristics for a sufficiently high number of adsorption/regeneration cycles;

➢ Cost of the sorbent: with the same capture performance those sorbents with lower costs are clearly preferable.

1.11 Adsorption of mercury by activated carbon

The only process of abatement of mercury vapors a result that is feasible at a full-scale albeit expensive level is adsorption by activated carbon fed directly into the flue gas and subsequently separated in the dedusting system. Activated carbons are materials of vegetable or mineral origin characterized by high values of specific surface area and pore diameter. The ability to retain organic contaminants is influenced by a large number of parameters including temperature, pressure, type and concentration of pollutants, their molecular weight and the presence or absence of humidity and particulates in the flow to be treated. (Montagnaro et al., 2015).Adsorption by activated carbon allows the removal of Hg^0 and Hg^{2+} from flue gas in a temperature range between about 120 and 220 °C. Mercury, after being adsorbed by activated carbon, is separated from the fumes by electrostatic precipitators or bag filters (Reddy et al., 2012). The removal of mercury by means of activated carbons occurs through surface functional groups, which can be oxygenated species (OH, C – O, C = O, COOH) or functional groups containing inorganic elements (Cl, S). The results showed that Hg^0 is physically adsorbed in non-impregnated activated carbon while chemiadsorption occurs in that impregnated with other species (Li et al., 2003).The presence of acids in the combustion fumes increases the adsorption capacity: in the presence of NO_2 a non-volatile mercury complex Hg (NO3)2 is formed, which binds to the basic sites of the coal; the presence of SO_2 influences the removal of Hg^0; in the presence of CO_2, the capture capacity decreases

because the surface conditions of the activated carbons favor the selective adsorption of CO_2 (Yan et al., 2003). However the process of adsorption of Hg^0 by activated carbon is complicated: no experimental results have been able to give a better understanding of the oxidation of Hg^0 to Hg^{2+}. For better performance some pre-treatments are carried out on the activated carbons: impregnation with sulfur and halogens in fact allows the chemiadsorption of mercury, increasing the adsorption capacity. These specific activated carbons have a significantly higher cost than those as they are, therefore their use could be assumed only when the concentrations of Hg^0 tend to rise and reach/exceed the legal limits. A premise to all this is the availability of a continuous mercury analyzer that controls the introduction of activated carbon additives only in the event of an increase in emission values. The limited use of these activated carbon would make the process economically sustainable. Activated carbons impregnated with sulfur have a greater capture capacity than materials as such due to the formation of HgS: with these specific activated carbons, abatement percentages between 90 and 93% are obtained in a temperature range between 25 and 150°C. Activated carbon impregnated with halogens can also be used. In the case of activated carbon impregnated with bromine, the adsorption of Hg^0 is faster and the capture capacity increases by 80 times when the catalyst is brominated at 0.33% wt (Hutson et al., 2007). Activated carbons impregnated with iodine remove mercury efficiently but, unlike bromine, the amount of Hg^0 captured is not proportional to the iodine concentration (Lee et al, 2004).

Although the presence of promoters on carbon has improved the adsorption capacity of activated carbon, the following abatement technique has multiple limitations:

➢ the process is mass transfer limited due to the very low concentrations present in the effluent stream, and the coal is not even fully exploited due to the limited contact times between the two phases;

➢ the activated carbon can be difficult to regenerate;

working at low temperatures (<200 °C), it is necessary to cool the flue gas stream;

➢ it is necessary to have a high carbon/mercury ratio;

➢ non-selectivity;

➢ disposal costs of fly ashes that cannot be reused in the common industry of binding materials due to the presence of C (Telesca et al., 2015, 2016, 2017).

The use of activated carbon as adsorbent material therefore involves high costs and difficulties in recovering both the sorbent and the ashes. There is a clear need to develop new strategies aimed at formulating new low cost materials with a high mercury capture capacity, and not containing carbon in such a way as not to alter the properties of the ashes.

1.12 Adsorption by transition metal oxides

Several studies in the literature suggest that an interesting path to pursue is the development of advanced materials with high adsorption capacity and regeneration possibilities. A further impetus to the study of these sorbents for the removal of mercury is the possibility of working at higher temperatures than activated carbon. Compounds that have promising properties of mercury capture and regeneration are noble metals, such as gold, silver, platinum and iridium: generally this capture occurs by amalgamation and release at high temperatures (Granite et al., 2000). But the pure noble metal is not suitable for this removal due to the low specific surface area, high density and difficulty of separation: the presence of a support, for example coal or a noble metal/mineral mixture, is necessary (Luo et al., 2010). Sabri et al. showed that glass beads coated with gold can actually adsorb elemental mercury thanks to the strong affinity between gold and mercury, by amalgamation (Sabri et al., 2009). The Ag sorbent/carbon nanotubes also showed complete mercury capture at a temperature of 150 °C and the presence of components such as SO_2, NOX, CO_2 and O_2 resulted in a negligible effect on capture performance (Luo et al., 2010).Other regenerable sorbents with a high ability to remove mercury vapors from combustion fumes are made of platinum or palladium supported on alumina, Pt or Pd/Al_2O_3, in a temperature range between 204 and 388 ° C. For both types of sorbent, the amount of mercury removed decreases with increasing temperature and metal load (Poulston et al., 2007). However, noble metals cannot be economically competitive, as previously noted, given their high cost. Transition metal oxides appear to be excellent candidates for the replacement of activated carbon due to their low cost and other important properties concerning, in particular, the possibility of regeneration in order to reduce operating costs and the possibility of capturing mercury at higher temperatures (200-400 °C). The latter feature

is very interesting in the case of integrated cycles that involve coal gasification, in which it would be extremely convenient to remove the mercury from syngas before its combustion in a gas turbine (Scala, 2004). Currently manganese oxide, MnOX, is arousing interest due to the good mercury capture potential, abundant availability, eco-sustainability and lower cost of activated carbon and noble metals (Ji et al., 2008; He et al., 2011; Scala et al., 2013).The supports used for this oxide were alumina and titania, Al_2O_3 and TiO_2 respectively. The MnOX/γ-Al_2O_3 sorbent is able to remove elemental mercury from combustion fumes both by adsorption and by catalytic oxidation: the first process plays a key role in the absence of HCl; the second process becomes dominant when HCl and Cl_2 are present in the combustion fumes. Scala et al. studied the mercury capture capacity of a synthetic MnOX/γ-Al_2O_3 sorbent, confirming its good adsorption capacity, regenerability at temperatures below 500 ° C and the possibility of being subjected to multiple adsorption / desorption cycles. As for the components of the combustion fumes, they have a non-negligible influence: CO and CO_2 reversibly worsen the performance of the sorbent; SO_2 has an irreversible negative impact on the performance of the sorbent; in the presence of HCl, the catalytic oxidation of Hg^0 becomes dominant. To improve the catalytic activity of this material at low temperatures (100-300 °C) various metal promoters have been impregnated on the sorbent: molybdenum and strontium perform better, even if Sr is inhibited by SO_2 (Li et al., 2010). The use of titanium dioxide, TiO_2, as a support has a considerable importance on the mercury abatement process. As regards the capture of elemental mercury by adsorption, several studies have been carried out on the sorbent with manganese oxides supported on titanium dioxide, MnOX/TiO_2, also used as a low temperature SCR catalyst. Ji et al. have prepared this compound by impregnation, studying the effect of MnOX and using two different fillers (10 and 20% wt): the X-Ray Diffraction analysis, XRD, indicated that the shape is well dispersed on the support and bothfillers have a degree of capture of about 90% (Ji et al., 2008). Cimino and Scala compared two

sorbents consisting of MnOX as the active phase and two different supports, spheres of γ-Al_2O_3 one and TiO_2 powder the other. Under the same conditions, the MnOX/TiO_2 sorbent showed a capture capacity per unit of mass of at least an order of magnitude greater than those measurable on the counterpart with γ-Al_2O_3 support. The first is more active and this is attributable to the greater presence of Mn4 + sites on titania than those present on alumina. Many studies have focused on understanding the interactions between mercury and a nanocrystalline sorbent, due to noteworthy physico-chemical properties: better surface chemical reactivity towards many adsorbates due to a larger surface area, smaller crystal diameter, unique morphology and porous material. Wang et al. they synthesized titanium dioxide nanotubes, TNT, by hydrothermal treatment and calcination at various temperatures, and then verified their ability to remove Hg^0 from a stream of flue gas (Wang et al., 2011). In non-calcined non-calcined, the nanotubular structure was evident, with a high surface area, while in those calcined at 500 ° C a rod-like structure. TNT irradiated with UV rays generated a large amount of hydroxide radicals, which reacting with Hg^0 formed HgO on the surface of the nanotubes. TNT calcined at 500 °C showed excellent removal efficiency more than 90% for 100 h.In some works, TNT have also been used as a support; Pappas et al. synthesized sorbents with manganese on TNT confirming the catalytic activity of this material due to the abundant Mn^{4+} acid sites (Pappas et al., 2016). The most accredited mechanism for this adsorption reaction is that of Mars-Maessen, in which mercury is captured by a metal oxide in the absence of halogen (Granite et al., 2000):

$$Hg\ (g)^0 + surface \rightarrow Hg\ (ads)^0 \quad (1.12)$$

$$Hg\ (ads)^{0} + MxOy \rightarrow Hg^{0}\ (ads) + MxOy^{-1}\ (1.13)$$

$$Hg\ (g)^{0} + surface \rightarrow Hg\ (ads)^{0}\ (1.14)$$

$$Hg^{0}(ads) + MxOy^{-1} + 0.5O_2(g) \rightarrow Hg^{0}\ (ads) + MxOy\ (1.15)$$

$$Hg^{0}\ (ads) + MxOy \rightarrow HgMxOy^{+1}$$

1.13 The selective catalytic reduction of NOX with NH3

The compounds mentioned in the previous paragraph can also act as heterogeneous catalysts, in particular several studies have shown that V_2O_5/T_iO_2, MnOX/γ-$Al_2O_3$and $MnOX/T_iO_2$ are catalysts of the Selective Catalytic Reduction (SCR) process (Cimino and Scala, 2015; Ji et al., 2008).The SCR reduction of NOX occurs through the reaction with ammonia (used in gaseous form, in aqueous solution, or as aqueous urea solution) to give harmless compounds such as nitrogen and water through the following main reactions:

$$4NO + 4NH_3 + O_2 \rightarrow 4N_2 + 6H_2O\ (1.17)$$

$6NO_2 + 8NH_3 \rightarrow 7N_2 + 12H_2O$ (1.18)

$NO + NO_2 + 2NH_3 \rightarrow 2N_2 + 3H_2O$ (1.19)

Reaction 1.17 runs quickly in the presence of a suitable catalyst in a temperature range between 250 and 450 °C and takes into account the overall stoichiometry of the process since nitrogen oxides are made up of more than 90% of NO. In fact, NO_2 is present in the combustion exhaust fumes in low percentages (around 5%), so reactions 1.18 and 1.19 play a secondary role in the SCR process. The term "selective" refers to the ability of ammonia to react with NO instead of being directly oxidized by the oxygen in the air; this characteristic is specific to ammonia as it has not been observed for other reducing agents such as hydrocarbons, H_2 or CO. The oxidation reaction of ammonia by oxygen is in fact highly undesirable as it reduces the efficiency of the denitrification process by subtracting the NH3 reagent and leading to the formation of NO and N_2O. In the case of fuels containing sulfur, during the combustion process SO_2 is formed which can be oxidized on the catalyst to SO_3 according to the reaction:

$SO_2 + 0.5O_2 \rightarrow SO_3$

This reaction is undesirable as the SO_3 produced reacts with ammonia and water to form sulfuric acid and ammonium sulphates. Ammonium sulphates can deposit and accumulate on the catalyst if the reaction temperature is not sufficiently high and are

deposited in the cold parts of the plant downstream of the catalytic reactor, where they give rise to corrosion problems and pressure drops. This forces periodic stops of the system for washing the recuperator itself. For these reasons, SCR catalysts must not be selective with respect to the SO_2 oxidation reaction. Compared to the typical operating conditions of industrial boilers (O2 = 2%, NO_X = 500-1000 ppm), in gas turbines the SCR catalysts operate with higher oxygen levels (approx. 15%) and lower concentrations of NO_X (approx. 25-42 ppm).These differences are attributable to the greater degree of dilution of the fumes and, in most cases, to the simultaneous presence of primary NO_X abatement treatments in the turbine (DLN or water/steam injection). Under typical operating conditions, the NO_X removal efficiency is around 75-85%, but it is also possible to reach efficiency values higher than 90%. The removal efficiency strongly depends on the NH_3/NO ratio in the feed; normally a value close to the stoichiometric one is used and equal to 1. High values of the NH_3/NO ratio allow to obtain high NO_X removal efficiencies but at the expense of undesired ammonia emissions (ammonia slip).One of the most critical parameters concerning the performance of catalysts is the working temperature. For low temperature values the conversion of NO_X is limited by the reactivity of the catalyst, while at high temperature the conversion decreases due to the onset of the NH_3 oxidation reaction. The temperature range in which the catalyst guarantees high NO_X removal efficiency (with complete selectivity towards N_2) is called the "working window" and represents the temperature range in which the catalyst can operate. Today, catalytic systems operating at low, medium and high temperatures are offered which cover the overall temperature range between 200 and 600 °C.The HTSCR catalysts, due to the high temperatures (300-400 °C), must be positioned upstream of the desulfurizers and/or particulate control devices to avoid heating of the combustion gas. However, this accelerates the deactivation of the catalyst by exposure to high concentrations of SO_2 and particulate matter. It is therefore desirable to develop low temperature SCR catalysts for the treatment of fumes operating

in the temperature range of 100-250 °C: this would make it possible to move the catalyst downstream of the desulphurizer and/or the particulate control device, in which the temperature it is normally below 150 °C. This configuration not only allows the removal of NO_X at relatively low temperatures but also makes it potentially feasible to remove elemental mercury from the fumes at the exit of the particulate collector. The MnO_X/T_iO_2 material is classified as an LTSCR catalyst and, having shown an adsorption capacity of mercury in the range of 50-250 °C, it is possible to perform a simultaneous reduction of NO_X and mercury in a single process unit. It was also highlighted how an appropriate control of the morphology of the catalyst, and in particular a good balance between the fraction of macropores (which favor the intraparticle diffusion of the NH_3 and NO reagents) and micropores (which increase the specific surface of the catalyst), can lead to significant improvements in catalytic performance.In any case, the volume of the macropores must not exceed a certain limit value in order not to compromise the mechanical properties of the catalysts.

1.14 Neurotoxic effects of mercury

Methylmercury (MeHg) is the etiological substance of Minamata disease, Minamata disease is associated with various neurological disorders such as sensory disturbances and ataxia and is the consequence of exposure to high concentrations of MeHg. Recently, the negative effects of relatively low MeHg levels have become a major issue in the MeHg toxicology study. MeHg accumulates in organisms at the higher levels of the food chain. Humans are mainly exposed to MeHg through the consumption of contaminated seafood. It has been suggested that dietary intake of a low MeHg level, through MeHg-rich fish, during gestation has negative effects on the fetus (S. Ceccatelli et al., 2013). Importantly, the nervous system is particularly sensitive to chemicals during development (Grandjean, Landrigan, 2006). Two previous representative epidemiological studies on the risk of low-level dietary MeHg in child development are a birth cohort study of the newborn population of the Faroe Islands and a child development study in the Seychelles. The cohort study on the birth of the Faroe Islands showed that low levels of MeHg can cause neuropsychological dysfunctions in the domains of language, attention and memory in the context of childhood neurodevelopment (Grandjean. 1997). However, in the Child Developmental Study in Seychelles, no significant association was found between low MeHg intake and the child's neuropsychic development (Davidson et al., 1998). Although several epidemiological studies such as the two reported above have been conducted, no unanimous conclusion has been reached on the risk of low-level MeHg exposure during fetal neurological development, also because, given the different origins of the populations studied, it is possible that a different inherited genetic pattern is at the basis of different effects, due to the expression of enzymatic isoforms, involved in the biotransformation of xenobiotics. Previous epidemiological studies have reported that low-level prenatal MeHg exposure may be associated with poor psychomotor

development (Choi, et al., 2014; Lederman et al., 2008). In vivo and in vitro studies have shown that exposure to low MeHg levels negatively affects the development of the cranial nervous system (N. Onishchenko et al., 2007; Tamm et al., 2006). Experimental studies focusing on the catecholamine system have indicated that relatively low concentrations of prenatal exposure to MeHg may be associated with functional changes in catecholaminergic neurotransmitters, rather than global morphological changes in the developing brain (Gimenez-Llort et al., 2001 ; Lindstrom et al., 1991). Furthermore, in vivo studies have shown that exposure to low MeHg levels can disrupt the catecholamine system (Shao et al., 2015; Zimmer et al., 2011). These studies indicate that MeHg exposure can lead to neurological disorders. However, the underlying toxicological mechanisms have yet to be elucidated. Recently, the idea of "Evolutionary Origin of Health and Disease (DOHaD)" has gained increasing recognition. The DOHaD hypothesis postulates that environmental conditions during the embryonic period are related to the risk of developing disease in adulthood (Silveira, et al., 2007). Therefore, it is necessary to consider not only the immediate impact of chemical exposure on the embryo or fetus, but also its possible effects in adulthood. The DOHaD hypothesis suggests that epigenetic changes, such as DNA methylation and histone modification, could be induced by environmental conditions during development. These epigenetic alterations persist into adulthood and increase the risk of developing disease (Gillman et al., 2007). Epigenetics is defined as the study of the transcriptional system that does not involve changes in the DNA sequence (Goldberg et al, 2007). Correct programming of epigenetic modifications on the genome during development is essential to successfully complete ontogenesis (Fagiolini et al., 2009). Therefore, epigenetics is an important factor when studying the molecular mechanisms of the effects of MeHg exposure during the embryonic period. As far as we know, the influences of MeHg exposure during development on epigenetic changes have not been previously studied. In an experimental investigation, the effects of MeHg exposure on neuronal differentiation in terms of

epigenetics were examined, using immortalized cells derived from the human fetal brain (LUHMES cells) as a model of differentiation.

2.1 Amyotrophic Lateral Slerosis

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease that appears in most cases after the age of 50 and leads to a degeneration of motor neurons or motor neurons. The disease is also known as Lou Gehrig's disease, named after the famous American baseball player who was affected, or Charcot's disease, named after the French neurologist who first described it in 1860. In most cases, beyond 90 percent, the disease is sporadic and there is still no certainty about its causes, despite the fact that in recent years numerous studies have been carried out and many hypotheses have been advanced. On the other hand, 5 - 10 percent of cases are of familial ALS, that is, they present previous cases in the family. The prevalence, that is the number of cases present in the population, is increasing: this is also thanks to the current treatments that allow to prolong the life of the patient. However, there are currently no treatments capable of stopping the disease and the outcome remains dire. Motor neuron diseases are characterized by a progressive deterioration of the nerve cells involved in the movements of the muscular system: this phenomenon, in turn, determines a considerable weakening of the muscles which are innervated, and therefore stimulated, by this type of neurons which, if affected due to illness, they are no longer able to perform their normal physiological role. Amyotrophic lateral sclerosis is the most common form of motor neuron disease. Muscles are usually weak and shrink in mass, resulting in atrophy, and movements become stiff, clumsy and clumsy. The doctor bases the diagnosis mainly on the results of their clinical evaluation, through the medical history and physical examination, and performs electromyography, nerve conduction studies, MRI and blood tests to help confirm the diagnosis. There is no cure, but medications can help decrease symptoms. Motor neuron diseases can involve the central nervous system (the brain and spinal cord) as well as the peripheral nervous system (the nerves outside the brain and spinal cord). For normal function, muscle tissue and nerve connections between the

brain and muscle must be normal. Muscle movement is initiated by nerve cells found in the spinal cord and in the front of the brain (the so-called motor cortex).Nerve cells in the motor cortex connect to the nerves in the spinal cord that stimulate muscle movement (so-called motor nerves). In motor neuron diseases, these nerve cells progressively deteriorate. As a result, the muscles weaken, shrink, resulting in atrophy, and can become completely paralyzed, although the muscles themselves are not the cause of the problem, but represent the anatomical locus that undergoes the effects of damage to motor neurons. The movement of a muscle usually involves communication between the latter and the brain, through the nerves. The stimulus to make a specific muscle, or a group of them, make a movement can originate in the brain, as when a subject consciously decides to move a muscle, for example to perform a precise and targeted action; or, the urge to move a muscle may originate in the sense organs. For example, special nerve endings in the skin (sensory receptors) allow people to feel pain or feel the temperature. This information is sent to the brain, which can send a message to the muscle telling it how to react. This type of exchange involves two complex nerve pathways: the pathway of the sensory nerves to the brain and the pathway of the motor nerves to the muscles. As regards, however, the onset and course of the disease, they vary greatly from individual to individual and depend on the form of ALS from which one is affected. Usually the initial symptoms are short muscle contractions, also called fasciculations, cramps or a certain stiffness of the muscles, weakening of the muscle tone that affects the functioning of a limb and an indistinct voice. Nonspecific manifestations, which can be confused with many other diseases, often delaying diagnosis. This type of onset concerns about 75% of ALS cases while the remaining 25% has an onset called bulbar, which manifests itself with difficulty in speech up to the loss of the ability to communicate verbally and difficulty in swallowing. The two types of onset depend on which motor neuron is affected first, whether the first is at the level

of the cerebral cortex (bulbar onset) or the second is at the level of the brainstem and spinal cord (spinal onset).

In the past it was believed that the patient while progressively losing the ability to move, speak, swallow and often even breathe independently could keep his cognitive abilities almost intact. However, recent studies conducted with imaging techniques show how in some cases the ALS can affect the fronto-temporal lobe, thus accompanying dementia. Up to 50% of cases, there are extra-motor manifestations, such as changes in behavior, executive dysfunction and speech problems. In 10% -15% of patients, these problems are severe enough to meet the clinical criteria for frontotemporal dementia (FTD). A common genetic cause is a repeated expansion of the hexanucleotide in the C9orf72 gene, responsible for 30% -50% of familial ALS and 7% of sporadic ALS. These expansions are also a frequent cause of frontotemporal dementia, underscoring the molecular overlap between ALS and FTD (Masrori, Van Damme, 2020). One of the most interesting factors for understanding the pathophysiological mechanisms of this serious disease concerns the changes associated with oxidative damage to both neurons and glial cells. Astrocytes are known to support the development of motor neurons. Oxidative damage is also known to lead to the expression of stress-sensitive genes and proteins, as well as to inflammation of glial cells. Chronic inflammation could be a key factor in the pathogenesis of ALS as it has been linked to motor neuron death. Pathophysiological research has confirmed the influence of some proteins on the prognosis of ALS. ALS is therefore typically a proteinopathy in which proteins aggregate in motor neurons. Furthermore, the excitotoxicity of glutamate has also been linked to ALS, with the mutated form of the enzyme superoxide dismutase (SOD1) which has been shown to be responsible for familial ALS. Regarding the pathogenesis of ALS, various phenomena have been examined such as: increase in the levels of specific serum compounds, reduced concentrations of myelin and changes in 5-

hydroxytryptamine, all phenomena that could represent key indicators of the pathogenesis, prognosis and therapy of the SLA.

When into ALS therapy, antioxidant treatment is potentially very important. It is also believed that exposure to heavy metals negatively affects ALS, as we will try to demonstrate in this PhD thesis. Evidence also suggests that good nutrition is a very important factor in treating the disease. From a pharmacological point of view, serotonin treatment appears to be a useful therapeutic agent (Holecek, Rokyta, 2018). The diagnosis of ALS is made mostly symptomatically, based on clinical evidence and takes place, on average, one year after the onset of symptoms, although there are cases in which the diagnosis is made in a much longer time. Only in recent years have biological markers been developed for diagnosis that appear to have a high degree - more than 90 percent - of accuracy. It is currently believed that ALS is a disease with multifactorial causes, that is, that its onset can be determined by a series of genetic and environmental reasons. Among the causes of ALS, recent studies have identified the mutation of a group of genes as a predisposing factor. In particular, the role of the SOD1 gene that produces the Cu / Zn superoxide dismutase is carefully studied. When this works properly, it has the role of clearing the cells of a particular free radical. When this is not done, a toxic build-up occurs which ends up killing the neuron cells. Particular attention is currently paid to environmental causes and lifestyles that can facilitate the onset of the disease in predisposed individuals. Factors include contact with pollutants and frequent head injuries. This study hypothesis has gained particular strength after various studies, about which there are still controversies, have shown a higher incidence of the disease among professional athletes in particular soccer and football. Currently the only drug approved and which has been shown to be effective in reducing the progression of the disease is Riluzole which has the function of reducing the release of glutamate, which studies show to accumulate in the plasma and cerebrospinal fluid of patients causing

death. progressively neurons. Other treatments for ALS are aimed at making symptoms less severe and improving the quality of life for patients. These palliative care is best provided by multidisciplinary teams as ALS is a disease that affects all functional areas of the patient. Most patients alternate periods of hospitalization with periods of home care that require continuous assistance, both medical and nursing, physiotherapy and speech therapy. Therefore, the very high social impact of the disease that affects the whole family should not be underestimated, most people who are affected by ALS, in fact, are in the midst of their work and emotional activity; often the spouse of the sick person leaves or reduces their work, the house must be adapted to the needs of the patient and the psychological impact is very strong, sometimes devastating, also due to the patient's inability to communicate with voice or gestures. needs and desires, therefore, day after day, bioengineering and bioinformatics support are being sought, dedicated to improving the quality of life of patients and reducing the load, both in material-welfare terms and in psychological terms, of caregivers. for the home management of patients, therefore, computer systems were also developed, equipped with electro-medical equipment, dedicated to monitoring the ordinary activities of daily life of patients with ALS. Objective symptom monitoring of patients with amyotrophic lateral sclerosis (ALS) also has the potential to provide an important source of information for assessing the impact of the disease on real-world aspects of functional capacity and daily living activities in the home. , providing useful objective outcome measures for clinical trials. One study, in particular, aimed to investigate the feasibility of using a new digital platform for remote data collection of multiple symptoms: physical activity, heart rate variability and digital language characteristics, in 25 patients with ALS in a context of observational clinical trial, in order to explore the impact of devices on the daily life of patients and to record the tolerability of the devices themselves and the study procedures over 48 weeks; patients used the equipment successfully and it was generally well tolerated. The amount of physical activity data for

home monitoring was less than expected, although it was sufficient to allow for the study of new physical activity endpoints. Good quality in-clinic speech data was successfully acquired for analysis (Garcia-Gancedo et al., 2019). Future studies that will use objective patient monitoring approaches, combined with the most current technological advances, could be useful to elucidate new digital biomarkers of disease progression. However, the fact remains that this pathology affects 360-degree figures ranging from computer science to bioengineering, up to the most disparate fields of neuroscience and welfare disciplines: all this indicates how much it remains, even today, a pathological state with many aspects still to be clarified in the various types of investigations, not only of a clinical nature, with the hope that future results will contribute to making this demanding pathological entity less aggressive and devastating.

2.2 Epidemiology and pathophysiology of ALS

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive and disabling neurodegenerative disease with a subtle onset and difficult to diagnose in the prodromal phases of the disease. It is a pathology of adulthood and can occur in two forms:

1 – Family member (5% of cases), ie in different members of the family nucleus with onset around 63 years of age;

2 – Sporadic (95% of cases) ie with unknown etiology with earlier onset between 40 and 60 years.

In general, there is a slight male prevalence with a ratio of about 1.2-1.5. The prognosis in 50% of patients is about 30 months from the onset of symptoms. 5-10% of patients survive for more than 8 years while cases of longer survival are rare. Death often occurs from paralysis of the voluntary respiratory muscles. The global incidence is 1.7 cases per 100,000 people/year, with about 1000 new cases per year in Italy. The global prevalence is currently estimated at around 200,000-300,000 cases, about 5000 in Italy, with a strong concentration in Lombardy, followed by Campania, Lazio and Sicily, although this could largely depend on a greater capacity for diagnosis of hospitals and local specialists. Environmental risk factors for ALS include: trauma; tobacco smoke; intense sporting activity; exposure to toxic and xenobiotic agents. The role of these factors is not fully understood and it is not excluded that the onset of the disease is due to the interaction between genetic and environmental factors. The incidence of ALS is about 1-2.6 cases per 100,000 people per year, while the prevalence is about 6 cases per 100,000. The median age of onset for ALS is currently 58-60 years and the median survival from onset to death is 3-4 years. Between October 19, 2010 and December 31, 2011, there were approximately 12,187 prevalent cases diagnosed with defined ALS only in the U.S. The incidence of ALS in European populations is two to three people per year for every 100,000 of the general population. In Europe, crude prevalences range from 1.1/100,000

inhabitants in Yugoslavia to 8.2/100,000 in the Faroe Islands. Important progress has been made in our understanding of the genetic causes of ALS while the contribution of environmental factors has been more difficult to assess and large-scale studies have not yet revealed a definitive and replicable environmental risk factor.

The only known risk factors to date are older age, male sex, and a family history of ALS. Median survival time from onset to death is usually 3 years from the first onset of symptoms. Older age and bulbar onset are consistently reported to have worse outcomes. However, there are conflicting data regarding gender, diagnostic delay and El Escorial criteria. The rate of symptom progression proved to be an independent prognostic factor. Psychosocial factors and impaired cognitive function are negatively related to the outcome of ALS, while nutritional status and respiratory function are also related to the prognosis of ALS. The effect of enteral nutrition on survival is still unclear, although non-invasive positive pressure ventilation (NIPPV) has been found to improve survival. These findings have relevant implications for the design of future studies (Couratier et al, 2016).

As regards the pathophysiological aspects of this devastating disease, it is currently known that the familial form and some forms of sporadic amyotrophic lateral sclerosis are due to mutations of genes involved in various pathophysiological mechanisms.

Today, from a genetic-molecular point of view, the best known are:

• the SOD1 gene whose mutations are represented in about 20% of the familial forms and in 2-3% of the sporadic ones. SOD1 is the first gene discovered in ALS and appears to be exclusively associated with this disease;

• the TARDBP gene whose mutations are present only in 3% of the familial forms and in 1-2% of the sporadic ones. TDP-43, the protein encoded by this gene is present in the protein aggregates found in neurons and motor neurons of 97% of patients with ALS;

• the C9orf72 gene whose mutations are currently the most represented both in familial ALS (40%) and in sporadic ALS (20%) as well as in some patients with frontotemporal

dementia. Unlike other genes, the C9orf72 mutation can cause both a loss of protein function and the acquisition of toxic effects.

So far there are more than 30 genes presenting a risk of association with ALS; however, there is an inheritance that also includes other gene variants in which the disease is evident only with the presence of more than one abnormal gene.

The genetic test on subjects at risk of family disease is carried out in multidisciplinary ALS centers: here a genetic consultant helps the patient and family members to assess the risk and discuss the impact, as there is currently no effective cure for this disease.

Genetic studies have shown that C9orf72, SOD1, TARDBP and FUS are the most common mutated genes in lateral sclerosis amyotrophic. A significant difference in the frequencies of mutations in major ALS genes was identified between European and Asian patients.

In European populations, the most common mutations were C9orf72 repeated expansions (FALS 33.7%, SALS 5.1%), followed by SOD1 (FALS 14.8%, SALS 1.2%), TARDBP (FALS 4, 2%, SALS 0.8%) and FUS mutations (FALS 2.8%, SALS 0.3%), while in Asian populations the most common mutations were SOD1 mutations (FALS 30.0%, SALS 1.5%) , followed by FUS (FALS 6.4%, SALS 0.9%), C9orf72 (FALS 2.3%, SALS 0.3%) and TARDBP mutations (FALS 1.5%, SALS 0.2%. These results demonstrated that the genetic architecture of ALS in Asian populations is distinct from that of European populations, which must be adequately taken into account when carrying out genetic testing on ALS patients (Zou et al., 2017). Sporadic ALS (90 -95%) constitutes the vast majority of cases, while the remaining 5-10% are hereditary and are referred to as familial ALS. Sporadic ALS is suspected to involve genetic susceptibility to environmental risk factors (Talbott, 2016). as great advances made in our understanding of the genetic causes of ALS, the contribution of environmental factors has been more difficult to assess.

Large-scale studies of clinical models of ALS, individual histories prior to the onset of ALS, and rates of ALS in different populations and groups have led to better patient care, but have not yet revealed a definitive environmental risk factor and replicable (Al-Chalabi, Hardiman, 2013). The cells of the anterior horn control all voluntary movements: motor activity, respiratory, speech and swallowing functions depend on the signals of the cells of the anterior horn. Diseases that damage the cells of the anterior horn therefore have a profound impact. Symptoms of anterior horn cell loss (weakness, falling, choking) lead patients to see a doctor.

Neurologists are the most accredited professionals in recognizing and diagnosing damage or loss of anterior horn cells. ALS, the prototype of motor neuron disease, demonstrates the impact of this class of disorders. ALS and other motor neuron diseases can present profound, but also challenging, diagnostic challenges. Neurologists are often called upon to support their patients step by step: coordinate care, arrange durable medical equipment, and lead end-of-life care discussions with patients and their caregivers. It is important for neurologists to be able to identify motor neuron diseases and to evaluate and treat affected patients (Tiryaki, Horak, 2014).

This disease, in fact, is fatal for motor neurons, being characterized by the focal onset of muscle weakness and incessant and worsening progression of the disease. While the presence of concomitant upper and lower motor neuron signs has been recognized as a pathognomonic feature of ALS, the pathogenic importance of upper motor neuron dysfunction has only recently been described. In particular, transcranial magnetic stimulation (TMS) techniques have established cortical hyperexcitability as an important pathogenic mechanism in ALS, related to neurodegeneration and disease spread. Separately, ALS exhibits a heterogeneous clinical phenotype that can lead to misdiagnosis, particularly in the early stages of the disease process. Cortical hyperexcitability has been shown to be a robust diagnostic biomarker of ALS, reliably

differentiating ALS from disorders mimicking other neuromuscular disorders (van denBos et al., 2019).

Unfortunately, ALS is an inexorably progressive and fatal disease, still with no curative therapies available to date. Symptomatic and palliative care, provided in a multidisciplinary setting, still remains the cornerstone of managing ALS.

However, our understanding of the molecular mechanisms underlying the disease has advanced significantly in recent years, offering new hope for the development of new diagnostic and therapeutic approaches (Riva et al, 2016). Genetic studies have helped to understand the implications of autophagy and / or mitophagy deficits in the onset of the disease.

Some authors have analyzed recent advances in our understanding of pathways for autophagy and mitophagy in neurons and how these pathways can be affected by mutations in genes including DCTN1, OPTN, TBK1, VCP, and C9ORF72. The researchers also considered the implications of autophagy modulation in ALS, highlighting both the potential for a possible treatment approach and the risks that could be connected to this type of therapeutic intervention (Evans, Holzbaur, 2019).

2.3 Cellular and molecular mechanisms involved in methylmercury neurotoxic damage

As is known, methylmercury is a neurotoxic agent highly present in the environment, both as a substance of natural origin and as a contaminant deriving from anthropic activities (in single way or in combination with other substances or molecules). Although the molecular mechanisms of methylmercury-mediated toxicity are not yet fully understood, a lot of of research indicates that the pro-oxidative properties of this substance are responsible for its toxic effects. Results obtained from experimental studies on the mechanisms of mercury toxicity in vivo and in vitro refer that the disruption of the antioxidant system may play an important role in the toxic effects of mercury. Furthermore, the accumulated evidence refers that the signal transduction, the protein and/or enzyme activity and gene regulation are involved in mediating the toxic and adaptive response to mercury exposure (Yang, Zhang et al. 2020).

About this, metalmercury appears to be a mild electrophilic agent interacting preferentially with nucleophilic groups oxidizing them, such as sulfur-containing and selenium-containing groups, belonging to biomolecules. This type of interaction has been provided to establish oxidative stress with the consequent compromise of various molecules together with different biological activities: membrane receptors, transporters, enzymes and structural proteins, membrane lipids (such as those associated with the phospholipid bilayer) or intracellular molecules with massager functions; also nucleic acids can be the target of the harmful action of metalmercury, thus contributing to the establishment of neurotoxicity mechanisms.

Some authors (Farina and Aschner, 2017) have studied a first general pattern, concerning the neurotoxicology of MeHg with particular attention to its pro-oxidative properties and its interaction with nucleophilic molecules containing thiol groups and selenols.

Recent studies have reported that the ubiquitin-proteasome system is involved in the defense against metalmercury toxicity through the degradation of proteins by synthesizing pyruvate. The mitochondrial accumulation of pyruvate can increase the toxicity of methylmercury.

Furthermore, methylmercury exposure induces several immune-related chemokines particularly in the brain and this biological phenomenon appears to be related to the onset of neurotoxicity (Lee, Hwang, Naganuma, Satoh, 2020).

According to researchers, experimental evidence shows that the symptoms (such as motor disability) resulting from exposure to MeHg are linked to its pro-oxidative properties, as well as to their molecular consequences (lipid peroxidation, disruption of glutamate homeostasis and/or calcium), the data concerning the relationship between molecular parameters under investigation and behavioral deterioration and others such as those relating to motor function (e.g. visual impairment, cognitive skills, etc.) are still not exhaustive, if one wishes to admit a cause-effect relationship between metalmercury and neurotoxic damage, also clinically objectionable.

2.4The epigenetic mechanisms of ALS

Epigenetic mechanisms control various functions throughout the body, from determining cell fate to developing immune responses and establishing inflammatory responses. Epigenetics therefore refers to heritable changes in gene expression unrelated to changes in the DNA sequence. Three major epigenetic mechanisms include DNA methylation, microRNA, and post-translational modification of histone proteins. Histone modifications occur in many amino acid residues and include phosphorylation, acetylation, methylation and other chemical moieties. Recent evidence points to a possible role of epigenetic mechanisms in the etiology of ALS. Studies have looked at recent advances linking ALS and epigenetics, with a strong focus on histone modifications. Both local and global changes in histone modification profiles are associated with ALS, drawing attention to potential targets for future Neuroinflammation is a major contributor to the initiation and progression of neurodegeneration in a variety of diseases, including ALS, Alzheimer's disease and Parkinson's disease. Since astrocytes are the largest population of glial cells, they represent an important regulator of CNS function, both in maintaining the regular homeostasis underlying the maintenance of health and in the various stages of disease processes. Studies have only recently begun to identify the epigenetic mechanisms that regulate the responses of astrocytes in neurodegenerative diseases. These epigenetic mechanisms, together with the epigenetic signs involved in the development of astrocytes, could elucidate novel pathways to potentially modulate neuroinflammation and astrocyte-mediated neurotoxicity in various neurodegenerative diseases. Some studies have examined the known epigenetic mechanisms involved in the regulation of astrocyte function, from development to neurodegeneration, linking these mechanisms to potential specific roles of astrocytes in neurodegenerative diseases with a focus on the potential opportunities for therapeutic intervention: in fact the pathophysiology of this

type of diseases involves a range of multifactorial pathogenetic elements, so it would be important to know, in addition to each single stage of the pathogenetic path, also the probable targets of this same process, in order to establish, apply and monitor adequate therapeutic strategies (Neal M, Richardson There are many biological molecules involved in epigenetic mechanisms that seem to be involved in the onset of various pathological processes, including neurodegenerative pathologies. Gene expression is epigenetically regulated through DNA methylation and covalent chromatin modifications, such as acetylation, phosphorylation, ubiquitination, sumoylation and histone methylation. The methylation state of histone is dynamically regulated by different groups of histone methyltransferases and demethylases. Trimethylation of histone 3 (H3K4) to lysine 4 is usually associated with activation of gene expression, while trimethylation of histone 3 to lysine 27 (H3K27) is associated with repression of gene expression. The polycomb repressive complex contains the H3K27 Ezh2 methyltransferase and controls the dimethylation and trimethylation of H3K27 (H3K27me2 / 3). The Jumonji-3 containing domain (Jmjd3, KDM6B) and the ubiquitously transcribed X chromosome tetratricopeptide repeat protein (UTX, KDM6A) have been identified as H3K27 demethylases that catalyze the demethylation of H3K27me2 / 3. The role and mechanisms of JMJD3 and UTX have been extensively studied for their involvement in development, cellular plasticity, neurodegenerative diseases, the immune system and neoplastic diseases (Burchfield et al. 2015).diagnostic and therapeutic approaches (Bennett, 2019).Reversing neurodegenerative symptoms and related disorders is the challenging task in which epigenetics plays a crucial role which includes DNA methylation, histone modification and chromatin remodeling and regulation, linked to the progression of various disorders neurodegenerative (NDD). The overexpression, for example, of various histone deacetylases (HDACs) can activate glycogen synthase kinase 3 which promotes the hyperphosphorylation of tau and inhibits its degradation. Although HDAC is important for maintaining neuronal morphology and

brain homeostasis, at the same time these enzymes promote neurodegeneration if it undergoes some dysregulation process. Several experimental models have also confirmed the neuroprotective effects caused by HDAC enzymes through the regulation of neuronal apoptosis, inflammatory response, DNA damage, cell cycle regulation and metabolic dysfunction. In addition to transcriptional regulation, protein-protein interaction, post-translational modifications of histone, non-histone protein deacetylation mechanism, and direct association with disease proteins have been linked to neuronal imbalance. Histone deacetylase inhibitors (HDACi) may be capable of altering gene expression and have demonstrated their efficacy in experimental models and in clinical trials for NDD and have proved to be a very promising therapeutic agent with certain limitations, for example, non-specific target effect, isoform-selectivity, specificity and limited number of expected biomarkers. Some authors have studied both the catalytic mechanism of the deacetylation process of various experimental models of HDAC in vivo and in vitro and the modalities through which HDACs are participating in neuroprotection and neurodegeneration, and the complete role of HDACi in maintaining neuronal homeostasis. and, finally, the possible therapeutic role of biomolecules to modulate HDAC, a molecule that, as mentioned, plays a truly crucial role in neurodegeneration processes, including ALS (Gupta et al, 2020).

3.1Protein structure and mechanism of action of REST

The REST (repressor element-1-silencing factor) or NRSF (neuron-restrictive silencing factor) protein is a transcription factor consisting of nine C2H2 zinc finger domains and binds DNA (and also RNA) by eight of them, into one specific region of a promoter or enhancer. The REST protein is made up of 1097 amino acids and the "zinc finger" domain, through which DNA binds, is followed by proline and lysine-rich domains inserted between the repressor domains (RD1-RD2) , located at the C and N terminal ends of the molecule. The REST protein binds specifically to a 21-23 base pair site of a binding sequence called Repressor Element 1 (RE1), of which there are approximately 1900 copies in the human genome. There are several variants of REST, including form 4, a specific neuronal variant, in which the C-terminal domain is absent. The REST 4 variant has a lower affinity for DNA than the normal form.

This truncated form is thought to inhibit REST-mediated repression through the prior recruitment of corepressors (Chong et al., 1995, Schoenherr et al., 1996), however the mechanism is still unclear. Although REST was originally identified as a transcription factor that silenced neuronal genes in non-neuronal tissues, numerous studies have shown that REST plays an important role in differentiating neuronal stem cells and in regulating the expression of genes also in adult neurons. In fact, REST protein was found in the neurons of the ventricular area of the neuroepithelium during the development of the neuronal tube and in specific brain regions (hippocampus, midbrain, hindbrain) of the adult brain. REST activity appears crucial in deciding the fate of neuronal stem cells; in fact, it is the best characterized protein that represses the transcription of neuronal genes in non-neuronal cells and in neuroblasts before terminal differentiation (Chong et al., 1995). In embryogenesis, during the process of determining and differentiating neuronal precursor cells, the REST protein is degraded before the expression of the REST gene becomes strongly reduced or even silenced (Ballas et al.,

2005). This suggests that a reduction in REST activity is necessary for the fate of a mature neuron and its function.

In neuronal stem cells, REST inhibits the expression of specific neuronal genes by keeping the cells in an undifferentiated state. This transcriptional repression is mediated by the N-terminal domain and induces the recruitment of histone-deacetylase (HDAC) via its corepressors: mSin3 and CoREST, along with other corepressors such as H3K9 methylase, the NADH-sensitive corepressor CtBP and G9a. The latter can be recruited directly from REST, but can also be associated with the CoREST complex. The CoREST complex is accompanied by two classes of histone deacetylases: HDAC1 and HDAC2, by the histone demethylase H3K4, which would be the LSD1 enzyme that remodels the chromatin, BRG1, the methylase G9a and the DNA binding protein MeCP2. Therefore, the REST protein, by interacting with several of the afore mentioned enzymatic activities, modifies chromatin. To repress the transcription of genes, REST coordinates chromatin modifications, which are obtained from the activity of distinct enzymes that modify chromatin, such as BRG1, an ATP dependent enzyme. BRG1 is recruited from the C-terminal domain of REST, as part of the CoREST complex, and stabilizes the binding of REST to the RE1 site. Once bound to chromatin, the BRG1 complex repositions the nucleosomes allowing REST to obtain a more stable interaction with DNA. From this interaction, REST mediates repression through the activity of histone deacetylase, histone demethylase and histone methylase. N-terminal histone modification by acetylation and deacetylation represents the main pathway for transcriptional regulation. The N-terminal domain of REST interacts with the mSin3 complex, which contains the histone deacetylases HDAC1 and HDAC2, and is important for the function of several repressors. Instead, the C-terminal domain of REST interacts with the CoREST complex, which is widely expressed in neuronal and non-neuronal cells. Although the N and C terminal domains of REST can function independently of each other, it is still unclear whether they should be considered distinct

and independent. In fact, both of them are able to repress the Nppa gene (atrial natriuretic peptide), but the simultaneous action of both complexes is necessary to repress the Nppb (brain natriuretic peptide) gene in the cell line of cardiomyocytes of rat H9c2 (Bingham et al., 2007). The mSin3 and CoREST complexes contain HDAC1 and HDAC2 and each of these corepressors is capable of deacetylating histone H3K9 and H3K4; once deacetylated, histone H3K9 is dimethylated by histone methylase G9a.

Through the action of its corepressors, REST mediates both transient gene transcriptional repression and the long-term silencing of specific genes in different tissues. The repression of some genes, such as Nppa in the heart tissue and Kcnn4 in the vascular musculature, requires the constant presence of the binding of REST to the RE1 sites (Bingham et al., 2007). On the other hand, in the case of Bdnf and Calb1, expressed in the brain, the repression is maintained following the loss of the binding of REST to its own sites. During neuronal differentiation, although binding of REST to RE1 sites is lost, the CoREST complex remains bound and continues to mediate repression of associated genes (Ballas et al., 2005, Ballas and Mandel, 2005). This continued repression appears to be facilitated by DNA methylation and the recruitment of MeCP2, which, together with CoREST, results in a stable repressive state containing methylated DNA. The mSin3 complex can also form stable interactions with chromatin, probably through the histone binding of the proteins RbAp48 and RbAp46, to maintain the repression due to the deacetylation of H3, regardless of the levels of acetylation of K4. These data highlight the potential mechanisms by which the REST protein, and other transcription factors that recruit mSin3 and/or CoREST, could mediate long-term gene repression following only transient binding of the repressor to its sequence, this binding being favored. by an initial stable corepressor-chromatin interaction. It is not yet known how long this repression lasts. Although REST can recruit a whole range of corepressors, not all complexes are recruited into all genes. The consequences of recruiting a complex rather than another will lead to obvious differences in chromatin

status. These differences in chromatin modification support the contention that REST can mediate both short-term repression and long-term gene silencing. The difference between the stable or transient repressor activity of REST is important when the levels of REST are reduced, as for example, in neuronal differentiation, cardiac hypertrophy and colon cancer (Ooi and Wood, 2007).

Despite the numerous studies performed to clarify the function of the REST protein, little is known about the mechanisms that regulate its expression. Analyzes of the gene structure of rats, mice, and humans have shown that the REST protein is made up of tre esons. The transcription of REST starts from one of the three alternately. The transcription of the first exon produces the largest share (about 80%) of mRNA; the second exon is responsible for the transcription of a share of mRNA equal to 20%; finally, the remaining 1% of transcript derives from the third exon. The presence of these three exons suggests that REST contains at least three promoters, although none of them appear to be cell or tissue specific. All REST promoters in mice and humans have multiple initiation sites for transcription and contain GC boxes which are binding sites for the Sp transcription factor family.

Two hypotheses have been made regarding the physiological function of REST: 1) levels of REST monitor regulated secretion (process in which proteins and neurotransmitters are stored in vesicles and released only in response to certain stimuli); 2) REST regulates the expression of microRNA. The hypothesis that REST regulates microRNA expression has been extensively validated (Bruce et al., 2009) it has been shown that REST regulates the expression of the mir-9, mir-124 and mir-132 genes in mice, which promote neuronal differentiation. REST itself can be a microRNA target such as mir-153, whose expression is also regulated (Mortazavi, A. et al., 2006) .These reciprocal repression mechanisms lead to the formation of a bistable switch which would lead to high levels or low REST, but not intermediate levels. In addition to the potential modulation by microRNA, it has also been proposed that REST can be modulated by

dsRNA. Kuwabara et al. have identified a dsRNA of 20 nucleotides that contains a RE1 sequence in neuronal stem cells of the adult rat hippocampus and which is involved in neuronal neurogenesis. This dsRNA is present at low levels in rat neuronal progenitor cells and at high levels in mature neurons; it does not appear to interact with the REST mRNA, but rather with the specific protein in order to alter its activity. The ectopic expression of this dsRNA in rat neuronal progenitor cells leads to the increased expression of genes containing RE1 (Gria2 and Stmn2). The mechanism of action of dsRNA is not yet fully understood, although the presence of Gria2 and Stmn2 requires both the presence of RE1 in the promoter and of dsRNA, suggesting that the latter converts REST from repressor to activator. However, dsRNAs do not alter the amount of REST that binds to the Gria2 and Stmn2 genes, instead their expression is associated with the presence of a CBP histone-acetyltransferase and an increased level of acetylation of these genes (Kuwabara et al., 2004).

3.2REST and neurological disorders

The REST protein is involved in various pathologies, thus underlining the important role it plays in allowing regular cell development. The deletion of the human chromosome 4q12, on which REST is located, is common in cases of colon cancer. In fact, an altered reading of the REST coding region with related synthesis of a truncated protein that lacks the C-terminal repression domain has been identified in a primary colorectal adenocarcinoma. Furthermore, even in the case of lung cancer and neuroblastoma there is a truncated form of REST that lacks the DNA binding domain and the C-terminal repression domain (Singh et al., 2008). These truncated forms of REST can act as "negative domains". An oncogenic role of REST has also been identified in medulloblastoma, an aggressive neoplasm of the cerebellum of neuronal origin that

affects children. In fact, it has been shown that high levels of REST are combined with an overexpression of Myc, which favors cell proliferation and tumorogenesis, rather than cell differentiation. Therefore, reduced REST function appears to be a common occurrence in different tissues during normal physiological responses. The REST protein is also implicated in various diseases affecting the Central Nervous System. Increased levels of REST were found in hippocampal cells of rats and in those of the cerebral cortex following seizures and ischemic episodes. In cerebral ischemia, elevated levels of REST are associated with a decreased expression of Gria2 which leads to a greater entry of calcium ions through the AMPA GluR-2 lacking receptors into the cell with consequent cell death (Calderone et al., 2003). In the CA1 region of the hippocampus, increased levels of REST are correlated with a lower expression of the gene of the Opmr1 receptor (opioid receptor), which determines a neuroprotective effect, probably as a consequence of an increased release of GABA (gamma amino butyric acid), which is known to reduce neuronal activity (Formisano et al., 2007). An important role of REST and its target genes has been identified in the pathogenesis of Huntington's disease. This is an autosomal dominant hereditary neurodegenerative disease that occurs around age 35 and involves psychosis, progressive movement and cognitive system disorders. Currently there is no effective treatment of the disease, but much progress has been made on the knowledge of the pathogenetic mechanisms, characterized by the involvement of various pathways controlling cellular homeostasis and the neuronal network. The gene responsible for Huntington's disease (HD) is located on chromosome 4 and encodes a protein of 3144 amino acids called huntingtin (58,1): the variation consists in an expansion, beyond 36 triplets, of a CAG trait present in the first exon of the coding gene. Both the wild-type and the mutated allele are transcribed and translated, so that a protein is generated from the mutated allele that possesses an abnormally elongated polyglutamine tract (poliQ), corresponding to the CAG gene expansion.

Numerous studies have shown that the mutated HT interacts with the transcriptional apparatus in a specific and related way to the length of the poliQ tract. Direct transcriptional targets of HT are the transcription activating factor Sp1, the cyclic AMP-responsive binding protein (CREB), and the transcription factors TFII and TFIIF, which are inhibited by mutated. A particular mechanism of transcriptional control that affects the pathogenesis of HD is that exerted by RE1 / NRSE gene repression or silencing sequences, which regulate some genes specifically expressed in neurons, such as genes for channel proteins, receptors for neurotransmitters, the corresponding enzymes to the synthesis, and structural and functional proteins of synaptic vesicles. The RE1 / NRSE sequences are controlled by the specific neuronal silencing factor (REST / NRSF) present in the cytoplasm, a protein that normally enters the nucleus and forms a complex that acts as a corepressor by binding to the RE1 / NRSE sites. These studies have shown that the ordinary HT sequesters in the REST / NRSF cytoplasm and therefore prevents the binding of these to the repressor, favoring the transcription of genes under the control of NRSEs. On the other hand, in the case of ht mutated, this function is lost, with consequent inhibition of the transcription of genes dependent on RE1 / NRSE elements (Zuccato et al., 2003, Buckley et al., 2010).

4.1Protein structure and mechanism of action of Sp transcription factors

Transcription is a concerted event that mediates the binding of transcription factors to specific DNA sequences and involves general transcription factors and RNA polymerase II, forming a complex that initiates transcription. The promoters of many genes transcribed by RNA polymerase II contain a TATA consensus sequence (called the TATA box), located approximately 25-30 nucleotides upstream of the transcription initiation site. These TATA sequences bind TFIID, which is an essential factor for the transcription of all mRNAs. However, many promoters lack the TATA box (approximately 50% of transcripts in Drosophila lack the TATA sequence). The TATA-less promoters usually contain binding sites for Sp (consensus sequence: GGGCGG), since the binding of Sp1 to its own sites tends to be stronger in TATA-less promoters than for those containing the TATA sequence. Activation of TATA-less promoters by Sp1 predicts a more likely recruitment of TFIID. Furthermore, in many of these promoters, Sp1 binding is involved in determining the transcription initiation site. The Sp transcription factor family consists of four members in humans: Sp1, Sp2, Sp3 and Sp4.

Sp1 is the first identified and the best characterized form. The four factors have similar structural domains: they contain three zinc finger domains, representing the DNA binding domain near the C-terminus, and glutamine-rich domains adjacent to serine/threonine, which elongate in the N-terminal region. The 81 aa that form the C2H2-type zinc finger domain represent the most conserved part of the protein.

Sp1 and Sp3 are ubiquitous factors involved in the control of a whole range of genes. Sp1, Sp3 and Sp4 compete for similar binding sites, and the relative transcription rate is affected by the outcome of this competition. Conversely, Sp2 is less similar to other members of the Sp family; it presents a 20-27% of overlap in the protein sequence, moreover, an important histidine residue in the first zinc finger domain is replaced by a

lysine residue. In agreement with this structural difference, it hes been found that it does not bind to the GC-box, but to a GT-rich region present within the 5'-flanking region of the T cell receptor gene. Finally, Sp4 has expression tissue-specific limited to the brain and nervous tissue.

The structure of the Sp1, Sp3 and Sp4 polypeptides consists of five domains. In Sp1, domains A, B, C and D are responsible for both homotypic and heterotypic protein-protein interactions. Supra-ordered or multiple structures of tetramers are formed through protein-protein interactions in domains A, B or D. These higher-order structures result in super-activation, in which multiple binding sites show greater transcriptional activity than the sum of the individual sites. Domains A and B are in the N-terminal half of the protein, which can be further divided into subdomains containing a serine and a threonine-rich zone followed by a glutamine-rich zone. The C-terminal domain contains a highly charged region, which is responsible for inhibition of transcription by Sp3. The actual DNA binding domain contains three zinc finger domains that recognize DNA at GGGCGG or CACCC binding sites with similar binding affinities. Each zinc finger pattern is composed of an alpha helix and a tetrahedral beta sheet structure that binds a zinc atom. Finally, domain D is positioned at the C-terminal end beyond the zinc finger domains and forms protein-protein interactions with other transcription factors (Suske, 1999).

4.2Sp1 and neurological disorders

Sp1 is a transcription factor that binds to the GC-boxes of the promoters of a series of genes expressed in various tissues. Phosphorylation of Sp1 and Sp1-binding adapter proteins regulate positive and negative effects in gene transcription. This transcription factor participates in a number of physiological processes, including cell cycle regulation and angiogenesis. Sp1 is related to Alzheimer's disease, the particular feature of which is

the abnormal deposition and aggregation of β-amyloid, responsible for the observed neurodegeneration, which results from the cleavage of the amyloid precursor protein APP by β and γ-secretases into the form β-amyloid and senile plaques. The key event that determines the alteration of the tau protein, from which the formation of neurofibrillar aggregates derives, appears to be the hyperphosphorylation of tau and the accumulation of hyperphosphorylated tau which contains neurofibrillated tangles. In vitro studies have shown that Sp1 is involved in the positive regulation of TGF-β dependent APP expression. Sp1 also regulates the expression of BACE1, the main β-secretase involved in the cleavage of APP, and BACE2, a homolog of BACE1. Finally, Sp1 also regulates the expression of tau (Santpere et al., 2006).

4.3Transcriptional activation of REST gene by Sp1 Huntington's disease

Huntington's disease is an autosomal dominant neurodegenerative disorder characterized by involuntary movements, psychiatric disorders, and cognitive decline. The pathology is characterized by severe atrophy of the striatum and this is due to an abnormal expansion of the CAG repeat in exon 1 of the HD gene, which is translated into an expansion of polyglutamines (PolyQ) in the huntingtin protein (58,1) 'N-terminal end. This disorder belongs to a group of 10 inherited neurodegenerative disorders, including 7 dominant spinocerebellar ataxias (SCAs) which are caused by the expansion of PolyQ into disease-specific proteins. PolyQ expansions are thought to impart toxic properties to mutant proteins by causing protein interactions and aberrant aggregations of proteolytic fragments containing PolyQ. Various studies indicate that the mutated protein ht disrupts the regular transcription process in diseased neurons. It was also noted that neurotransmitter levels were altered post-mortem in mice that had these disorders and a series of dysregulated genes were found, including a series of genes present in striatal neurons, in fact, this is the region most affected by the disease. However, the

mechanisms leading to gene repression in HD are unclear. MHtt has been found to alter many factors of transcription regulation including Sp1. Several studies suggest that mHtt binds Sp1 and causes loss of its function on the promoters it acts on Other studies report that the level of the Sp1 protein is increased in HD and that genetic or pharmacological methods that decrease the level or activity of Sp1 have a beneficial effect on HD mice. Consequently, the role of Sp1 in neuronal gene repression is far from clear (Ravache et al., 2010).

AIM OF THE STUDY

5 Aim of the thesis

Since MeHg exposure: (1) hastens the onset of amyotrophic lateral sclerosis-like fhenotype in SOD1 G93A mice activating glutamate receptors (Johnson et al., 2011) and (2) determined neuronal cell death by increasing REST expression (Guida et al., 2017a; Guida et al., 2018), we investigated the possible role of MeHg to accelerate reduction in cell survival in NSC-34 motor neuron-like cells transiently transfected with G93A-SOD1 construct via REST up-regulation. Furthermore, we studied the transcriptional mechanism increasing REST expression via its well-known transcriptional activators Sp1, Sp3, CREB and JunD. Additionally, we identified the epigenetic mechanism by which MeHg regulated REST gene. Since REST overexpression in neurons determined cell death by activating necroptosis, we investigated the role of MeHg to activate necroptosis in NSC34 cells overexpressing G93A-SOD1 construct.

MATERIALS AND METHODS

6.1 Material, cell cultures, drug treatment and small interfering RNAs (siRNAs) transfections.

The NSC-34 cells were purchased by Tebu-BIO (Magenta, (MI) Italy). Cells were used between 5–20 passages. Each type of experiment was performed on the same passage. Cells were cultured in RPMI in the presence of 10% inactivated fetal bovine serum and penicillin/streptomycin at 37 °C, 5% CO2 and 95% air, in a cell culture incubator Methylmercury (II) chloride (MeHg) (cod: 442534 stock solution 100 mM) and Necrostatin-1 (Nec) were purchased from Sigma (cod. N9037; stock solution 20 mM) both obtained from Sigma–Aldrich (St. Louis,MO) were dissolved as previously reported (Guida et al., 2017a). Culture media and sera were purchased from Invitrogen (Milan, Italy). All chemicals were diluted in cell culture medium. Synthetic oligonucleotides were from Eurofins Genomics. siRNA for REST (siREST) (EMU029201) siRNAs for Sp1 (siSp1) (EMU061231), and KMT2A (siKMT2A) (EMU180041) was purchased from Sigma-Aldrich. whereas the scramble (siCTL) (001910-10-05) were purchased from Dharmacon. For Nec experiments NSC-34 cells were pre-incubated for 2 h with the drug at 1, 5, 10 and 20 mM followed by MeHg 100 nM for 24 hours. siRNAs transfection in cells were performed 24 hours before MeHg exposure with Lipofectamine LTX (15338–100, Invitrogen, Milan, Italy), in accordance with the manufacturer's protocol, as previously reported (Guida et al., 2015a). The plates used and the density of cells plated have been previously published (Guida et al., 2017b).

6.2 Cell viability assessment

Cellular function is related to mitochondrial function which was assessed by measuring the activity of mitochondrial dehydrogenases. The soluble compound 3- (4,5-dimethylthiazol-2-yl)-2.5, diphenyltetrazolium bromide (MTT) was used as substrate. Mitochondrial dehydrogenases reduce MTT by converting it into formazan, a compound that is insoluble in water and optically active at a wavelength of 540 nm. The amount of formazan produced is directly proportional to the metabolic activity of mitochondrial dehydrogenases and therefore, indirectly, to cell viability. At the end of the exposure to MeHg, the culture medium was removed and the cells were incubated in 2 ml of a PBS solution containing MTT at a concentration of 0.5 mg/ml for one hour at 37°C in an atmosphere consisting of 5% CO2 and 95% air. At the end of the hour, the incubation was interrupted by removing the solution and adding 1 ml of dimethyl sulfoxide (DMSO), to solubilize the formed formazan salts. The results were obtained by spectrophotometric analysis and were expressed as the percentage of survival of cells treated with MeHg compared to the values obtained from cultures exposed to the vehicle (Veh) to which the 100% survival value was attributed.

6.3 Determination of cell death

Cell death was identified with the lactate dehydrogenase (LDH) cytotoxicity kit from Cayman, as previously reported (Guida et al., 2017c). NSC 34 cells had been treated with MeHg 100nM for 24 hours alone or after 2 hours pretreatment with Nec at 1, 5, 10 and 20 µM. Cells treated with 1% Triton X-100 (Sigma) was considered 100% of cell death.

6.4 Quantitative Real Time PCR

Total RNA was extracted using Trizol according to the manufacturer's instructions and quantified spectrophotometrically. The retro-transcription was performed as previously reported (Guida et al., 2018). The reaction mixture was incubated for 60 min at 42°C and 2 min at 94°C, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. Normalization of the results was performed by Beta-Actin (Actin). The oligonucleotides sequences were: REST (m): (FW 5′-TCCGCATGTGTCGCGTTA-3′ and RV 5′-GTTCAGCACGTGCGAACTCA-3′); for Sp1 (m): (FW 5′-GCCTGTTAGGAGGTCCCTGAA-3′ RV 5′-AGGCTGCCCATTTGTACTCATTTA-3′); for Sp3 (m): (FW 5′-CCTGACACAGACCCCTGTTGA-3′ and RV 5′-CAGTGCTCGCATCTGTGGAA-3′); for KMT2A (m): (FW 5′-GACCGAATCGGACTAAACACTTTC-3′ and RV 5′-GTGACCTTGCCTAGTAATCGAACTT-3′); for Actin (m): (FW 5′-AACCCCTTTTCAGCCTAGTCAGT-3′ and RV 5′-CGAATCGTGTTGCCAGAGAA-3′). Changes in gene expression between groups were represented as the mean of the relative quantification (RQ) values, that was calculated as the difference in threshold cycle (ΔCt) between the target gene and the reference gene ($2^{-\Delta\Delta CT}$=RQ).

6.5Western Blotting and Immunoprecipitation

Protein expression was evaluated using standard Western Blotting methods. After the treatments, the cells were collected and centrifuged for 30 minutes at 15000 rpm. The cells were washed only once with PBS and a lysis buffer was added TRIS-HCl pH 7.5 (1 M), NaF (0.5 M), NaCl (4 M), PMSF (100 mM), Vanadate (100 mM), NP-40 (20%) +

protease inhibitor. The determination of the protein content of the samples was carried out according to the Bradford method. Aliquots containing 50 µg of proteins were fractionated by polyacrylamide gel electrophoresis (BIO-RAD Laboriatories, Milan, Italy) in SDS (0.1%) (SDS-PAGE) and the proteins thus separated, were transferred to a nitrocellulose filter. The filters were blocked for 2 hours at room temperature in a blocking solution prepared with 0.1% Tween-20 (FlukaChemie GmbH, Italy), in 5% casein (Bio-Rad Laboratories, Milan, Italy), dissolved in TBS (Bio-Rad Laboratories, Milan, Italy). Immunoprecipitation of KMT2A in MeHg-treated cells was performed as previously described (Guida et al., 2015b). Briefly, cell lysates (1,5 mg) were immunoprecipitated overnight at 4°C using KMT2A antibody (3 µg) and IgG as a negative control. Protein A/G-agarose beads (25 µl) (sc-2003 Santa Cruz Biotechnology) were used to precipitate the bound protein. The antibodies used were: anti-REST (07-579, Millipore, 1:1000) anti-Sp1 (sc-14027, Santa Cruz Biotechnology, 1:500), anti-KMT2A (sc-374392, Santa Cruz Biotechnology, 1:500), H3K4-3me (PA5-17420, Invitrogen, Milan, IT), anti-caspase-8 (sc-6136, Santa Cruz Biotechnology, 1:1000), RIPK1 (sc-41169, Santa Cruz Biotechnology, 1:500) and anti-Tubulin (T5168, Sigma-Aldrich, 1:15000). The protein bands under examination were visualized using a chemoluminescence system (ECL Western Detection Kit; Amersham, Arlington Heights, IL, USA). The bands were quantized by densitometry. Normalization of the results was ensured by hybridizing the blots with anti-tubulin antibody.

6.6Statistical analysis

All experiments were performed in triplicate. The data were represented as means ± SEM. For the analysis was used an unpaired Student's t test for statistic between two experimental groups and the one-way ANOVA with Tukey's post hoc test for more than two experimental conditions.

RESULTS

7.1 Identification of protein levels of human SOD1 wild type and G93 A mutant in NSC34 cells

NSC34 cells were transiently transfected with the following vectors: (1) empty vector (EV), (2) SOD1-WT and (3) SOD1-G93A, separately. As shown in Fig1A, SOD1-WT and SOD1-G93A showed an increase of SOD1 protein compared to EV cells. Interestingly, a significant reduction of cell viability started at 48 hours and increased at 72 hours but not at 24h of seeding cells in SOD1-G93A cells, compared to SOD1-WT and EV groups (Figs 1B-D). These experiments suggest that exogenic human SOD1 protein was transiently expressed in NSC34 cells and that cell death occurs at 72 hours in the SOD1-G93A cells, but not in EV and SOD1-WT group.

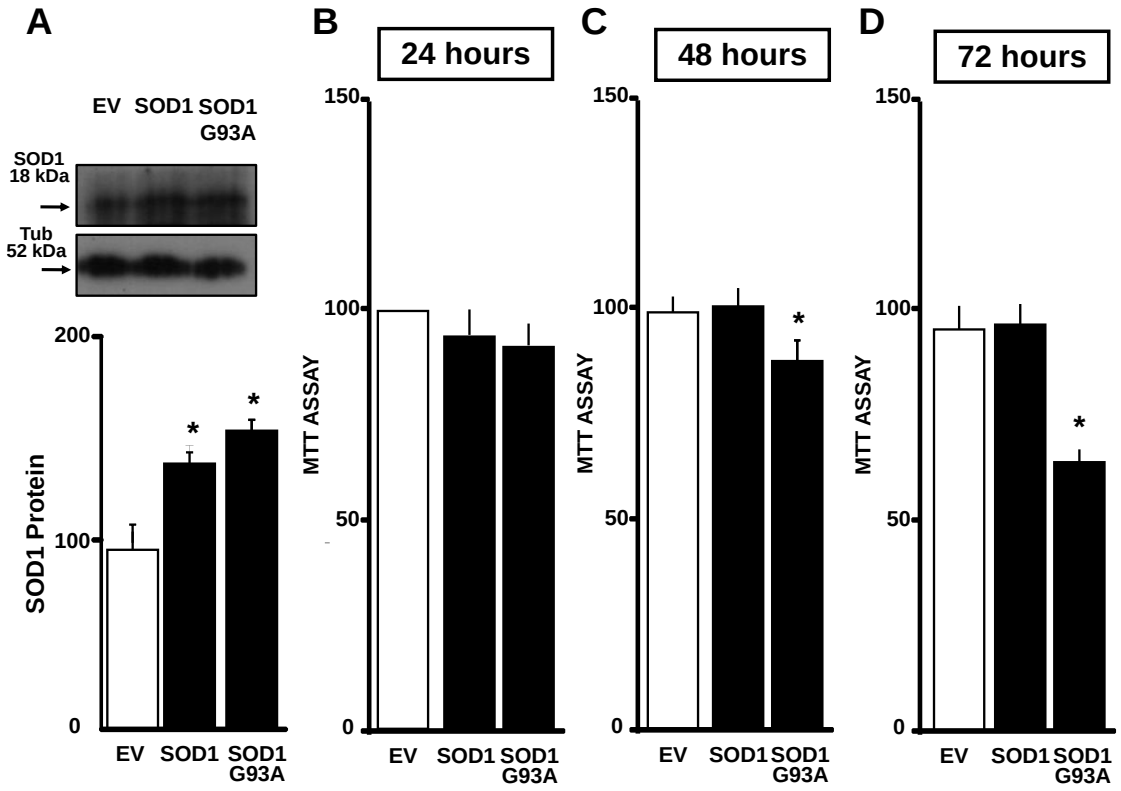

Fig. 1

Figure 1**REST mRNA and protein levels and cell viability in NSC34 transiently expressing the empty vector, SOD1 and SOD1G93A constructs.**

(A) Western Blot of SOD1 in NSC34 transiently transfected with the followed vectors: (1) empty vector (EV), (2) wild type human SOD1 (SOD1) and (3) SOD1 containing the G93 A mutation (SOD1G93 A). Graphs show quantification of ratio of SOD1 and Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus EV. (B-D) Effects of EV, SOD1 and SOD1G93 A vectors on cell survival, measured as MTT assay after 24 , 48 and 72 hours seeding. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus EV and SOD1

7.2MeHg induced cell death via REST at 72 hours in EV and SOD1-WT cells and at 24 hours in SOD1-G93A cells.

To evaluate the role of MeHg to anticipate cell death EV, SOD1-WT and SOD1-G93A groups were exposed for 24, 48 and 72 hours with toxic concentration of MeHg (100 nM) (Chapman and Chan, 1999). Notably, in EV and SOD1-WT groups a significant reduction in cell survival was revealed after 72 hours, but not at 24and 48 hours of MeHg exposure in parallel with an increase in REST gene and protein (Figs. 2A-F). On the other hand in SOD1-G93A group the increase in cell death after MeHg treatment started at 24 hours and became more evident at 48 and 72 hours, similarly with an up-regulation of REST mRNA and protein (Figs. 2G-I). These results suggest that SOD1-G93A cells are more sensible to MeHg-induced neurotoxic effect. Afterward, we investigated the role of MeHg by activating REST to reduce cell survival in: (1) EV and SOD1-WT cells at 72 hours and in (2) SOD1-G93A cells at 24 hours. As shown in Figs 3A-F REST knocking-down reverted MeHg-induced REST protein increase and its consequent neurodetrimental effect at 72 hours in WT (Figs. 3A-C) and SOD1 (Figs. 3D-F) cells and at 24 hours in SOD1-G93A cells (Figs. 3G-I). These results indicate that MeHg-induced cell death in EV and SOD1-WT groups via REST up-regulation and that in SOD1-G93A group the effect of MeHg to anticipate cell death is REST mediated.

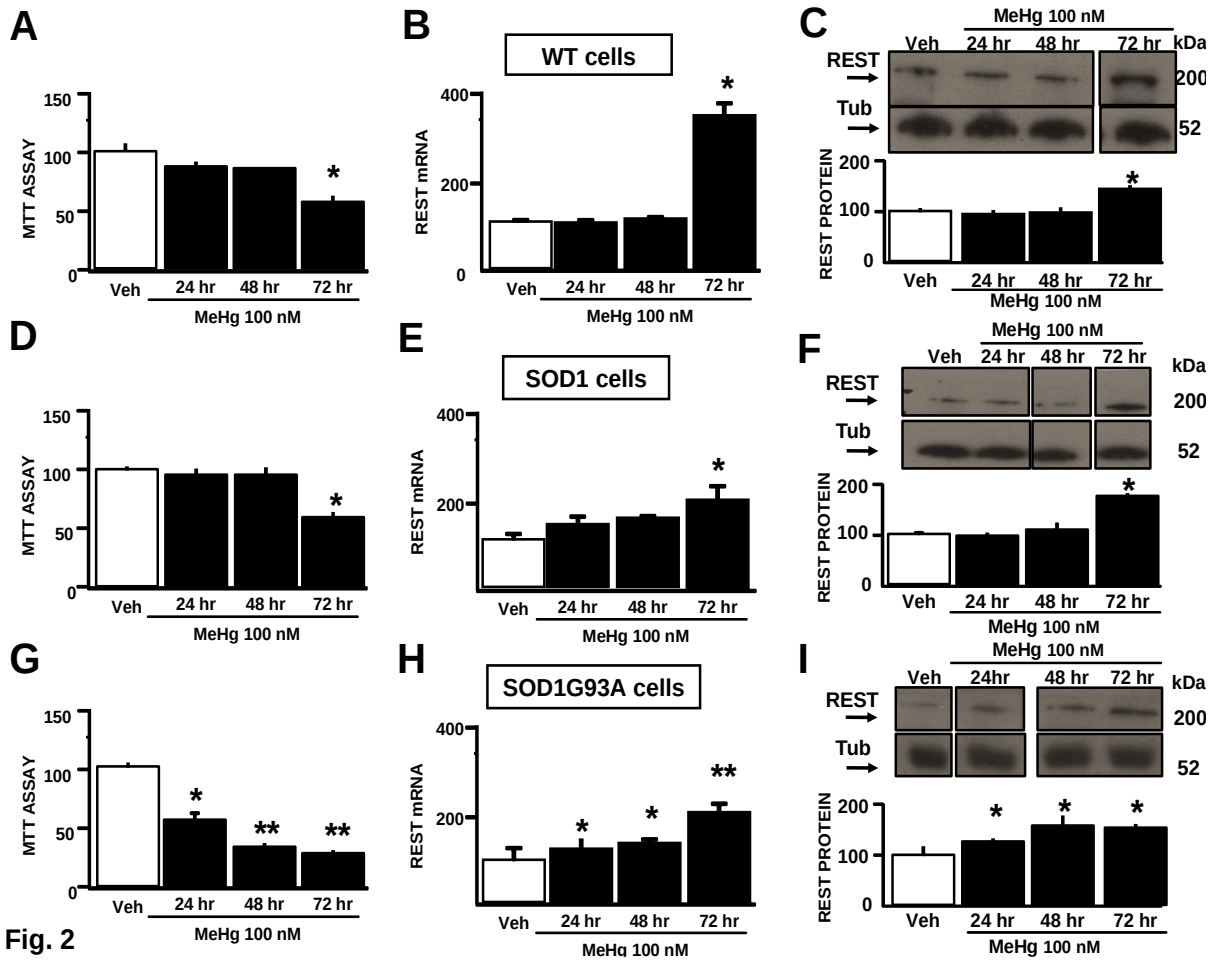

Fig. 2

Figure 2 **Effect of MeHg (100 nM) at 24, 48 and 72 hours on neurosurvival and on REST mRNA and protein levels in WT, SOD1 and SOD1G93 A cells.**

(A, D and G) Effect of 24, 48 and 72 hours of MeHg (100nM) exposure on mitochondrial activity in: (A) WT, (D) SOD1 and (G) SOD1G93 A cells. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus vehicle (Veh); **p ≤ 0.05 versus MeHg 48 hours. (B, E and H) Effect of 24, 48 and 72 hours of MeHg (100nM) exposure on REST mRNA levels in: (B) WT, (E) SOD1 cells and (H) SOD1G93 A cells. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus vehicle (Veh) **p ≤ 0.05 versus MeHg 24 and 48 hours. (C, F and I) Effect of 24, 48 and 72 hours of MeHg (100nM) exposure on REST protein levels in: (C) WT, (F) SOD1 and (I) SOD1G93 A cells. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus vehicle (Veh).

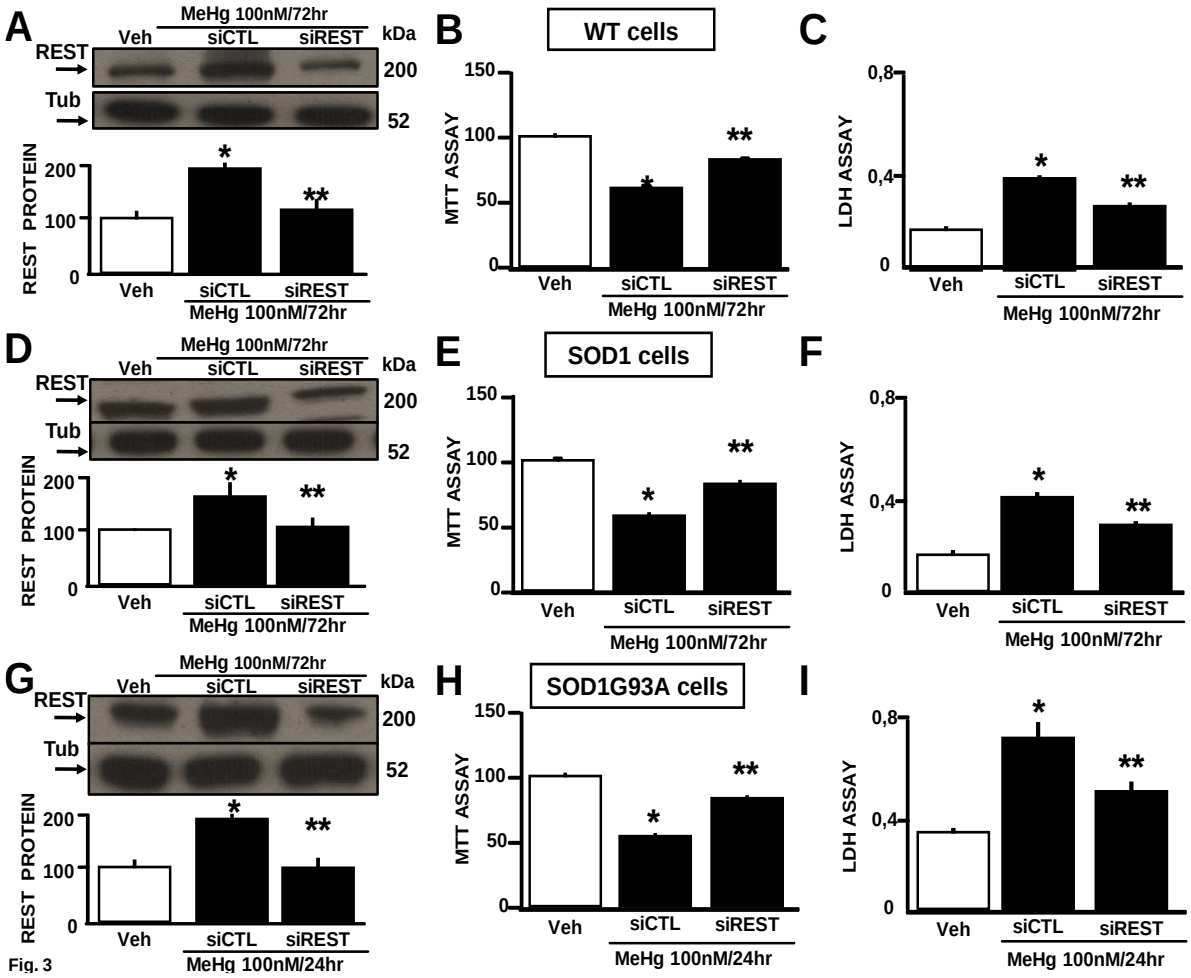

Figure 3 **Effect of REST knocking-down to counteract MeHg-induced cell death in in WT, SOD1 and SOD1G93 A cells.**

(A-F) Effect of 72 hours of MeHg (100nM) exposure on REST protein levels, mitochondrial activity and LDH release in: (A-C) WT, and (D-F) SOD1 cells. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus vehicle (Veh), **p ≤ 0.05 versus MeHg+siCTL. (G-I) Effect of 24 hours of MeHg (100nM) exposure on REST protein levels, mitochondrial activity and LDH release in (G-I) SOD1G93 A. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus vehicle (Veh), **p ≤ 0.05 versus MeHg+siCTL.

7.3MeHg-induced REST mRNA and protein increase is determined by Sp1 activation that forms a complex with the histone lysine methyltransferase KMT2A

Since it has been reported that REST gene is transcriptionally modulated by Sp family transcription factors Sp1 and Sp3, CREB, and JunD (Guida et al., 2015a; Guida et al., 2017c), we evaluated in SOD1-G93A cells the effect of MeHg in modulating mRNA levels of Sp1, Sp3, CREB and JunD. In these cells 24 hours of MeHg 100 nM induced an increase in Sp1, but not of CREB, JunD and Sp3 genes expression (Figs. 4 A–D). Notably, MeHg induced an increase in Sp1 protein expression (Fig. 4 E). Afterward, the role of Sp1 transcription factor to modulate REST gene and gene product levels after MeHg treatment was investigated. To this aim SOD1-G93A cells were silenced with a siRNA for Sp1 (siSp1), that reduced its mRNA levels by 40%, compared to cells transfected with siCTL (Fig.4F). As shown in Fig. 4G, siSp1 significantly counteracted the effect of MeHg to increase REST mRNA.

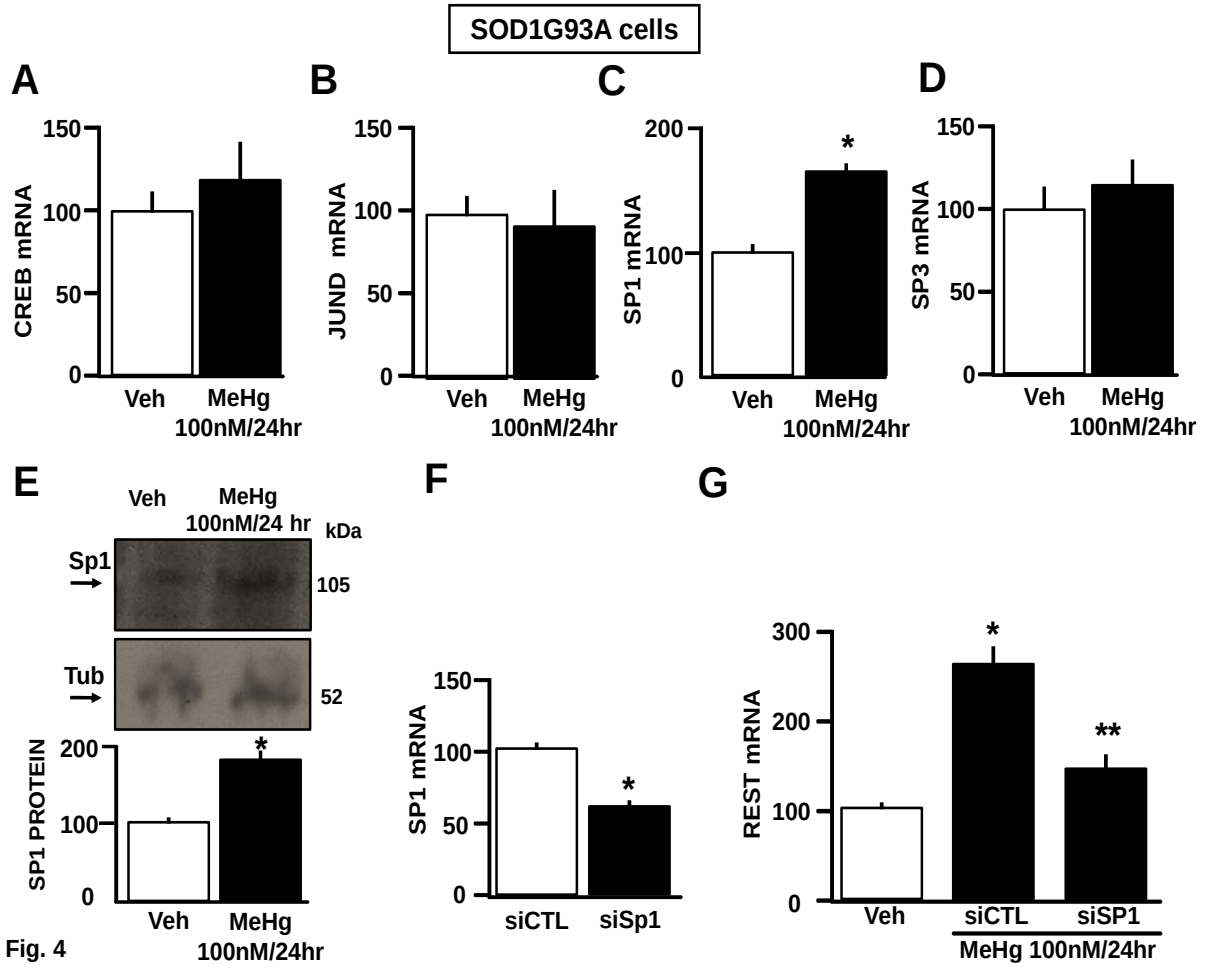

Fig. 4

Figure 4 **Effect of Sp1 knocking-down on REST gene in SOD1G93 A cells exposed to MeHg (100 nM) at 24 hours.**

(A-D) qRT-PCR of CREB, JUND, SP1 and SP3 genes in SOD1G93 A cells treated with MeHg. Graph shows the quantification of the ratio of CREB, JUND, SP1 and SP3 to Actin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Veh.. (E) Western Blot of SP1 in SOD1G93 A cells treated with MeHg. Graph shows the quantification of the ratio of SP1 to Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Veh.. (F) qRT-PCR of SP1 in SOD1G93 A cells treated with siSP1. Graph shows the quantification of the ratio of SP1 to Actin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus siCTL.. (G) qRT-PCR of REST in MeHg-exposed SOD1G93 A cells transfectced with siSP1. Graph shows the quantification of the ratio of REST to Actin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Veh, **p ≤ 0.05 versus MeHg+siCTL.

7.4 Sp1 forms a complex with the histone lysine methyltransferase KMT2A down-regulating REST

Given that Sp1 interacts with KMT2A in the breast cancer cell line MCF7 cells (Xu et al., 2017), we investigated by immunoprecipitation assay the possible interaction between Sp1 with KMT2A in SOD1-G93A exposed to MeHg. As shown in Fig. 5A, Sp1 bound the epigenetic writer KMT2A in SOD1-G93A MeHg-treated cells. Furthermore, we studied the role of KMT2A to modulate REST expression. Transfection of siRNA against KMT2A reduced its mRNAs expression by 70% (Fig. 5 B) and the trimethylation of the lysine-4 on the histone-3 (H3K4me3) by70% (Fig. 5 C), both compared to siCTL (Fig. 5 B). As shown in Figs 5D, E siKMT2A, counteracted MeHg-induced REST mRNA and protein. Notably, also siSP1 blocked the effect of MeHg to increase REST protein levels (Fig. 5 E)

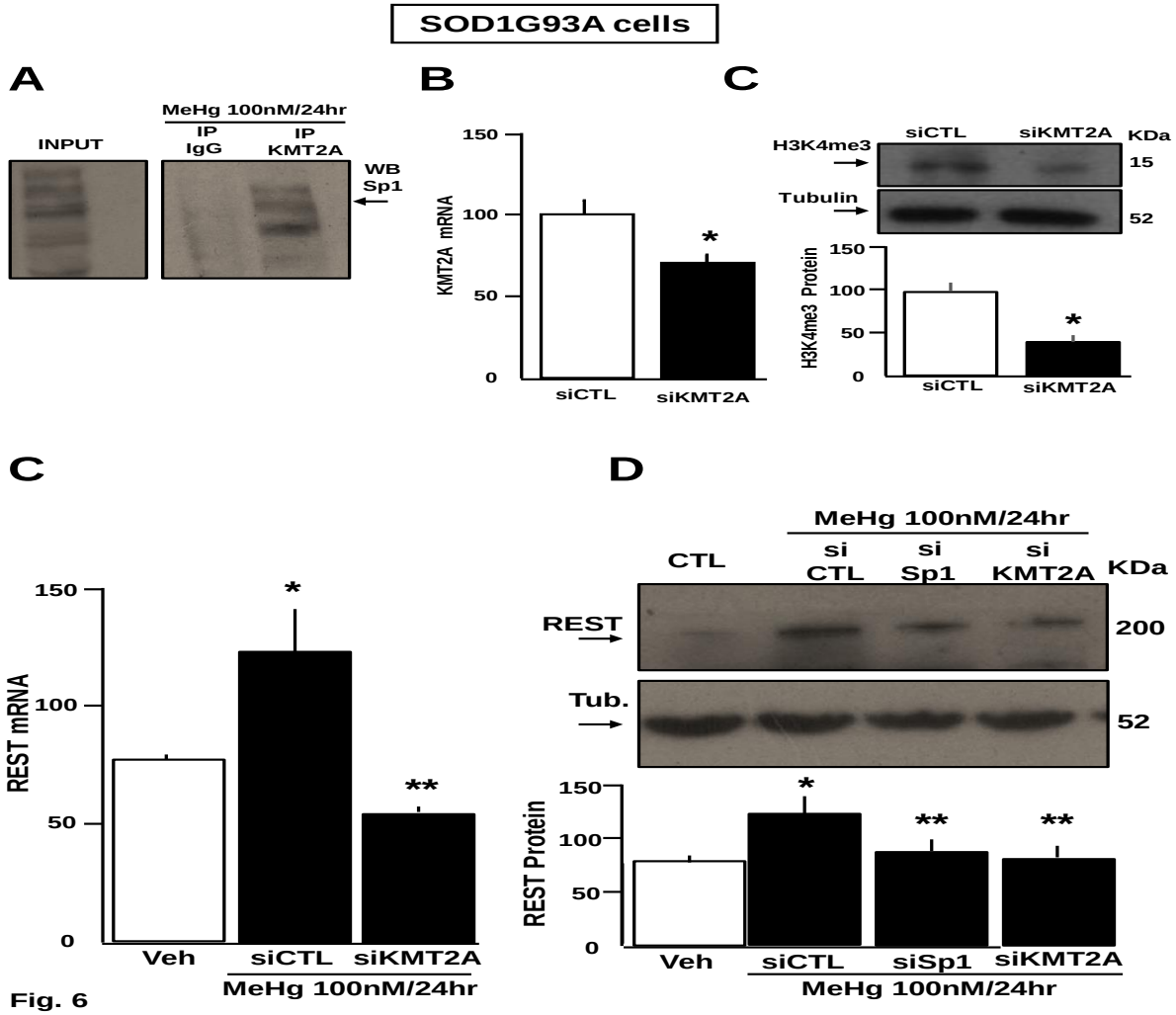

Fig. 6

Figure 6 **Sp1 increased REST gene levels in SOD1G93 A cells exposed to MeHg (100nM) via KMT2A recruitment at 24 hours.** (A) Representative Western Blotting showing immunoprecipitation between KMT2A and Sp1. (B) qRT-PCR of KMT2A in SOD1G93 A cells treated with siKMT2A. Graph shows the quantification of the ratio of KMT2A to Actin. Bars represent mean ± S.E.M. n = 3 per group. *$p \leq 0.05$ versus siCTL. (C) Representative Western Blotting with quantification of trimethylated-H3K4 (H3K4me3) in SOD1G93 A cells transfected for 24 hours with siCTL and siKMT2A. Graph shows the quantification of the ratio of H3K4me3 to tubulin. Bars represent mean ± S.E.M. n = 3 per group. *$p \leq 0.05$ versus siCTL. (D) qRT-PCR of REST in MeHg-exposed SOD1G93 A cells transfected with siKMT2A. Graph shows the quantification of the ratio of REST to Actin. Bars represent mean ± S.E.M. n = 3 per group. *$p \leq 0.05$ versus Veh, **$p \leq 0.05$ versus

MeHg+siCTL. (E) Western Blot of REST in MeHg-exposed SOD1G93 A cells treated with siSp1 and siKMT2A. Graph shows the quantification of the ratio of REST to Tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Veh, **p ≤ 0.05 versus MeHg+siCTL.

7.5REST knockdown prevents MeHg-induced necroptotic cell death

Since it has been demonstrated that necroptosis contributes to motor neuron cell death in ALS (Yuan et al., 2019) and that the neurotoxicant PCB-95 activates the necroptotic pathway in cortical neurons via REST up-regulation (Guida et al., 2017c), the effect of REST knocking down by siRNA transfection (siREST) on RIPK1 and caspase-8 protein expression in MeHg-treated SOD1-G93A cells was evaluated. Interestingly, siREST significantly counteracted MeHg-mediated: up-regulation in RIPK1 and down-regulation in cleaved-caspase 8 protein expression (Figs. 6A;B). Importantly, MTT and LDH assays demonstrated that when SOD1-G93A-Mehg-treated cells were pre-exposed with the specific inhibitor of necroptosis Necrostatin (Nec) at the following concentrations (1, 5, 10 and 20μM), a significant reduction of cell death occurred, compared to SOD1G93A cells exposed to MeHg alone (Figs. 6A, B).

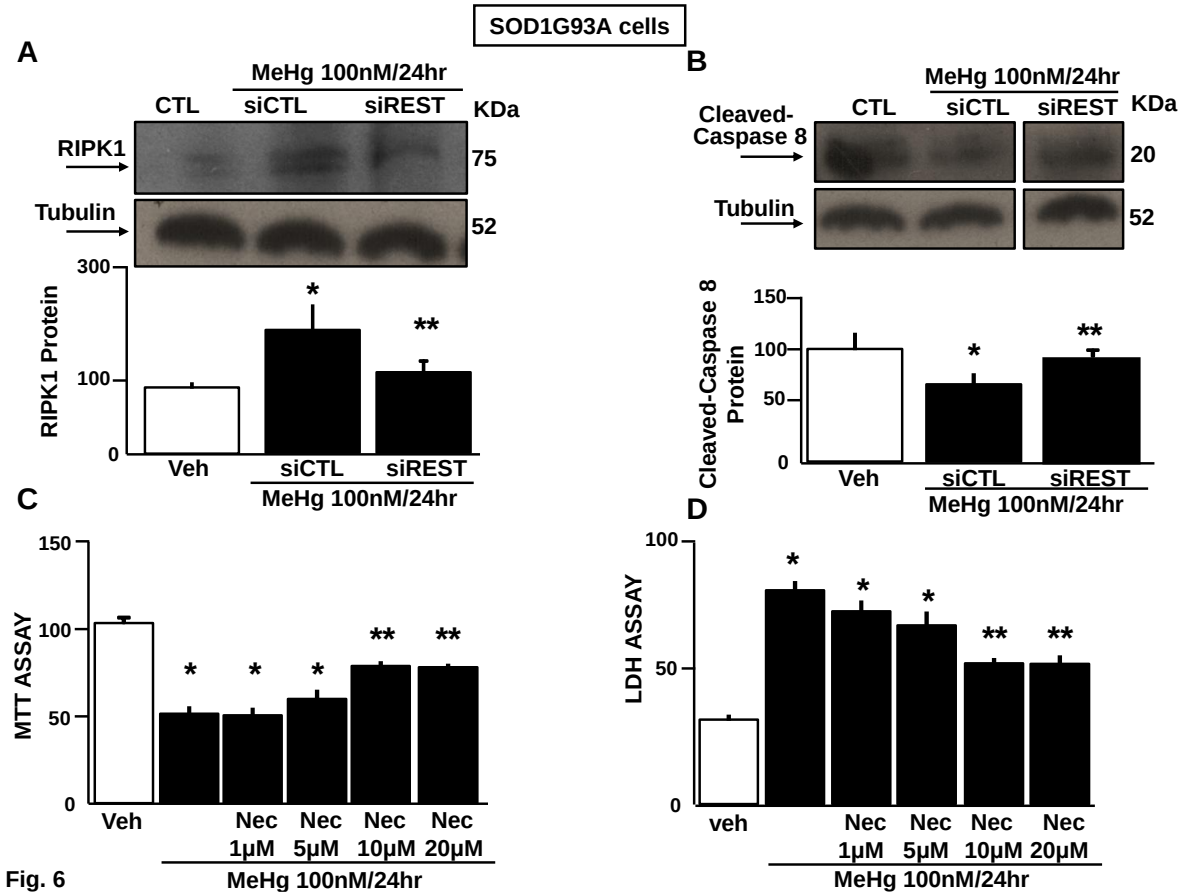

Fig. 6

Figure 6 Effect: (1) of REST knocking-down on RIPK1 and cleaved-caspase 8 protein levels and (2) of Necrostatin-1 (Nec) on neurosurvival, in SOD1G93 A cells exposed to MeHg (100nM) at 24 hours.

(A,B) Western Blot of REST in MeHg-exposed SOD1G93 A cells transfected with siREST. Graph shows the quantification of the ratio of RIPK1 and cleaved-caspase 8 to tubulin. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Veh, **p ≤ 0.05 versus MeHg+siCTL. (C,D) MTT assay and LDH release in SOD1G93 A cells pre-treated for 2 hours with Nec at 1, 5, 10 and 20 µM and with MeHg (100nM) for 24 hours. Bars represent mean ± S.E.M. n = 3 per group. *p ≤ 0.05 versus Veh, **p ≤ 0.05 versus MeHg+siCTL.**p ≤ 0.05 versus MeHg+siCTL, Nec 1 and 5 µM.

8. Discussion

Collectively these results indicate that MeHg determined necroptotic cell death in SOD1-G93A cells by increasing the transcriptional repressor REST. Several papers demonstrated the possible relationship between methylmercury exposure and the development of ALS (Johnson and Atchison, 2009; Johnson et al., 2011; Trojsi et al., 2013). Importantly, the molecular mechanisms of cell death identified after MeHg exposure and ALS are closely related, such as glutamate neurotoxicity, where has been found an increase of intracellular calcium concentrations due to the activation of AMPA receptors (Bailey et al., 2017). Furthermore, after MeHg poisoning there are symptoms like ataxia, muscle weakness, and inability that are similar to those revealed in ALS patients (Eto et al., 2010). Herein, we found that toxic concentrations of MeHg anticipate cell death via REST up-regulation in NSC34 cells transiently transfected with the G93A mutant of SOD1 but not in NSC34 cells expressing SOD1 wild type construct. Furthermore, we showed that the transcriptional factor Sp1 was the protein responsible of the REST mRNA and protein up-regulation. In addition we identified that necroptosis was the cell death mechanism induced by REST after MeHg exposure. The motoneuron-like cell line NSC-34, have been already used to study the molecular mechanisms related to ALS (Grad et al., 2014) and MeHg neurotoxicity (Chapman and Chan, 1999). Interestingly, MeHg at the concentration of 100nM reduced cell survival in NSC34 wild type (WT) and SOD1 cells at 72 hours whereas in SOD1G93A cells already at 24 hours neuronal death was identified. Intriguingly, MeHg-induced cell death resulted in parallel with REST mRNA and protein up-regulation. The modifications in REST expression is associated to cell death, as evidenced by the down-regulation of REST by siRNA transfection reduces MeHg-induced neurodetrimental effect in NSC34 SOD1G93A cells. These results, are in accordance with previous studies demonstrating the

neurotoxic role of REST after MeHg and PCB exposures (Guida et al., 2017b; Guida et al., 2018; Formisano et al., 2020). Furthermore, it has been identified the transcriptional factor Sp1 as the molecular determinant up-regulating REST gene after MeHg exposure. Indeed Sp1 protein levels were up-regulated during MeHg exposure and REST gene and protein increase was counteracted by the knocking-down of this transcription factor. These results are partially in accordance with those obtained by Ravache et al. (2006), where in NG108 cells mutant huntingtin (mHtt) via Sp1 up-regulates REST by binding its promoter sequence whereas Sp3 is a REST transcriptional repressor. Interestingly, it has been reported that REST is up-regulated by the following transcriptional factors Sp1, Sp3, CREB, and JunD (Guida et al., 2015a; Guida et al., 2017b), in our experimental model MeHg increased Sp1 gene and protein whereas CREB, Sp3 and JunD mRNA levels were unmodified. REST promoter sequence contains three alternative promoters A, B, and C that modulate the exons a, b and c that in turn splice with the exon d containing the ATG translation site (Koenigsberger et al., 2000; Ravache et al., 2010). Because, it has been found that Sp1 and Sp3 were specifically bound to the A promoter region of the mouse REST gene in undifferentiated NG108 cells, but were absent from B and C regions (Ravache et al., 2010), it could be speculated that Sp1 up-regulates REST gene by binding REST promoter A sequence. Furthermore, immunoprecipitation experiments demonstrated that the transcriptional factor Sp1 interacted with the epigenetic writer KMT2A after MeHg exposure. Interestingly, KMT2A regulates the survival, proliferation, and differentiation of subventricular zone neural stem cells in postnatal mouse brains that differentiate into glial lineages (Huang et al., 2015) and promoted neuron apoptosis in ischemic penumbra in an in vivo model of brain ischemia (Feng et al., 2020). Since, KMT2A catalyzes the reaction of trimethylation of the lysine 4 on the histone protein 3 (H3K4me3) , it could be hypothesized that Sp1 by recruiting KMT2a on REST gene promoter sequence activates REST transcription by inducing an increase of the tri-methylation status on the lysine 4 of the histone protein 3. This

hypothesis is supported by the fact that, siKMT2a reverted MeHg-increased REST mRNA and protein in SOD1G93A cells. Furthermore, necroptosis was the cell death pathway related to REST up-regulation, indeed MeHg-mediated increase in RIPK1 and reduction in caspase-8 proteins expression was counteracted by transfection of silencing for REST, thereby suggesting that by up-regulating REST expression MeHg treatment modulates transcription of necroptosis pathway genes. These results are consistent with our previous study demonstrating that the neurotoxicants PCB95 induced necroptotic cell death in cortical neurons by increasing REST expression and that caspase-8 is REST gene target (Guida et al., 2015a). Necroptotic cell death mechanism has been identified as a relevant neurotoxic pathway in in vitro and in vivo models of ALS and the RIPK1- specific inhibitor Nec-1 was found to increase cell survival in both ALS models (Yuan et al., 2019). Herein, it has been identified that MeHg-induced necroptotic cell death was reverted in dose dependent manner and that 10 µM was the concentration having the maximum inhibitor effect to reduce neuronal death. Collectively, these results demonstrate that motor neuron-like NSC 34 cells transiently overexpressing human SOD1G93 A construct are more sensible to MeHg neurotoxicity. Furthermore, the findings of this PhD thesis identify that the transcriptional activator Sp1, by recruiting KMT2A, up-regulates REST in SOD1G93 A cells through a possible increase of H3K4me3 on REST gene promoter sequence. Additionally, the transcriptional complex Sp1/KMT2A caused neuronal death through the increase activation of REST gene transcription and consequent repression of the REST target gene caspase 8 levels, determining necroptotic cell death.

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