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Sporadic Amyotrophic Lateral Sclerosis in patients with common geographical origin: a multidisciplinary study

[BIO/10]

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a late onset, fatal, neurodegenerative disorder that selectively affects motor neurons. It leads to the degeneration of both upper and lower motor neurons, respectively in the motor cortex and in the brainstem and spinal cord. Different mechanisms have been proposed to explain the pathogenesis of the disease: protein aggregation, oxidative stress, impairment of mitochondrial function, transcription dysfunctions, alterations in the proteasome pathway, inflammation and excitotoxicity.

A wide phenotypical variability is described, likely attributable to a combination of genetic and environmental factors, able to modify the clinical expression of the disease. However, the difficulty to focus on one specific environmental factor, the variability of such exposure in space and time and the interaction between environment and genetic background, hampered the evaluation of their possible role in ALS etiology.

The study of geographical clusters of ALS patients has been helpful - in the past years - to get more insights into the pathology. Studying individuals exposed to the same environment, thus ideally subjected to the same exogenous stressors, could be very valuable by limiting one of the most confounding variables in ALS studies. Indeed, a diverse environmental exposure is almost always necessarily present when analyzing the large cohorts of patients that are usually required to reach statistically significant numbers of cases in epidemiological-type studies.

This PhD thesis presents a multidisciplinary study performed on a small cohort of sporadic ALS patients, all originating from a restricted and defined geographical area. Focusing on a very limited geographical area, gave the chance to consider the characteristics of the surrounding environment and allowed to raise hypotheses on the possibly involved stressors acting on the local population, being those environmental or dietary contributions.

In detail the experiments performed can be divided into five main themes:

- To assess possible differences in the diet of ALS subjects and healthy controls and to provide information as for whether the consumption of one or more foods were associated to the disease status by an exploratory questionnaire submitted to the subjects involved in the study. There were no evident differences in the nutrition habits of ALS subjects with respect to healthy controls.

- To determine concentrations of a selected panel of metals in serum and whole blood with ICP-MS. Metals analyzed have been chosen based on their biological relevance and previous works. Concentrations of As, Al, Mn, Se, Ni, Pb, Hg, Cu, Fe, Zn, Co, Cr, Ba, Sn, U, V, Sr have been evaluated in serum, while an additional analysis of Cr, Mn, Co, Cu, Zn, As, Se, Pb and Hg has been performed on whole blood. The most striking result comes from the association of lower levels of serum As with the disease status, an occurrence reported for the first time.
- To analyze the serum proteome for the first time in ALS studies, through two-dimensional electrophoresis, to dissect the possible links between circulating proteins and circulating metals and to exploit this technique to look for a possible panel of easily accessible disease biomarkers. Proteins in which was registered an alteration are involved in the Acute Phase Response. Indeed, the different expression with respect to controls could be related to the disease status of the subjects. Alterations in some proteins related to lipid homeostasis have been detected, that is consistent with a proposed metabolic shift towards an increased peripheral use of lipids in ALS. Deregulation of lipid homeostasis proteins seems to be more directly linked in modulating the disease progression, as supported from literature data.
- To evaluate the genetic background of patients by analyzing the most frequently mutated genes (*SOD1, FUS, TDP43* and *C9orf72*) to exclude a genetic cause of the disease, giving more relevance to the environmental exposure as a risk factor. *APOE4* and *PON* genotypes have been evaluated in the light of the results obtained from the proteomic studies. The allelic frequency for the APOE*4 allele, associated to neurodegeneration, is 3-fold higher as well as APOE*3/APOE*4 genotype in the patients' group than in control's.
- To evaluate the DNA oxidative stress by Comet Assay, since it is well known that cellular oxidative stress is involved in the disease. Furthermore, metals impaired homeostasis may exacerbate oxidative stress via Fenton reactions. In literature at present there are few works evaluating this aspect with this approach. The levels of endogenous DNA damage did not differ between ALS and control group.

To overcome the obvious limits related to the small number of subjects necessarily involved in this study, advantage was taken from the application of a multifactorial statistical evaluation, based on a machine learning software. This software, belonging to the artificial neural networks architecture, was specifically designed to analyze complex problems, where the number of variables significantly exceeds the amount of subjects involved in the study, as usually is in the case of rare diseases.

Great amount of data is, nowadays, a limit in understanding results of experiments performed with more and more sophisticated technologies, especially in complex diseases such as ALS. The application of new statistical analyses, based on machine learning, where data are the basis of the creation of models to interpret interactions among variables, would be the key to translate raw data into understandable models.

The lack of an effective pharmacological treatment in ALS leads to rapidly looking for alternative approaches to modulate this devastating disease. To this purpose, a nutritional intervention, with the development of specific nutritional formulas, would be an effective tool to intervene on the patients' lipid profile and to contrast potential metal's homeostasis impairment. Thus, development of such formulations is strongly supported from the evidences raised from this study, together with its application in clinical trials.

INTRODUCTION

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder with unknown etiology and adult onset. It is characterized by the progressive degeneration of motor neurons in the motor central cortex, in the brain stem, and in the anterior horn of spinal cord. Motor neuronal death leads to muscle weakness, gradual loss of muscle functionality and total paralysis, leading to death, generally in 3 to 5 years from symptoms onset, due to respiratory failure.

The most characteristic clinical feature is motor weakness starting locally. In two-thirds of the cases first symptoms are cramps and limb weakness, the remaining third presents bulbar problems, like dysarthria and dysphagia, a condition that usually leads to death more rapidly. In rarer cases, first symptoms could be represented by respiratory failure, cognitive impairment or behavioral disturbances (Ajroud-Driss and Siddique 2014).

The term Lateral Sclerosis, refers to the autoptic characteristics of the spinal cord, whose lateral columns result hard to palpation. This is consequent to the occurring reactive astrogliosis, that leads to the alteration of the corticospinal tracts where the axons of damaged motor neurons are localized. The term Amyotrophic refers instead to the neurogenic muscle atrophy. The loss of innervation leads to muscular weakness, and degeneration of fibers up to paralysis of limbs and muscles involved in deglutition and speech (Wijesekera and Leigh 2009).

ALS has an overall estimated annual incidence of 1.5-2.7/100,000 and a prevalence of 3-5/100,000 (Al-Chalabi and Hardiman 2013). The mean age at onset is 55-65 years, only a few percentage of cases presents an early onset, before 30 years of age (Wijesekera and Leigh 2009). The mean duration of disease is about 32 months, but it can vary from 23 to 48 months. However, 20% of patients can survive up to 5 years and a lower amount (10%) is still alive after 10 years from onset (Harms and Baloh 2013).

No evident family history is present in most of the cases (90%, defined as sporadic ALS or SALS), while only the remaining 5-10% have a family history with a Mendelian inheritance (familial ALS or FALS) (Li and Wu 2016). A rarer third form of the disease, called juvenile ALS or jALS, has an onset usually before 25 years of age (Dion et al. 2009); it is distinct from the other forms of ALS since it

generally is familial and with a relatively benign course of disease. The other different forms of the disease (SALS and FALS) are clinically undistinguishable, even if generally the spectrum of symptoms in ALS is very broad. The only difference resides in the age at onset, usually earlier in familial forms (46 years of age), whereas sporadic forms have a mean age at onset at 56 years (Kiernan et al. 2011). In the familial form of the disease usually the survival is about 5 years longer than in sporadic patients (Wijesekera and Leigh 2009).

Despite great efforts in research, the etiology of the disease is still unknown and an effective treatment is still not available. More than 100 drugs have been tested up to now, some of them even in Phase II and III of human clinical trials, but resulted inefficient and inapplicable in the clinical practice. At date the only drug approved by FDA is Riluzole, that showed only modest effect by prolonging lifespan of patients of few months (Miller et al. 2007).

Clinical features

ALS is a progressive neurodegenerative disease affecting selectively motor neurons. It represents the most common form of the so-called Motor Neuron Diseases (MNDs). This definition groups different progressive diseases that have as a common feature, namely, the final loss of the first and/or the second motor neurons (Mancuso and Navarro 2015). The most characteristic symptom is the slowly progressive, painless, muscular weakness in one or different regions of the body, without changes in the ability to feel. Together with weakness, also spasticity, fasciculation, and cramps may be present (Wijesekera and Leigh 2009).

Diagnosis of ALS requires presence of both upper and lower motor neurons defects and progression of disease. When Lower Motor Neurons (LMN) are involved, involuntary muscle twitching (fasciculation) may occur; other typical signs of LMN impairment are cramps, muscular hypotonia, and atrophy. Subjects with Upper Motor Neurons (UMN) involvement present generally stiffness and hyperreflexia. Other UMN symptoms are spasms and sudden straightening movements of lower limbs (spasticity). Babinski's (anomaly of the cutaneous plantar reflex) and Hoffman's (same for the upper limbs) signs are variably present in the early stages of disease. These signs are the result of the degeneration of upper and lower motor neurons in brain and spinal cord. Small interneuron loss in the motor cortex and in the spinal cord may also occur, affecting muscles that control eyes movement and urinary sphincters (Pasinelli and Brown 2006).

The disease may present bulbar and spinal onset forms (Figure 1). About 75-80% of patients have the spinal onset form. Symptoms are related to focal muscle weakness, starting either distally or proximally, more frequently in upper than in lower limbs. Patients with upper limbs onset may present reduced finger dexterity, cramping, stiffness and weakness of hand muscles. Those with lower limbs onset may suffer from stumbling, tripping or difficulty in running. Focal muscle atrophy involves the muscles of hands, forearms or shoulders in the upper limbs, and proximal thigh or distal foot muscles in the lower limbs. The Hoffmann's and Babinski's signs may be positive (Wijesekera and Leigh 2009). Usually the disease onset is asymmetrical, but limbs weakness and wasting inevitably spreads. Most patients with time develop respiratory symptoms.

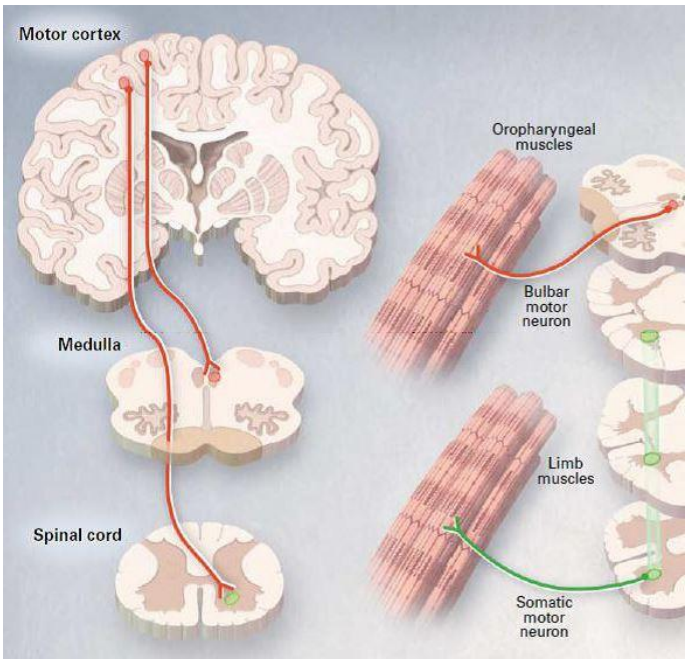


Fig.1. Motor neurons selectively affected in ALS patients

Occasionally encountered symptoms include bladder dysfunction, sensory symptoms, cognitive symptoms and multi-system involvement (e.g. dementia, Parkinsonism).

The remaining 20-25% of cases present bulbar onset form. Common features of these subjects include dysarthria or dysphagia for solids or liquids, up to complete loss of verbal communication and fasciculation in the tongue. Limbs symptoms may occur simultaneously or within 1-2 years. Almost all patients present sialorrhea due to difficulty in swallowing and bilateral facial weakness in the lower part of the face. The fatal event is commonly denervation of respiratory muscles, occurring within 5 years from disease onset (Valdmanis and Rouleau 2008).

In ALS patients cognitive abnormalities are present, ranging from executive dysfunctions to Fronto-Temporal Dementia (FTD), present in 5-15% of patients (van Langenhove et al. 2012). FTD indicates a heterogeneous group of neurodegenerative dementias, different from Alzheimer's Disease, characterized by progressive deterioration of the frontal and temporal lobes in the brain (Sieben et al. 2012). Progressive FTD signs are: personality abnormalities, lack of interest in personal care, obsessive and stereotyped attitudes, reduction of empathy, disinhibition, delusions and hypersexuality (Phukan et al. 2007).

In a large group of subjects with FTD, inclusion of TDP-43 in cortical neurons have been described, similar to those found in ALS patients' motor neurons (Neumann et al. 2006). These cases, presenting clinical features of both the pathologies are referred to as ALS/FTD. Furthermore, several molecular biology studies have found that ALS and FTD may be caused by mutations in the same genes: *C9ORF72* (Chromosome 9 open reading frame 72), *TARDBP* (TAR DNA-binding protein), *FUS* (FUsed in Sarcoma/Translated in LipoSarcoma), GRN (Progranulin), *VCP* (Valosin Containing Protein), *MATR3* (Matrin 3), *CHCHD10* (Coiled-coil-Helix-Coiled-coil-Helix Domain containing 10), *TUBA4A* (Tubulin Alpha 4A) e *TBK1* (TANK-binding kinase 1) (Sabatelli et al. 2016). Nowadays is accepted that there are common pathological processes in ALS and FTD, and that the two diseases are part of a continuous spectrum of disease with overlapping symptoms (Figure 2).

Epidemiology

Usually ALS is classified in 3 categories: Sporadic ALS, Familial ALS and Western Pacific ALS.

Sporadic ALS (SALS)

SALS represents the most common form of the disease and it is diagnosed when the loss of the first and/or the second motor neuron occurs in only one member of a family (Mitchell and Borasio 2007). Etiology is still unknown and disease is supposed to be caused following a complex interaction of genetic and environmental factors; however, genetic influence is far from being completely understood, since only 10% of sporadic cases presents a pathological mutation in the known responsible genes (Renton et al. 2014). Incidence in North America and Europe is similar, at about 1.5-2.5/100,000 person/year (Chiò et al. 2013). Incidence in Italy is 2.64/100,000 inhabitants per year with very small differences in data collected from different groups in different regions (Scialò et al. 2016).

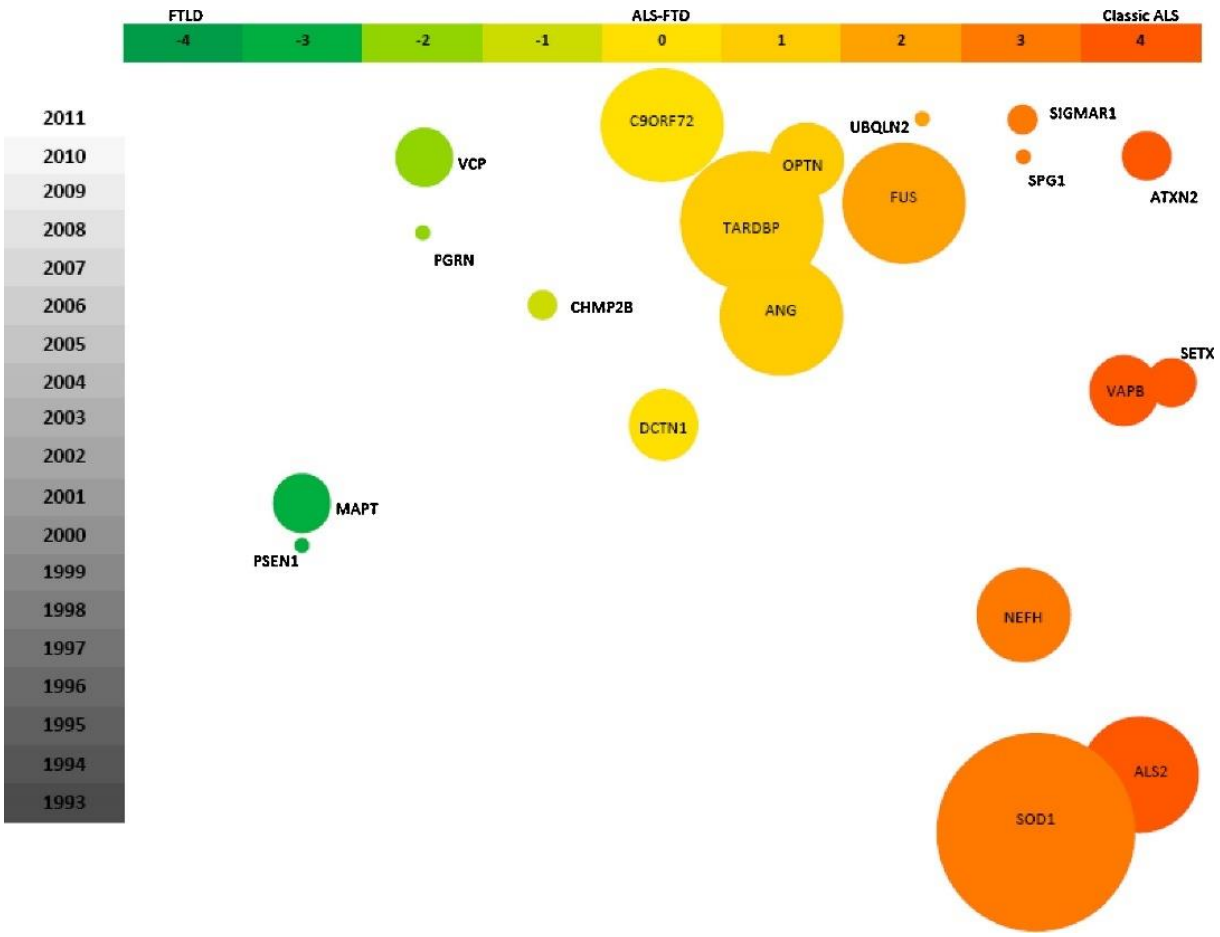


Fig. 2. ALS and FTD causative genes. Size of circles reflects frequency of mutation (Eisen and Krieger 2013)

An increment of 46% of incidence and 57 % of mortality rates has been described in the last years of the past century, with respect to the previous decades (Worms 2001), particularly in Mediterranean countries, in the female population, and in people with more than 75 years of age. This likely could be dependent from an increase in life expectancy of population in the last decades and from a better understanding of the disease, leading to a better case ascertainment. Recent studies showed that in the last ten years prevalence of the disease did not increase (Zarei et al. 2015). The prevalence of ALS is estimated at 4-6/100,000 persons with a uniform distribution across the world.

Mean age at onset is 55-65 years of age, with 80% of subjects presenting the first symptoms in the ages between 40 and 80 (Chiò et al. 2009). Incidence shows a great variability in different age groups, reaching its peak in ages 60-79 and rapidly decreasing after 80 years of age (Logroscino et al. 2005). This is likely related to the fact that elder patients may present symptoms easily mistaken as signs of aging or that may be masked by other diseases. Conversely, an age-specific peak in

incidence curve suggests that a time dependent exposure to environmental risk factors could be involved in the disease. Early onset might represent a consequence of harsher exposure to these risk factors (Sabatelli et al. 2013).

Survival is commonly of 3-5 years from symptoms' onset. It can be prolonged by ventilation support that can ease breathing problems. Although it does not affect progression of the disease, it can improve or at least maintain quality of life in people with non-severely compromised bulbar function (Radunovic et al. 2010). However, about 10% of patients has a survival longer than 10 years.

Familial ALS (FALS)

ALS is defined familial in case of the presence of at least two affected relatives in less than four generations; in 50-75% of cases only two affected subjects are reported within the same family (Sabatelli et al. 2013). This form of the disease presents a wide phenotypical and genetic heterogeneity, even in the presence of the same mutation within the same family.

The autosomal dominant inheritance pattern is the most common when the onset is in adult age; recessive transmission is more frequent in juvenile onset (Ajroud-Driss and Siddique 2014). A dominant X-linked inheritance pattern is described only in one family (Wilkins et al. 1977). Between 1958 and 1996 several epidemiological studies have been performed, indicating about 0.8-13.5% of patients with familial ALS. However, the frequency of FALS indicated in these studies is very variable, due to several reasons:

- non-homogeneous diagnostic criteria,
- incomplete genealogical investigations,
- inadequate recognition of different subtypes of ALS in different family members,
- reluctance of patients to report a hereditary disease,
- loss of contact between family members,
- early death in family members due to causes different from ALS,
- earlier age at onset in patients of the successive generations (anticipation),
- incomplete disease penetrance,
- misdiagnosis of ALS in some family members,
- illegitimacy.

Nowadays it is established that cases of authentic FALS account for the 5-10% of total cases (Ajroud-Driss and Siddique 2014). Symptomatology of familial ALS is overlapping to that of the sporadic form of the disease, except for little differences (Li et al. 1988):

- earlier age at onset (about 10 years before),

- Gaussian distribution of age at onset with respect to an age-dependent incidence in SALS,

- both genders are affected in the same proportion,

- generally longer disease duration,

- lower limbs onset is more frequent,

- degeneration of the posterior columns of the spinal cord, spino-cerebellar traits, and dorsal nucleus of Clarke is evident in 70% of patients with familial ALS.

Mean age at onset is 45.7 ± 11.3 years with a median survival of 24 months. Usually, pathology is focal and asymmetric at onset, then tends to spread in a contiguous way. As in the sporadic form, phenotypic heterogeneity is present: age at onset may vary up to 30 years, duration of illness and signs at onset may vary as much (Ajroud-Driss and Siddique 2014).

Western Pacific ALS

In the Western Pacific area, the prevalence of ALS is 50-100 times higher than in the rest of the world. Four well studied geographical clusters are known: the Guam island in Micronesia, the Japanese Kii peninsula, the Western coast of former West Papua New Guinea and an isolated tribe at Anguru on Groote Eylandt in the Gulf of Carpentaria (North Australia). In the 1960s, in Guam, incidence of ALS was about 179/100,000 for men and 60/100,000 for women. Scientists refer to this form of disease as Amyotrophic Lateral Sclerosis/Parkinsonism-Dementia Complex (ALS-PDC) because subjects are prone to manifest atypical Parkinsonism, dementia, motor neuron disease, or a combination of these three phenotypes. The cause of the disease is still unknown, but some evidences point towards a complex interaction of genetic susceptibility and a strong environmental influence. Indeed, despite a positive family history for 45% of patients, it was not possible to identify a mendelian inheritance pattern. The annual incidence declined since 1955, indicating that causes may have ended decades ago (Kurland 1988; Plato et al. 2003).

Two environmental hypothesis - related to nutritional habits - have been proposed. High levels of aluminum and manganese and low levels of calcium and magnesium were found in drinking water

and soil samples in Guam, the Kii Peninsula and Western New Guinea. Deposition of aluminum in affected neurons was described (Perl et al. 1982); deficiency of calcium and magnesium has been hypothesized to increase aluminum uptake as alternative source of cations, thus possibly leading to neuronal damage and death (Garruto et al. 1989). Alternatively, higher iron levels, following aluminum accumulation, may cause oxidative stress in vulnerable neurons.

Another hypothesis relates to the possible presence of a neurotoxin in the affected area. Neurological symptoms in monkeys fed with large doses of beta-methyl-amino-L-alanine (BMAA), an unusual, neurotoxic, excitatory amino acid were described in the late 1980s (Spencer et al. 1987). This toxin is present in low quantities in *Cycas circinalis* seeds and it is easily removed after washing, thus doses to achieve neurotoxicity were unrealistic to achieve. However, Cox and Sacks raised the theory of bio magnification of this toxin exerted by flying foxes (*Pteopus ariannusmariannus*). This species of megabats consume large amounts of Cycads leaves and seeds and Chamorro indigenous of Guam, in turn, traditionally feasted on these animals (Cox and Sacks 2002). In the last 30 years ALS gradually disappeared from Guam, in coincidence with huge and rapid ecological and socio-economic changes, following deforestation for the construction of huge military bases and westernization of lifestyle, that changed food collection and dietary habits, possibly removing the ALS-PDC exogenous factors (Chen 1995).

Etiology

Despite intense efforts in research, pathogenesis of ALS is still unknown and it is not clear if it is the same across single individuals. It is believed that to trigger the disease, the interplay of multiple genetic and environmental factors is required (Oskarsson et al. 2015).

Genetics

ALS is considered a complex genetic disease in which multiple cellular mechanisms, not mutually exclusive, can be affected (Shaw 2005). The number of genes involved in the disease increased in the last years, along with the hope of identifying common pathways related to the disease. Most frequently mutated genes code for proteins involved in antioxidant response, axonal transport, angiogenesis and RNA processing (Al-Chalabi and Hardiman 2013).

Nineteen genes located in twenty-one chromosomal regions have been associated with ALS, jALS and ALS/FTD (Li and Wu 2016) (Table 1). Genetic architecture of ALS is complex: mutations in *SOD1*, *FUS* and *TARDBP* genes are considered at high risk of causing the disease; mutations in other genes are less frequent (Leblond et al. 2014). An expansion of six nucleotides in *C9ORF72* gene, nowadays, is the most frequent genetic alteration associated with ALS (DeJesus-Hernandez et al. 2011; Renton et al. 2011). The improvement of genetic analyses has made a large number of genes considered as "candidate genes"; however, they present a low contribute in ALS, and association studies are still required (Leblond et al. 2014). The so called modifier genes are able to alter ALS expression and the phenotype (age at onset, disease course, prognosis) given by the mutated causative gene; among these there are *SMN* (Survival Motor Neuron), *CNFT* (Ciliary Neurotrophic Factor), *KIFAP3* (Kinesin associated Protein 3), *UNC13A* (Unc-13 homolog A), *ZNF512B* (Zinc Finger Protein 512B), *APOE* (Apolipoprotein E), *PON* (Paraoxonase), *HFE* (Hemochromatosis) *MAOB* (Monoamine Oxidase B), *EPHA4* (EPH receptor A4), *VEGF* (Vascular Endothelial Growth Factor) (Marangi and Traynor 2015).

SOD1 was the first gene associated to ALS in 1993 (Rosen et al. 1993). It is ubiquitously expressed and its nucleotide sequence is highly conserved (Wang et al. 2006). *SOD1* gene codes for the Cu-Zn Superoxide Dismutase enzyme, a protein localized in cytosol, nucleus, peroxisomes and mitochondria; it is widely expressed in mammalian central nervous system (Pardo et al. 1995). It functions as a homodimer protein, where each subunit is composed of 153 amino acids and binds its own copper and zinc ions in the active site. Its function is to catalyse the conversion of the Superoxide radical ($O_2^{\cdot-}$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), that in turn are removed by the glutathione peroxidase and catalase enzymes (Bunton-Stasyshyn et al. 2014) (Figure 3). Mutations generally affect either amino acids in the active site, involved in the binding of cations, or alter the protein folding and stability (Andersen et al. 1997).

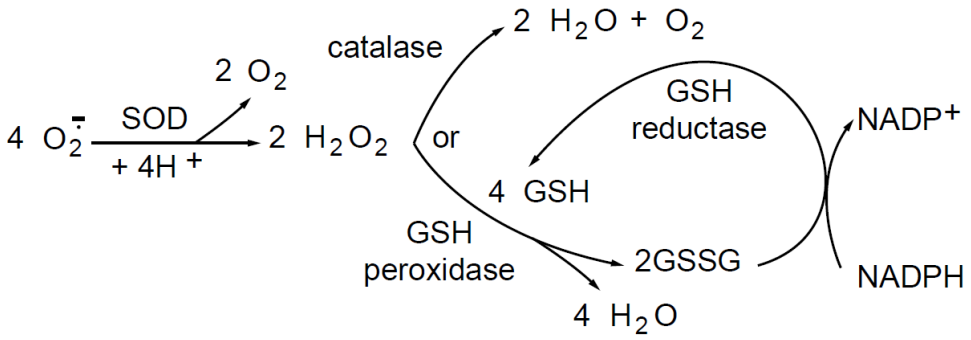


Fig. 3. Function of SOD1 enzyme.

Type	*Gene*	*Chr*	*Protein*	*Function*	*Heredity*	*FALS*	*SALS*	*FTD*
ALS1	SOD1	21q22.1	Cu-Zn Superoxide Dismutase	Antioxidant enzyme	AD, AR, *de novo*	Y	Y	Rare
ALS2	ALS2	2q33.2	Alsin	CT, ESC	AR	Y +jALS	N	N
ALS3	UN	18q21	UNK	UNK	AD (one family)	Y	N	UNK
ALS4	SETX	9q34	Senataxin	RNA processing	AD	Y +jALS	Y	N
ALS5	SPG11	15q21.1	Spatacsin 11	UNK	AR	Y +jALS	N	Rare
ALS6	FUS	16p11.2	Fused in sarcoma/translated in liposarcoma	RBP	AD, AR, *de novo*	Y +jALS	Y	Y
ALS7	UNK	20p13	UNK	UNK	AD (one family)	Y	N	UNK
ALS8	VAPB	20q13.3	Vescicle-associated membrane protein-associated B and C	CT, ERGP	AD	Y	N	N
ALS9	ANG	14q11.2	Angiogenin	RBP, Angiogenesis	AD	Y	Y	Y
ALS10	TARDBP	1p36.22	TAR DNA-binding protein 43	RBP	AD	Y	Y	Y
ALS11	FIG4	6q21	FIG4 phosphoinositide 5-phosphatase	PRD, ERGP, CT	AD	Y	Y	N
ALS12	OPTN	10p13	Optineurin	PRD, ERGP, CT	AD, AR	Y	Y	Y
ALS13	ATXN2	12q24	Ataxin 2	Triplet Expansion	AD	N	Y	N
ALS14	VCP	9p13	Valosin containing protein	PRD	AD	Y	N	Y
ALS15	UBQLN2	Xp11.21	Ubiquilin 2	PRD	XR	Y +jALS	Y	Y
ALS16	SIGMAR1	9p13.3	Sigma receptor 1	ERGP	AD	Y +jALS	N	Rare
ALS17	CHMP2B	3p12.1	UNK	PRD, CT, ESC	AD	Y	Y	Y
ALS18	PFN1	17p13.2	Profilin 1	Actin polymerization	AD	Y	Y	Y
ALS19	ERBB4	2q33.3-q34	Erb-b2 receptor tyrosine kinase	CT	AD	Y	N	N
ALS20	hnRNPA1	12q13.1	UNK	RBP	AD	Y	Y	Y
ALS21	MATR3	5q31.3	Matrin 3	RBP	AD	Y	Y	Y
ALS/FTD2	C9ORF72	9p21.2	UNK	CT, hexanucleotide expansion	AD	Y	Y	Y
ALS/FTD	GRN	17q21.31	Progranulin	Cellular growth regulation	AD	N	Y	Y

Table 1: UNK: unknown; CT: cellular trafficking; ESC: endosomal sorting complex; ERGP: endoplasmic reticulum-golgi pathway; RBP: RNA Binding Protein; PRD: protein degradation; AD: Autosomal Dominant; AR: Autosomal Recessive; XR: Chromosome X-linked.

Mutations in the *SOD1* gene account for the 20% of FALS cases and 1-2% of SALS cases (Li and Wu 2016). The genotype-phenotype correlation is not clear, as there is a great variability both in disease onset and course, clinical features, age and site at onset; survival may vary within the same family (Penco et al. 2011). Animal model studies indicate a possible pathologic gain of function of mutated SOD1, leading to a lowering of antioxidant function of the enzyme and a higher tendency to aggregation and mislocalization (Kaur et al. 2016).

FUS gene mutations in ALS have been described in 2009 (Kwiatkowski et al. 2009; Vance et al. 2009). *FUS* is a gene ubiquitously expressed, coding for a 526-residues protein "fused in sarcoma / translated in liposarcoma. The protein has a nuclear localization, it is able to bind DNA and RNA and it is involved in RNA splicing, transport, and maturation (Lagier-Tourenne et al. 2010). The protein is composed of several different domains and the most frequently mutated residues lie within the C-terminal nuclear localization signal (Andersen and Al-Chalabi 2011).

FUS is found mutated in 3-5% of FALS cases and in 1% of SALS cases (Chen et al. 2013). Subjects harbouring a FUS mutation usually present an earlier age at onset and a more rapid progression of disease; bulbar onset is more frequent (Li and Wu 2016).

The *TARDBP* gene codes for a ubiquitously expressed protein of 43 kDa called TDP-43. The protein is found ubiquitinylated in high amounts in neuronal cytoplasmic inclusions in subjects with ALS and/or FTD (Neumann et al. 2006). Similarly to FUS, TDP-43 exerts its physiological function in nucleus, where it is involved in RNA transcription, splicing, transport, and maturation (Lagier-Tourenne et al. 2010). Mutations in the *TARDBP* gene account for the 4% of FALS cases and 1% of SALS cases (Li and Wu 2016). Most of the mutations are located in a region essential for protein solubility and cellular localization (Pesiridis et al. 2009). TDP-43 mutations are found also in patients with FTD and Parkinson Disease (Li and Wu 2016). In 2011 two independent groups identified the *C9ORF72* gene as associated with ALS and FTD (DeJesus-Hernandez et al. 2011; Renton et al. 2011). Differently from other ALS genes, where exonic mutations are usually missense and nonsense, the pathological genetic event in *C9ORF72* is a hexanucleotide expansion (G_4C_2) in a non-coding region (Figure 4).

The region where this alteration is localized is highly conserved through different species (Leblond et al. 2014) and, depending on the protein isoform, it can lead to missed transcription of the gene (loss-of-function) or to the transcription of the repeat itself, leading to a toxic gain-of-function through the accumulation of transcripts in nuclear RNA *foci* (Woollacott and Mead 2014).

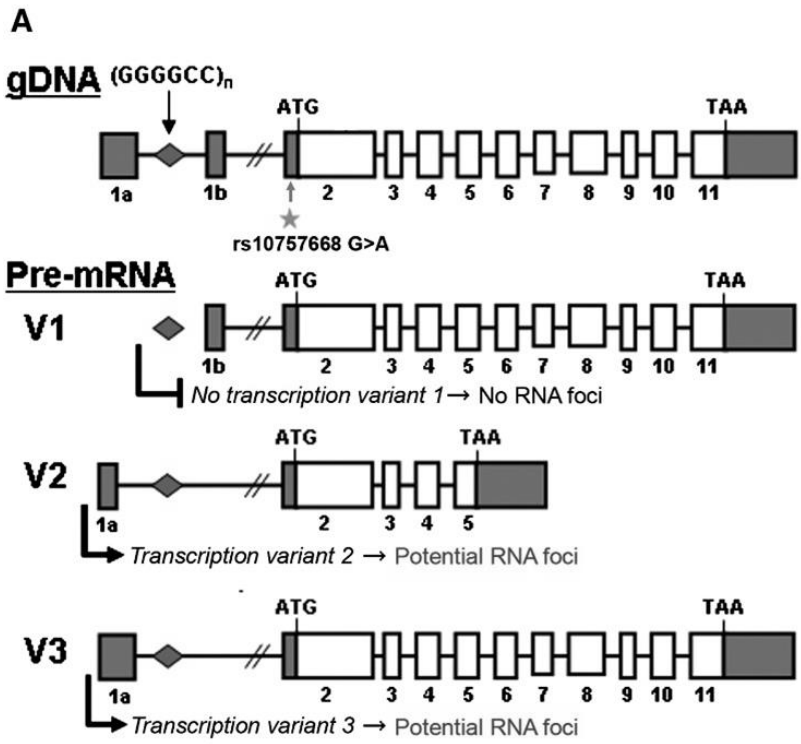


Fig. 4. C9ORF72 gene structure. V1, V2, V3 represent the three alternative transcripts. Grey boxes represent non coding exons, white boxes represent coding exons. Localization of the hexanucleotide expansion is indicated (DeJesus-Hernandez et al. 2011).

At present little is known about the function of the coded protein; bioinformatic analyses predicted a possible role in endosomal trafficking and autophagy (Levine et al. 2013). The amount of expansions is highly variable but in healthy subjects it usually does not exceed 25 repeats, whereas in affected subjects there are more the 30 (and up to hundreds/thousands) repeats (Rohrer et al. 2015). *C9ORF72* expansion is the most frequent cause of ALS and FTD: it has been identified in 40% of FALS cases, 7% of SALS cases, 25% of familial cases of FTD and 6% of sporadic FTD. ALS patients bearing a *C9ORF72* expansion show a great phenotypic heterogeneity. Subjects present bulbar onset and cognitive impairment more frequently, have a rapid progression and a lower survival (Li and Wu 2016).

Despite most of the mutations show a typical mendelian Autosomal Dominant pattern of inheritance, a monogenic model of heredity does not fully explain the genetics of ALS (Singleton et al. 2010). Indeed, interactions between different multiple genetic variants may contribute to ALS susceptibility. This could also explain the huge phenotypical differences between subjects bearing the same causative mutation and the incomplete penetrance of mutations found within the same family (Marangi and Traynor 2015). Different studies support this model of oligogenic disease by

describing patients with simultaneous mutations in two different causative genes (Chio et al. 2012; Van Blitterswijk et al. 2012).

Oxidative Stress

Oxidative stress is the result of an imbalance between production of Reactive Oxygen Species (ROS) and the cell ability of scavenging them through a variety of mechanisms. ROS are - in most cases - the result of the leakage of electrons from the mitochondrial respiratory chain, leading to the production of superoxide anion ($O_2^{\cdot\cdot}$) and hydrogen peroxide (H_2O_2). Neither superoxide nor hydrogen peroxide is highly reactive, but each undergoes further reaction to produce more potent oxidants (Barber and Shaw 2010).

The hypothesis of an involvement of oxidative stress in ALS is supported by the fact that SOD1, one of the most important antioxidant enzymes, is mutated in almost 20% of familial cases. This protein is a key regulator of intracellular red-ox equilibrium and its alteration may trigger a cyclic process in which ROS can lead to cellular damage and thus neurodegeneration (Ferrante et al. 1997). Post-mortem tissues from ALS patients reveal a broad oxidative damage to lipids, proteins and DNA (D'Amico et al. 2013). Oxidative stress may be caused by several endogenous and exogenous influences. The main endogenous sources of ROS are mitochondria, so intense efforts have been done in studying mitochondria related pathogenic mechanisms (Cozzolino et al. 2015). Oxidative stress may also be linked to the other proposed pathogenic pathways, in ways not fully clear yet, by causing RNA dysmetabolism, which in turn can promote oxidative stress itself and affects protein folding, creating a complex network of deleterious interactions (Bozzo et al. 2016) (Figure 5).

Mitochondrial dysfunctions

Mitochondria are the cell's energy production sites through oxidative phosphorylation. They are the deposit of intracellular calcium and are involved in apoptosis. Several studies have demonstrated altered morphology in mitochondria located in proximity to the end of axons in motor and sensory neurons, in muscles, and in other tissues (Siklós et al. 1996). Impaired activity of Complex IV and a higher mutation rates in mitochondrial DNA have been reported in spinal motor neurons (Shaw 2005). Mitochondrial dysfunctions also relate to impaired clearance of damaged organelles, defects in transport or morphology, induction of mitochondrial mediated apoptosis, and calcium storage defects (Cozzolino and Carrì 2012).

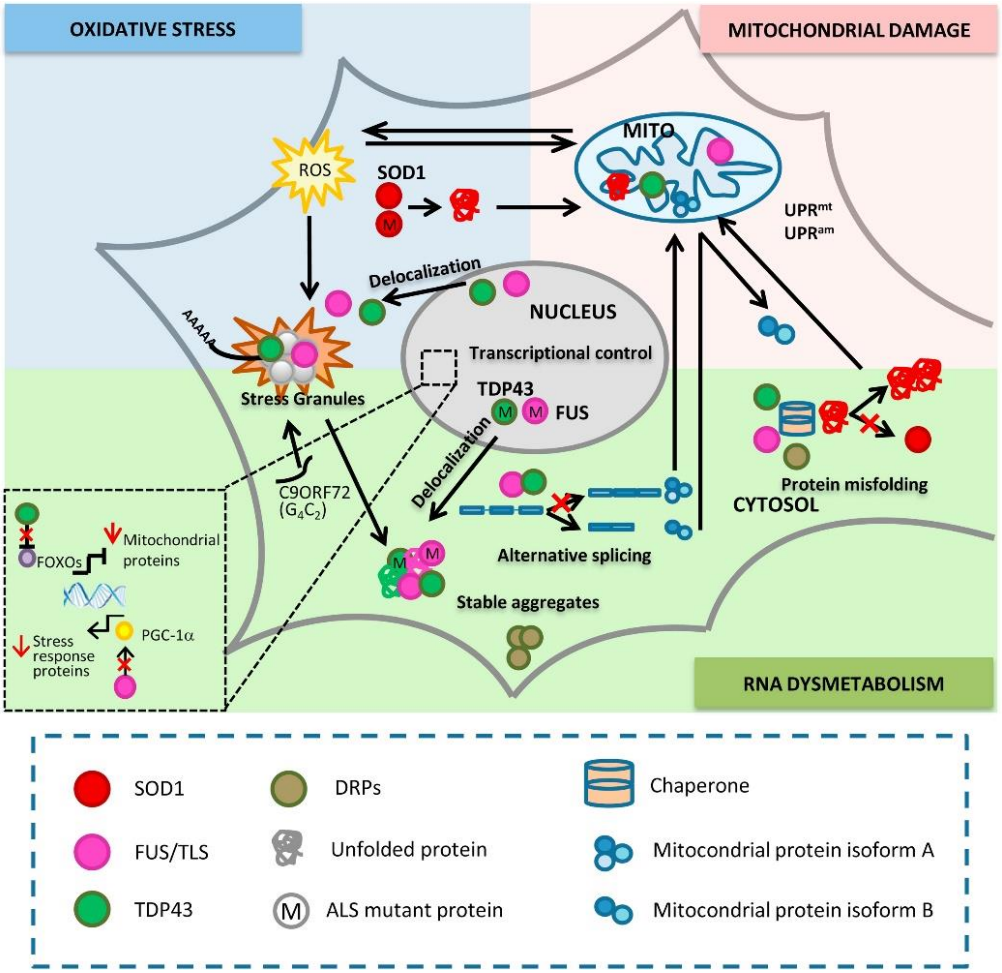


Fig. 5. Schematic representation of the pathogenic vicious cycle underling motor neuron degeneration in ALS. Oxidative stress and mitochondrial impairment are linked to protein unfolding and aggregation and to altered RNA metabolism (Bozzo et al. 2016).

Disruption of mitochondria may be triggered by aggregation of products of mutated ALS genes as well as by RNA dysmetabolism and oxidative damage. In transgenic mice, mutant SOD1 localizes in spinal cord mitochondria before symptom onset, and this has been associated with decreased respiratory activity and increased oxidative damage (Higgins et al. 2002; Mattiazzi et al. 2002). Physical interaction between mitochondria and the endoplasmic reticulum, usually occurring at specific sites called Mitochondrial Associated Membranes (MAMs), is crucial for the correct functioning of both compartments in calcium signalling, in lipid metabolism and mitochondrial bioenergetics, as well as for morphology, transport and clearance. Disturbances of these interactions in ALS and other neurodegenerative diseases have been documented (Manfredi and Kawamata 2015; Carrì et al. 2016).

Protein aggregation

Correct protein folding and a preserved native three-dimensional structure are crucial to ensure biological function. Under certain circumstances, enhanced by mutations and environmental conditions, proteins tend to misfold. Protein misfolding could lead to the formation of insoluble, toxic protein aggregates, a hallmark of several different neurodegenerative diseases. These inclusions have been detected in several neural compartments in ALS animal models and in *post-mortem* patient's tissues as well (Al-Chalabi et al. 2012). In the familial form of the disease, these protein aggregates are mainly composed by those encoded by the mutated genes (one of the most present is ubiquitinylated TDP-43). When a pathological *C9ORF72* repeat expansion is present, repeated-associate non-conventional (RAN) translation occurs (Zu et al. 2013), resulting in the production of dipeptide repeat proteins (DPR). DPR are considered as mediators of toxicity, since they can aggregate and cause endothelial reticulum (ER) stress (Ash et al. 2013; Wen et al. 2014). In addition, wild-type forms of TDP-43, SOD1 and FUS misfolded proteins have also been detected in inclusions of SALS patients (Neumann et al. 2006; Blokhuis et al. 2013).

With so many evidences towards protein misfolding to be crucial in ALS, proteostasis impairment is likely to be a central feature in the disease. Indeed, several disease-causing genes code for protein whose function is linked to proteostasis (Parakh and Atkin 2016) and particularly in ubiquitin-proteasome system (UPS), in chaperone mediated autophagy (CMA), and in macroautophagy (Ciechanover and Kwon 2015). Unfolded protein response (UPR) is a cellular mechanism able to reduce the load of misfolded proteins, and in the last years its role in ALS pathogenesis has been widely discussed (Kanekura et al. 2009) since markers of ER stress and UPR have been found in elevated levels in spinal cords of SALS patients (Atkin et al. 2008).

Axonal transport dysfunction

Intracellular inclusions containing neurofilaments have been found in degenerated motor neurons (Shaw 2005). Neurofilaments represent the major component of neuronal and motor neuronal cytoskeleton, responsible for maintaining cell shape and axon calibre. They are formed upon aggregation of subunits, assembled in cellular *soma* and transported in the axon with a slow movement characterized by progressive phosphorylation and anterograde/retrograde transport systems. They play a crucial role in maintaining neuronal structure and axonal transport, so a malfunctioning of these finely tuned processes could lead to motor neuronal degeneration (Perrot et al. 2008). Mutations in the *DCTN1* gene - coding for the protein dynactin, part of dynein-dynactin molecular motor - are a rare cause of ALS. Problems in microtubule binding and defects in vesicular

trafficking resulting in ER morphology abnormalities and autophagy activation have been observed (Münch et al. 2005; Laird et al. 2008). Rare mutations have also been detected in NFH protein in a site where a crucial phosphorylation occurs, resulting in toxicity due to neurofilament disorganization (Brettschneider et al. 2006). Also profilin 1, an actin binding protein intervening in actin polymerization, has been found mutated in ALS patients (Wu et al. 2012).

Excitotoxicity

The only approved pharmacological treatment for ALS consists in Riluzole, an inhibitor of glutamate release. High levels of glutamate have been found both in plasma and in CSF of ALS patients. Defects in glutamate transporters localized in glial cells, whose function is to rapidly inactivate glutamate action after release, have been described both in animal models and in human patients and could possibly lead to higher levels of this excitatory neurotransmitter in the extracellular compartment. All these evidences led to hypothesize that abnormal levels of L-glutamate could trigger a signalling cascade leading to motor neuronal death (Struzyńska 2009). In detail the hyper-activation of glutamate receptors could lead to an uncontrolled calcium influx in cells, that could trigger nucleic acid damage, lipid peroxidation and mitochondrial disruption, leading in the end to cellular death.

RNA processing

Several ALS associated genes have a role in RNA processing (*FUS, TARDBP, SETX, hnRNPA1, MATR3, ANG*). This complex pathway includes transcription, intron removal through splicing, RNA maturation, editing, transport and degradation. Proteins coded by these genes are physiologically located into the nucleus, with only a small fraction in the cytoplasm. Mutated proteins, instead, are mostly found in cytoplasm, with only minor presence in the nucleus. In spite of the still unclear role of the protein encoded by *C9ORF72* gene, it has been demonstrated that the hexanucleotide expansion associated to the disease could promote the formation of RNA *foci* into the cytoplasm - where RNA binding proteins are sequestered - lowering the amount of available functional proteins (Barber and Shaw 2010). It remains to be clarified if the pathological event in these processes is in a cytotoxic gain of function or a depletion from the nucleus of those proteins involved in RNA processing (Peters et al. 2015).

Inflammation

Microglial and dendritic cells are involved in early processes of motor neuron degeneration, as their activation leads to inflammatory status in the neural compartment (Glass et al. 2010). Studies in mice expressing mutated SOD1 protein showed that the expression of the mutated protein is not

sufficient to promote motor neuronal degeneration by itself, but the involvement of other non-neuronal cells is required. Conversely, wild-type motor neurons surrounded by *SOD1* mutated cells may present signs of the pathology (Clement et al. 2003). ROS released from damaged motor neurons can activate glial cells. Activated microglia becomes toxic resulting in release of further ROS, of reactive nitrogen species, and of pro-inflammatory cytokines that activate, in a self-sustaining loop, neighbouring glial cells. Mediators released by motor neurons enhance the switch from neuroprotective and anti-inflammatory microglia to the release of neurotoxic and pro-inflammatory cytokines such as IL1β, TNFα, and IL-6 (Shaw 2005).

Potential Environmental Factors

Nowadays, environmental contribution to the etiology of ALS remains elusive. There is no specific environmental factor proven to cause ALS, but a lot of hypotheses have been raised in the last years, since genetics explains only a small percentage of the cases. Even among clear-cut genetic cases, variability is extremely high and may be influenced by different environmental exposures. In ALS, it is highly probable that both known and unknown susceptibility genes interact with environmental factors to modulate disease risk. Challenges in assessing influence of environmental factors are hard to overcome. The variables to consider, in evaluating the exposure, are potentially infinite both in space and time. Specific exposures can be evaluated only in the presence of a prior hypothesis (Al-Chalabi and Hardiman 2013). However, several factors have been discussed to be involved in ALS etiology, but most of them in a controversial way.

Gender

Male gender is one of the strongest risk factors for SALS. In fact, males have 1.5 time increased risk to develop ALS, with respect to females (Mehta 2015). Genetics, as seen, may play an important role, but up to now only a very rare mutation in the Ubiquilin 2 gene lies in the X-chromosome. Hormone influences may account for the differences in ALS risk between gender. The sex hormone testosterone has been suggested as a risk factor for ALS, and higher prenatal circulating levels of testosterone possibly influence motor-neuron vulnerability in later life (Vivekananda et al. 2011). Hormone influence is supported also by the fact that incidence of ALS becomes equivalent between the genders after menopause. Pregnancy has been described - in few studies - to have a possible role in unmasking latent ALS, probably due to a decrement of estrogens levels especially in patients

lacking the ability to inactivate efficiently superoxide radicals as a result of mutations in the SOD1 gene (Chiò et al. 2003; Lunetta et al. 2014). Indeed, estrogens are known to have a potential neuroprotective effect in Parkinson's and Alzheimer's diseases (Tang et al. 1996; Benedetti et al. 2001). Beyond the biological differences, males and females also differ in activities leading to different environmental exposures (Oskarsson et al. 2015).

Geographic clusters

Modern epidemiology is more and more focusing in investigating geographical clusters in order to dissect complex diseases. However, such a methodology is difficult to apply in a disorder as ALS since usually latency between disease onset and time of exposure is unknown or hard to assess. Most of the studies conducted on geographical clusters are aimed at epidemiologically identifying them, rather than at evaluating potential causes of this phenomenon. Indeed, the most used approach relies on performing long and detailed prospective, longitudinal observations over a lifetime in large numbers of people (Al-Chalabi and Hardiman 2013).

This approach led to increase knowledge of the disease in the case of ALS-PDC syndrome in the island of Guam, as described above. In two studies performed on Spanish population, ALS frequency - adjusted for age, sex, and mortality rate - showed a North-South gradient (Veiga-Cabo et al. 1997; Alonso et al. 2011). Clusters based on residence at diagnosis or death have been identified in different ALS population from studies based on mortality data from Italy (Uccelli et al. 2007; Ragonese et al. 2012; Nicoletti et al. 2016), UK (Keren et al. 2014), Finland (Sabel et al. 2009), New Jersey (USA) (Henry et al. 2015) and France (Boumédiène et al. 2011). However a considerable fewer number of studies have aimed at evaluating the possible environmental causes of the origin of these clusters (Vinceti et al. 2012; Kihira et al. 2013; De Benedetti et al. 2016). At last, geographical clusters might be suspected in a case of a local genetic founder effect, as it has been hypothesized in the case of Kii Peninsula in Japan, where the higher prevalence of ALS has been partially explained by the presence of *C9ORF72* hexanucleotide repeat expansion (Ishiura et al. 2012).

Smoking

Several studies have included tobacco smoking among the potential risk factors for ALS (Kamel et al. 1999; Armon 2009; De Jong et al. 2010). However, findings are not conclusive, since seven studies showed an increase ALS risk in smokers, whereas 14 showed no increased risk. If a risk exists, it seems to be confined to female smokers, particularly in post-menopausal women (Al-Chalabi and

Hardiman 2013). It is not clear if the hypothesized risk could be associated to increased oxidative stress or to nicotine and other toxic substances contained in tobacco smoke.

Diet and BMI

An inverse association between dietary antioxidant intake and lower risk for ALS has been widely investigated. Among antioxidants, Vitamin E was one of the most studied, both in terms of regular use and duration of supplementation, in different cohorts of subjects (Ascherio et al. 2005; Veldink et al. 2007; Okamoto et al. 2009). A more recent, huge study performed on Finnish male smokers, hypothesized a protective effect of Vitamin E on ALS risk, by measuring serum concentrations on a long, double-blind, placebo-controlled trial (Michal Freedman et al. 2013). However, using Vitamin E in addition to Riluzole therapy, was not effective in extending survival in ALS patients, although an improvement in rate deterioration function was observed (Desnuelle et al. 2001). Polyunsaturated fatty acids are another class of antioxidants associated to a lower risk of ALS, possibly because they are capable to modulate lipid metabolism and inflammatory processes (Veldink et al. 2007; Fitzgerald et al. 2014).

On the basis of clinical data, there is the impression that ALS patients have higher levels of physical fitness and thus a body mass index (BMI) lower than average (Huisman et al. 2013). Low BMI and malnourishment have been associated with shorter survival (Bouteloup et al. 2009). Conversely, overweight and obese subjects show a reduced risk to develop ALS (O'Reilly et al. 2013). Thus, increased fat and cholesterol intake may represent a protective factor (Paganoni and Wills 2013). Other evidences stem from different studies assessing that high carbohydrate and low fat intakes are associated with higher ALS risk (Okamoto et al. 2007). Finally, a 10 year follow-up study reached the conclusion that high fat content is associated with a lower risk of developing ALS (Gallo et al. 2013).

Physical exercise

Before showing symptoms of the disease, ALS patients often show high levels of physical fitness. It is well known that one of the first well described ALS affected patient is Lou Gehrig, a famous baseball player of the beginning of the 20th century (Lewis and Gordon 2007). Furthermore, media have always raised the attention on sport celebrities who developed ALS at the top or near the end of their career. In consequence, much interest was raised on the hypothesis that physical activity could be a risk factor for developing ALS (Al-Chalabi and Hardiman 2013). Several studies have shown that - before symptom's onset - subjects that developed ALS were leaner than healthy

controls, and that many performed semi-professional sport activities during their college years (Scarmeas et al. 2002).

However, despite self-reported physical activity well correlated to objectively measured data, other studies showed no significant excess of extreme exercise in premorbid period of ALS patients (Huisman et al. 2013) and no dose-response relationship between physical activity and ALS could be assessed. Indeed, it seems to be that athletic prowess and ALS risk are two distinct aspects of a particular predisposition that confers both athletic advantage and increased risk of neurodegeneration (Al-Chalabi and Hardiman 2013). Furthermore, ALS is a pathological condition usually expressing in a period of life when other diseases of advanced age manifest. Consequently, those people affected by ALS are usually those that did not die by cancer or heart diseases, among which is likely to find subject who in the previous years exercised and had a better fit (Sutedja et al. 2011). A higher incidence of ALS cases has been described among football players in the top Italian professional leagues and this may support the involvement of physical exercise. Other proposed causes may be soccer specific trauma or micro trauma, the use of illegal toxic substances or chronic misuse of drugs and dietary supplements, as well as exposure to pesticides used on playing fields (Vanacore et al. 2006). However, thus not excluding a potential effect of several factors acting together, conclusive scientific evidence of a link between ALS and soccer (or other professional sports) is lacking.

Occupation

Possible occupations where workers may be subjected to a higher risk to develop ALS include veterinarians, athletes, hairdressers, power-production plant operators, and armed forces personnel (Al-Chalabi and Hardiman 2013). However, no causal factors have been identified and common denominators among such different occupations are hard to identify. Several studies highlighted that US army personnel, especially those deployed in the Gulf Wars, were at risk for developing ALS. Soldiers are exposed to various potential harmful agents such as trauma and toxic substances (heavy metals and chemicals). However, drawing conclusions as for the role of military service in the etiology of ALS seems premature (Beard et al. 2016).

Electric shock

Magnetic fields, electrical fields, contact currents, micro shocks, and both perceptible and imperceptible electric shocks all contribute to occupational exposure to extremely low frequency electromagnetic fields (EMF) (Ingre et al. 2015). Higher risk of ALS has been associated to workers

involved in occupations leading to electric exposure (Vergara et al. 2013). The hypothesis that electric shocks are linked to ALS is intriguing because of the effects of electricity on the neuromuscular system, and the assumed excitotoxicity that would follow. However, evidence of a causative relationship is poor (Al-Chalabi and Hardiman 2013).

Pesticides and chemicals

Many pesticides are known to be neurotoxic at high levels and substances as organophosphates have a direct effect on the lower motor neuron synapse due to their ability to inhibit acetyl cholinesterase, the enzyme responsible for terminating the biological activity of acetylcholine. Most of these compounds are able to induce oxidative stress, mitochondrial dysfunction and neuronal loss. The main pesticides for which exposure is frequent, not only in professional workers as farmers, are insecticides, fungicides, herbicides and rodenticides (Bozzoni et al. 2016). Several reviews and meta-analyses associated pesticides exposure to increased ALS risk with different strengths (Sutedja et al. 2009; Kamel et al. 2012; Malek et al. 2012; Capozzella et al. 2014). Again, interaction of genetics with environment may play an important role. High interest was posed in evaluating the role of the paraoxonase gene cluster (*PON*) coding for enzymes able to detoxify organophosphate pesticides. Some *PON1* polymorphisms have been identified as involved in lowering enzymatic activity. It is possible that *PON1* mutation predisposes to ALS by reducing pesticide hydrolysis and promoting oxidative stress processes. There are strong evidences indicating a role for pesticides in ALS, as well in other neurodegenerative diseases, for which the association is more clearly established (Bozzoni et al. 2016).

Metals

The role of metal influence in ALS following environmental and/or occupational exposure has long been studied. Evidences that metal metabolism may be impaired in other neurodegenerative diseases like Alzheimer disease, Parkinson disease, neurodegeneration with brain iron accumulation (NBIA), multiple sclerosis, Huntington's disease, Wilson's disease, Menkes disease, and Friedreich's ataxia all strongly supported this hypothesis and promoted studies in this direction (Roos et al. 2006; Rouault 2013). However, taken together, the studies performed in order to dissect metal contribution to ALS produced inconsistent results, probably due to limited exposure assessment (Sutedja et al. 2009). The samples analyzed were very heterogeneous, spanning from serum, to Cerebrospinal Fluid (CSF) to hair, to neuronal cells (Pamphlett and Kum Jew 2013; Roos et al. 2013; Bocca et al. 2015). In addition, exposure to metals is highly dependent on the environment, so that selecting a proper cohort of subjects and controls is crucial. Accordingly, another factor to lead to

inconsistent results could be the inclusion in the study of groups of subjects originating from different geographical areas.

Transition metal induced toxicity has been proposed to be involved in the pathology (Carrì et al. 2003) and higher concentrations of metals and proteins that regulate metal homeostasis have been described in ALS patients (Goodall et al. 2008; Nadjar et al. 2012; Roos et al. 2013). Several studies have shown a deregulated homeostasis of metals like Fe, Cu and Zn in ALS; a deleterious effect of Zn on TDP-43 expression also has been demonstrated (Caragounis et al. 2010). Accumulation of these elements in motor neurons could exacerbate the oxidative stress, responsible for the cell death. However, it is not clear whether deregulation of these elements is a cause or a consequence of the pathology.

Lead (Pb) is a well-known neurotoxic element that can cause neuropathies with the involvement of motor nerves (Oskarsson et al. 2015) and its association with ALS is a long-standing hypothesis. Lead levels in bone and blood were associated with ALS (Kamel et al. 2002 and 2003). Blood lead levels reflect environmental exposition, but are also influenced by the mobilization of lead from bones. Lead accumulates in human bones over the course of a lifetime, making them a possible endogenous source of Pb (Bocca et al. 2015). A study that considered also lead turnover from bone adjustment, still described higher levels of this element in ALS patients (Fang et al. 2010). However, the connection between lead and ALS is still unclear. Many of the lead-related studies have described also prolonged survival in patients with ALS with high lead levels. This led to the intriguing - but questionable - hypothesis that lead may represent a risk factor for developing ALS and a protective factor that slows ALS disease progression (Oskarsson et al. 2015).

Manganese (Mn) toxic properties are known, as well its ability to cross the blood brain barrier and to accumulate in nervous tissues, prolonging its half-life (Dobson et al. 2004). Occupational exposure is documented in welders, that may present motorial deficit symptoms (Bowler et al. 2006). Data on the association between Mn and ALS are quite scattered (Hozumi et al. 2011; Roos et al. 2013; Garzillo et al. 2014; Kihira et al. 2015). The heterogeneity of the results obtained in these studies may depend on the type of analysis, the tissue that was chosen, and the selection of the population involved in the study.

Mercury (Hg) in all its forms (elemental, inorganic, and organic) has neurotoxic properties due to its ability to interfere with the glutathione pool, to damage mitochondria, and to enhance oxidation of lipids, proteins and DNA, all relevant factors in ALS etiology (ATSDR 1999; Carocci et al.

2014). In the case of Hg too, literature is insufficient to draw definitive conclusions. The association with ALS could be difficult also because exposure to high levels of Hg may lead to develop neurological and motorial symptoms that resemble ALS pathology.

Iron (Fe) is the most abundant transition metal in human body, and an important cofactor in many biological processes like oxygen transport to tissues and oxidative phosphorylation in mitochondria. Given its importance and its toxicity as a potential catalyst of the formation of ROS species, its metabolism is very finely tuned. Different studies showed a correlation between high serum ferritin levels and ALS, probably reflecting general iron overload (Goodall et al. 2008; Nadjar et al. 2012). Other neurodegenerative disorders are characterized by iron accumulation with concomitant deregulation of iron homeostasis (Hadzhieva et al. 2014). Among ALS patients, increased iron concentration has been reported in the ventral spinal cord and in the motor cortex (Kasarskis et al. 1995).

Exposure to selenium (Se), and particularly to its inorganic forms, has been hypothesized as a risk factor for ALS. Selenium is an essential element and its biological activity mostly depends on its chemical form. Inorganic selenium is indeed more toxic than the organic species (Vinceti et al. 2010). The suggestion that selenium may be involved in ALS raised from the study of a cluster of patients resident in a seleniferous area of South Dakota (USA), and from an Italian study that extensively investigated a well-defined ALS case series of residents of the Emilia-Romagna region (Kilness and Hochberg 1977; Vinceti et al. 1997; Vinceti et al. 2010). A study performed on selenium species in the Italian cohort found an association of increased ALS risk with higher concentrations in CSF of inorganic selenium, as selenite, and of human serum albumin-bound selenium, confirming results obtained in a cohort from Norway (Roos et al. 2013; Vinceti et al. 2013). How and whether these findings may be generalizable to other populations seems to deserve further investigation (Ingre et al. 2015).

Aluminum (Al) can easily cross the blood/brain barrier and reach the brain, where it can accumulate after chronic exposure. High levels of Al in the brain may activate glial cells to produce inflammatory cytokines and ROS, possibly leading to neuronal cells loss (Becaria et al. 2002). These effects were confirmed after studies on animal models, with a particular focus on motor neuronal degeneration (Tanridag et al. 1999; Kihira et al. 2002). In studies performed on ALS patients, Al was found slightly increased in the blood of patients respect to controls, decreased in patients' hair (Bocca et al. 2015), and increased in CSF (Roos et al. 2013). Other reports did not detect differences in Al concentrations between patients and heathy controls (Bergomi et al. 2002; Garzillo et al. 2014).

Other metals with potential relevance for ALS are copper, arsenic, cadmium, cobalt, zinc, vanadium, and uranium, all of which have been found in significantly elevated concentrations in the CSF of ALS patients when compared with healthy controls (Roos et al. 2013). In conclusion, single metal evaluations may underestimate their relevance for health risks. It is more likely that all these metals interact with one another with an additive or even a synergistic effect (Andrade et al. 2015). In consequence, it is important to consider the possible complex interactions between single metals in evaluating their potential association with ALS as it has been demonstrated, for instance, that a mixture of arsenic, cadmium and lead has the ability to compromise blood brain barrier, by acting with synergistic toxicity (Rai et al. 2010).

Biomarkers

Biomarkers are defined as objectively measurable parameters that can be used as an indicators of a biological condition or the effect of a treatment (Bowser et al. 2006). To be applied to diagnostic procedures, such as predicting disease susceptibility, monitor disease progression or evaluating therapies' toxicity (Figure 6), biomarkers must have high levels of sensitivity and selectivity (Ryberg and Bowser 2008).

ALS still lacks of diagnostic tests and its diagnosis is usually late; however, the fast developments in proteomics may facilitate the discovery of new protein biomarkers that could also help in providing insights in the pathogenic mechanisms of the disease. Prognostic biomarkers could further indicate different stages and modifiers of disease progression (Krüger et al. 2013).

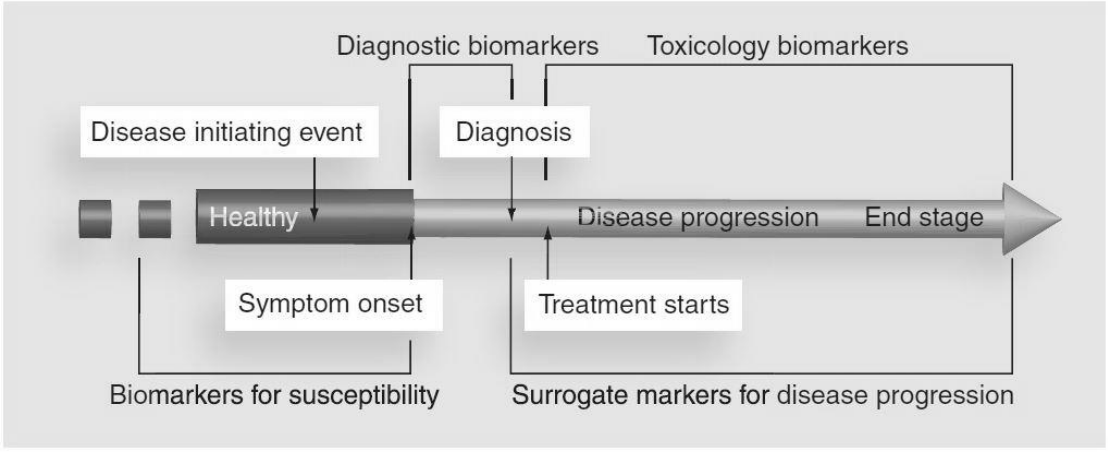


Fig. 6. Disease key clinical events and their relation to different types of biomarkers (Ryberg et al. 2008).

Blood and cerebrospinal fluid (CSF) are the most accessible biofluids. The disadvantage relies in the fact that a potential protein biomarker released from a specific cell type, would be necessarily diluted in the large volumes of blood or CSF: it would also be of difficult detection and may reflect processes different from what it is interested. Indeed, biochemical constituents of these body fluids may be altered also by secondary pathological effects.

Plasma and serum are the preferred biofluids for biomarkers discovery and a wide number of researches pointed to find out one or more circulating protein biomarker. However, it often resulted in conflicting and inconclusive results mostly due to differences in analytical assays and samples together with small study group sizes. Proteins investigated as serum biomarkers belong to several different groups, such as growth factors or hormones, cytokines and other inflammatory proteins, but also enzymes and enzyme inhibitors (Ryberg and Bowser 2008).

CSF is an important source for proteomic-based biomarker research, since it represents a diagnostic window to the central nervous system. However, high invasiveness, heterogeneous sampling volume and low protein abundance represent a considerable limit. Anyhow, due to functional and physical proximity of motor neurons to CSF, it may better reflect protein abundance alterations than other body fluids (Krüger et al. 2013). CSF biomarkers candidate can be classified into inflammatory markers, glial response markers, axonal damage and apoptosis markers (Tumani et al. 2008).

The state of art of all the current studies related to the research for biofluids biomarkers in ALS is excellently presented by Krüger et al. and by Robelin and De Aguilar in their recent reviews (Krüger et al. 2013; Robelin and Gonzalez De Aguilar 2014).

Finally, ALS diagnosis is strongly hampered also from the wide heterogeneity of its phenotype. Thus, it could be more effective to apply a concerted set of biomarkers or to assess the ratio of different proteins, instead of focusing on a single biomarker, that could be not so specific for discriminating the disease from other pathological conditions.

Therapy

At present, no therapy is available to treat ALS. Treatment of patients is mostly symptomatic, aimed at improving quality of life and maintaining patient's autonomy as long as possible. Dysphagia is a common symptom, leading to malnutrition, weight loss and dehydration. Malnutrition is usually associated to a worsened prognosis and to a reduced survival. When autonomous nutrition is no longer possible, percutaneous gastrostomy is recommended (PEG). When respiratory muscles become too weak, the intervention with artificial breathing machines to support ventilation is required (Kiernan et al. 2011).

The only available drug, approved by the Food and Drug Administration (FDA), is Riluzole. It is a neuroprotective compound whose efficacy is to extend survival (typically, 3/6 months) and to delay the intervention with artificial breathing (Leigh et al. 2003). However, Riluzole has no effects on motor and pulmonary function, and it is ineffective when treatment is done in the last stages of the disease (Rowland and Shneider 2001). Riluzole acts as a blocker of calcium channels. It activates potassium channels, inhibits dopamine and acetylcholine release, and blocks GABA reabsorption. Riluzole ability to lower presynaptic glutamate release makes it able also to control excitotoxicity (Wijesekera and Leigh 2009).

At present, the most promising therapeutic targets are excitotoxicity, oxidative stress and neurotrophic factors modulation. Gene and stem cells therapy represent the newest and most promising approaches to treat the disease, although clinical trials are still needed for assessing their safety and efficacy.

AIM OF THE STUDY

ALS is a complex disease in which genetics and environmental factors interplay in different and still unknown proportions, both in giving rise to the pathology and in modifying the clinical phenotype. The study of geographical clusters of ALS patients, such as those described in the Western Pacific region, has been helpful - in the past years - to get more insights into the pathology. In medical terms, a cluster is a “mini-epidemic” distribution of a pathological condition within a well-defined region, where it accounts for a higher-than-expected disease prevalence (i.e., above the estimated national prevalence) (Malaspina et al. 2002). Studying individuals exposed to the same environment, thus ideally subjected to the same exogenous stressors, could be very valuable by limiting one of the most confounding variables in ALS studies. Indeed, a diverse environmental exposure is almost always necessarily present when analyzing the large cohorts of patients that are usually required to reach statistically significant numbers of cases in epidemiological-type studies.

This PhD thesis presents a multidisciplinary study performed on a small cohort of sporadic ALS patients, all originating from a restricted and defined geographical area. Focusing on a very limited geographical area, gave the chance to consider the characteristics of the surrounding environment and allowed to raise hypotheses on the possibly involved stressors acting on the local population, being those environmental or dietary contributions.

The study area includes four municipalities – Casarza, Lavagna, Cogorno and Sestri Levante – with a total population of about 36,000 individuals over an area of 83.5 km^2, located between two narrow mountain valleys and the Mediterranean seaside, belonging to the Genoa district of the Liguria region in northwestern Italy. ALS point prevalence in the Liguria region is 7.85/100,000 (95% CI 6.54–9.36) people (Scialò et al. 2016). The study enrolled seven patients (4 men and 3 women) and five controls (2 men and 3 women, age-matched with the patients (69 ± 12 years vs. 64 ± 6 years) and living in the same geographical area). Besides the seven patients enrolled in our study, we are aware of the presence of additional affected subjects, making prevalence of ALS in this area markedly higher than in the rest of the region.

To overcome the obvious limits related to the small number of subjects necessarily involved in this study, advantage was taken from the application of a multifactorial statistical evaluation, based on a machine learning software. This software, belonging to the artificial neural networks

architecture, was specifically designed to analyze complex problems, where the number of variables significantly exceeds the amount of subjects involved in the study, as usually is in the case of rare diseases. These approaches had been successfully applied in previous studies of a complex disease such as ALS (Buscema et al. 2012).

In this multidisciplinary work two main lines of research were followed. Although both rely on assessing specific components in the blood of patients and controls, these two lines are methodologically distinct and provide independent information (Figure 7).

A first research line aimed at investigating the role of circulating metals in both serum and whole blood, trying to point out differences in metals' levels between ALS patients and healthy control subjects. The analytical data were processed to unravel possible associations with clinical features of the disease, eating habits, and DNA oxidative damage. Data on environmental pollution were not collected in this study, but the presence of a mine within this area led to focus the investigations firstly on the already proposed role of metals in ALS. This Cu and Fe mine, closed in 1962, is located 8 km inland from the study area, within the basin of a creek, whose waters are reported to be strongly polluted due to Acid Mine Drainage. Waste rock dumps can store significant amounts of potentially deleterious metals that can be released to solutions during transformation processes, induced by variations in the physicochemical parameters or by ageing. This could lead to the release, to the circulating solutions, of high amounts of potentially toxic metals during weathering of primary sulfide ores (Fe, Cu, Zn, etc.) and of the accompanying mine tailings (Cr, Ni, V, etc.) (Marescotti et al. 2010).

The other line of research pointed at evaluating the serum proteome, and used bi-dimensional electrophoresis (2DE) as the main approach. Despite 2DE being an already mature methodology, literature data on ALS subjects are still scarce. This research aimed at describing the serum proteome of this peculiar group of patients, with a particular focus on possible disease biomarkers, actually badly needed for diagnostic purposes. ALS diagnosis is usually difficult and may take up to 16 months from the onset of the symptoms (Kraemer et al. 2010). Only the identification of selective and sensitive biomarkers could allow an earlier diagnosis, which would in turn lead to a better care of the patients, since it is by now clear that the timelier the treatment with Riluzole, the more successful its outcome. The availability of usable biomarkers would be most valuable also in clinical trials, in which at present the only meaningful indicator is the survival rate (Robelin and Gonzalez De Aguilar 2014). In this perspective, great efforts have been made during the last years for identifying possible biological markers and risk factor indicators, including different proteomic

investigations on tissues of different availability (Dengler et al. 2005; Ranganathan et al. 2005; Brettschneider et al. 2008; Brettschneider et al. 2010; Nardo et al. 2011; Forseen and Corey 2012; Collins et al. 2015; Chen et al. 2016). However, heterogeneity of investigated biological samples and technologies applied, hampered the definition of conclusive results.

Finally, the proteomic analysis was integrated by the evaluation of ALS associated genes that resulted relevant from these experiments. This work is, at present, the first attempt to describe the serum proteome of a geographic cluster of ALS patients, with the aim of limiting the variability, necessarily present in the cohorts of subjects described in literature, in the most accessible biological sample.

The multidisciplinary approach applied in this study was possible due to the limited number of subjects involved. This, in turn, gave the opportunity to consider simultaneously different aspects of this pathology, trying to get a more comprehensive understanding of the disease.

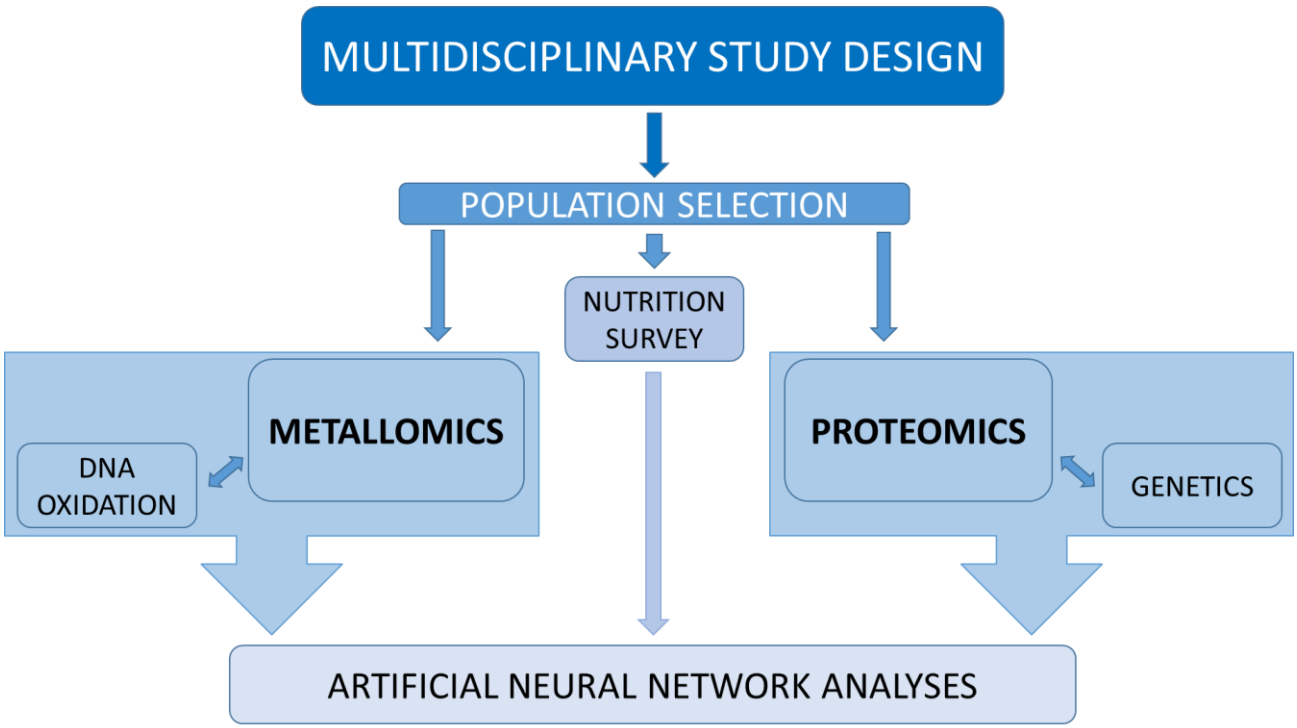


Fig. 7. Schematic representation of the design of the study presented.

MATERIALS AND METHODS

Blood sampling

Blood was collected in a vial for serum separation and in two vials for whole blood and genetic analyses. Sampling was performed after an overnight fast and, after clotting, serum was separated by centrifugation at 3000 rpm for 15 minutes. Aliquots of serum were stored at -80°C. Vials for whole blood analyses were stored at -80°C without any treatment. To minimize contamination during blood sampling, the vials to be used for trace metal analysis were the last ones collected.

Nutrition survey

Both patients and controls were administered a survey to collect information about lifestyle and nutrition habits. The questionnaire mostly focused on intake of several foods with frequency indication assessed as: never, once a week, two or three times a week, every day. Patients were instructed to fill the questionnaire with reference to their eating habits before the disease onset.

Inductively Coupled Plasma - Mass Spectrometry

Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) is a technique used for elemental determinations with high detection capability for rare elements at concentrations as low as one part in 10^{15} (ppq). The instrument combines a high-temperature inductively coupled plasma source - that converts the atoms of the sample's elements in ions - with a mass spectrometer that separates and quantify those ions. An inductively coupled plasma is a plasma made by partially ionizing argon gas ($Ar \rightarrow Ar^{+} + e^{-}$), energized by inductively heating the gas with an electromagnetic coil, and containing a sufficient concentration of ions and electrons to make the gas electrically conductive. Ions extracted from the plasma flow, through a series of "cones", into a mass spectrometer, usually a quadrupole. Separation of ions occurs on the basis of their mass-to-charge ratio and a detector receives an ion signal proportional to the concentration. The concentration of a sample can be determined through calibration with a certified reference material, such as a single or a multi-element reference standard. For the analyses of both serum and whole blood samples, ^{45}Sc, ^{89}Y and ^{159}Tb were used as

internal reference at a final concentration of 20 µg L^{-1}, and were added to other samples from a stock 2 mg L^{-1} solution.

Serum samples were diluted 1:20 with 0.05% Triton X-100 in MilliQ water. Typical analytical interferences were removed by using Collision-Reaction-Interface (CRI) with an H_2 flow of 70 mL min^{-1} through the skimmer cone. Seronom™ Trace Elements Serum L-1 and L-3 were used to build appropriate calibration curves. Samples of whole blood were diluted 1:100 with 1.3% HNO_3 and 0.05% Triton X-100 in MilliQ water. Since whole blood has a pronounced matrix effect, the standard addition method illustrated above was used to control and validate the results. Typical analysis interferences were removed by using CRI, but in this case the H_2 flow through the skimmer cone was increased to 93 mL min^{-1}. Seronom™ Trace Elements Whole Blood L-3 was used to control and validate the results.

Two-dimensional Electrophoresis

Two-dimensional gel electrophoresis (2DE) is a well-known technology and a very valuable, powerful and widely used method to assure resolution and pattern recognition for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. Furthermore, it provides a very wide spectrum of customizable solutions with minimal changes in the work pipeline.

This technique separates proteins in two steps, Isoelectric Focusing (IEF) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The first-dimension (IEF) run separates proteins according to their isoelectric points (pI), while the second dimension consists in a SDS-PAGE that separates proteins according to their molecular weight (MW) (Figure 8 and 9):

A. The sample, composed by a heterogeneous mixture of proteins, is placed close to the electrode, at one end of a strip with a gradient of pH.
B. At the end of the IEF run the proteins have migrated and stopped at their pI, where they have no electric charge. Proteins of different sizes may migrate at the same pI.
C. After the equilibration step, each protein is coated with SDS, that confers a homogeneous negative charge per mass unit.
D. To run the second dimension, the IEF strip is set to the top of a SDS-PAGE gel where proteins are separated according to their size and thus to their molecular mass.

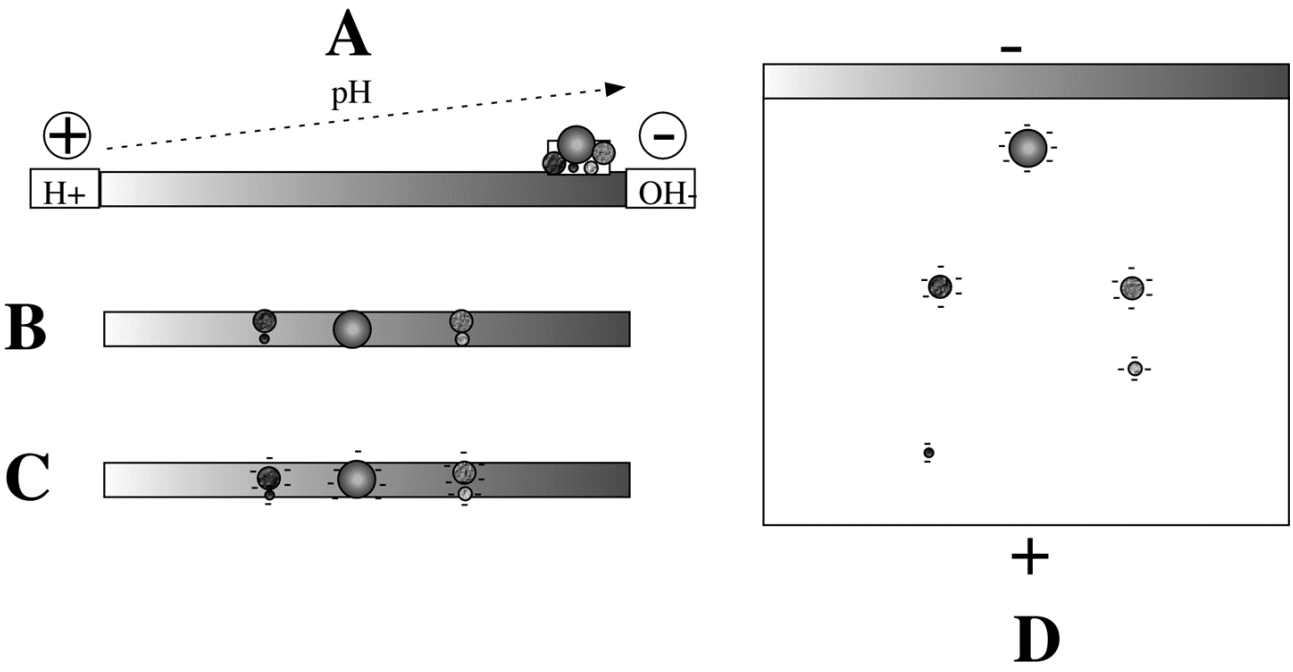


Fig. 8. Schematic representation of 2DE (Rabilloud and Lelong 2011).

Immobilized pH Gradient (IPG) strips were prepared following the procedures described in The Protein Protocols Handbook (Walker 2009). A Non Linear (NL) IPG in the 4-7 pH range was prepared by mixing appropriate volumes of 0.2 M Acrylamido Buffers with different pK_a values (available from Sigma-Aldrich), acetic acid 1 M (Sigma-Aldrich), T30C4 acrylamide stock (BioRad), ammonium persulfate solution (Sigma-Aldrich), N,N,N',N'-tetramethylethylenediamine (BioRad) and glycerol (Merck). After polymerization at 50°C for 1h, the slabs were washed in 1% glycerol for an additional hour. After drying, the gel was rehydrated in a solution of 8 M Urea containing 0.5% carrier ampholytes in the 4-7 pH range. Gel strips 16 cm x 0.8 cm in size were cut for individual sample loading. Serum aliquots corresponding to 600-700 µg of proteins were loaded near the cathode, with or without the addition of 1% 2-mercaptoethanol, depending on whether reducing or non-reducing conditions were desired. Reducing and non-reducing conditions optimize the resolution of different sets of proteins over a crowded area of the 2DE map of human serum, so that their combined use enhances the potential for an accurate quantitative evaluation of most serum proteome components (Wait et al. 2005).

IEF was run in a Multiphor II horizontal electrophoretic chamber with movable electrodes (Amersham Bioscience). The electric conditions were: 1 h at 200 V, 1 h at 300 V, 1 h at 400 V, 1 h at 500 V, 1 h at 600 V, 700 V overnight, 1 h at 2000 V and 1 h at 3000 V. After equilibration in 2% SDS,

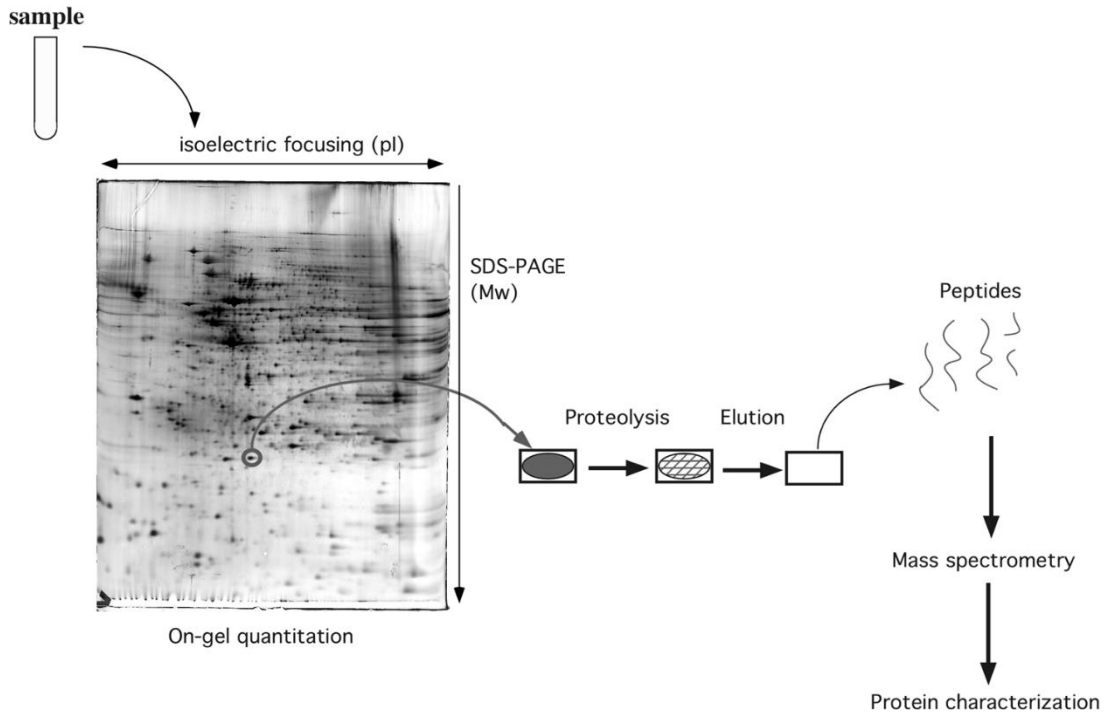


Fig. 9. Workflow of how 2D gel electrophoresis is used in a proteomics analysis(Rabilloud and Lelong 2011)

with or without the addition of 1% 2-mercaptoethanol, SDS-PAGE was run on individual strips in slab gels consisting of a 7.5-17.5% T gradient polyacrylamide gel cast in Laemmli buffers (Laemmli 1970), at 75 mA per gel. Image analyses of the Coomassie Blue stained patterns were carried out with Image Master Software ver. 5.0 (Amersham Biosciences). The gel with the highest number of spots among controls and patients was designated as the reference. The remaining gels were matched to the reference in order to identify the differences in spot volume (absorbance integrated over area). Most of the spots were automatically matched across gels by the software; the correctness of each matching was checked manually adding, where needed, "*de novo"* spots. In order to minimize any possible variation that might have been caused by differences in loading, staining, and destaining, the volume of each protein spot on a 2DE gel was normalized by computing its percentage over the total protein volume in that gel.

Mass spectrometry identification of proteins from 2-DE gels

The gel pieces were firstly washed and destained by two sequential rinses with 25 mM NH_4HCO_3 and 50% v/v aqueous acetonitrile (ACN), followed by washing them three times with a 1:2 mixture of ACN and 50 mM NH_4HCO_3. Finally, the gel pieces were completely dehydrated with 100% ACN, and the solvent removed in a SpeedVac for about 20 min. Samples removed from the SpeedVac were treated with a small volume of trypsin (Promega, Milan, Italy, 12 µg mL^{-1} in 50 mM NH_4HCO_3).

After trypsin absorption (30 minutes on ice) the gel pieces were completely covered with a few microliters of 50 mM NH_4HCO_3 and incubated overnight at 37°C. Formic acid (0.1% final concentration) was used to stop trypsin activity.

The samples were then analyzed by means of LC-ESI-MS/MS. MS spectra were acquired on a hybrid quadrupole orthogonal acceleration time-of-flight (Q-Tof) mass spectrometer (SYNAPT-MS G1, Waters Corporation, Milford, MA, USA) equipped with a TRIZAIC source and connected to a nanoACQUITY UPLC system. The samples were injected onto a TRIZAIC nanoTile (Acquity HSS T3, Waters Corporation, Milford, MA, USA), that integrates a trapping column (5 µm, 180 µm x 20 mm) for desalting and an analytical column (1.8 µm, 85 µm x 100 mm) for highly reproducible peptide separation. The elution was performed at a flow rate of 450 nL min^{-1} by increasing the organic solvent concentration from 3 to 40% B in 30 min. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in ACN. The TOF analyzer was externally calibrated using [Glu^1]-fibrinopeptide B from 50 to 1990 m/z. Data were post-acquisition lock-mass corrected, using the monoisotopic mass of the doubly charged precursor of [Glu^1]-Fibrinopeptide B (m/z 785.8426) infused into the mass spectrometer at a flow rate of 100 nL min^{-1} through a NanoLockSpray interface using the auxiliary pump of the nanoACQUITY system. A survey scan over the m/z range of 350-1990 was used to identify protonated peptides with charge states of 2, 3 or 4, automatically selected for data-dependent MS/MS analysis (MassLynx v 4.1 SCN833, Waters Corporation, Milford, MA, USA). All raw MS data were processed with ProteinLynx Global SERVER software (PLGS v 2.5.1, Waters Corporation, Milford, MA, USA) and the proteins were identified by correlating the interpreted spectra with entries in UniProt database.

The UniProt database (release 2015-3; number of human sequence entries, 20199) was used for database searches of each run. Carbamidomethylation was considered as fixed modification and methionine oxidation as variable, one missed cleavage per peptide was allowed. The mass tolerance window was set at 25 ppm for peptide precursors and 0.05 Da for fragments. In parallel, the spectra were also searched against the Uniprot database by using Mascot (Matrix Science, London, UK). Valid identification required two or more peptides independently matching the same protein sequence, with a significant peptide score (higher than the identity score from Mascot).

Comet assay

The level of oxidized DNA bases was determined through the comet assay, by assessing the sites sensitive to formamido-pyrimidine DNA glycosylase (FPG), which detects oxidized purines, primarily 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and ring-opened formamido-pyrimidine nucleobases. The standard protocol for the evaluation of DNA damage is performed in peripheral blood mononuclear cells. Recent studies reported the possibility to use also frozen whole blood (Akor-Dewu et al. 2014). In the present study, only aliquots of frozen whole blood samples were available for the evaluation of DNA damage; thus, the protocol was highly standardized and performed according to Akor-Dewu et al. with slightly modifications.

Aliquots of whole blood (5 µl, thawed from frozen stock at -80°C) were mixed with Low Melting Point (LMP) agarose (1.5% wt/vol) in Tris/acetate-EDTA buffer at pH 7.4 at 37°C and immediately pipetted onto a frosted glass microscope slide (Richardson Supply Co., London, UK) precoated with a layer of 1% (wt/vol) Normal Melting Point (NMP) agarose in Tris/acetate-EDTA buffer. The following steps were performed as previously described (Del Bo' et al. 2015). For each sample, two slides were prepared. Cells were lysed by placing the slides in a lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, 1% Triton X-100, 1% DMSO, and 1% N-lauroylsarcosine sodium salt, pH 10) for 1 h at 4°C in the dark. Then, slides were washed three times in a buffer solution (40 nM HEPES, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg ml^{-1} bovine serum albumin, pH 8) and successively, incubated for 45 min at 37°C with buffer containing FPG (100 ng ml^{-1}). FPG was omitted in control runs. The slides were then placed in an alkaline solution (0.3 M NaOH, 1 mM Na_2EDTA) for 40 min at 4°C in the dark. Electrophoresis was performed at 1.1 V cm^{-1} for 20 min. Slides were successively neutralized (0.4 M Tris/HCl, pH 7.5) for 15 min at 4°C in the dark, stained with ethidium bromide (2 µg ml^{-1}), washed in PBS, drained and coverslipped. One hundred images per slide were electronically captured using an epifluorescence microscope (Olympus CX 41; Olympus Italia) attached to a high-sensitivity CCD video camera (CFW 1808M; Scion Corporation, Germany) coupled to an image analysis system (Cometa 1.5; Immagini e Computer, Bareggio, Milan, Italy). For each sample, the percentage of DNA in the tails of control cells (not treated with FPG) was subtracted from that in tails of the FPG-treated cells.

Genetic analyses

Genomic DNA was extracted according to standard procedures (Miller et al. 1988). Coding exons and intron-exon boundaries of the *SOD1* gene, exons 5, 6, 13, 14 and 15 of the *FUS* gene, exon 6 of the *TARDBP* gene, an amplicon containing both rs429358 (C112R) and rs7412 (R158C) from the

APOE gene, one including rs854560 (L55M) and another with rs662 (R192Q) from the *PON1* gene were amplified through PCR (Veriti™ Thermal Cycler, Applied Biosystems™, Waltham, MA, USA), sequenced (Big-Dye Terminator sequencing kit 1.1 or 3.1, Applied Biosystems™, Waltham, MA, USA) and run on an ABIPrism 3730 genetic analyzer. An amplicon containing rs7493 (S311C) from the *PON2* gene was PCR amplified and digested with *DdeI* enzyme; the presence of the polymorphism abolishes a cut site for the restriction enzyme. The product of digestion reaction was loaded on a 4 % agarose gel and run at 70 V for 3 hours. In the presence of the polymorphism only two bands (207 bp + 33 bp) were present, whereas the wild type sequence was cut in two sites, yielding three bands (140 bp + 67 bp + 33 bp).

The possible presence of the G_4C_2 hexanucleotide repeat expansion in the first intron of *C9ORF72* was evaluated with fragment length analysis. This technique consists in a PCR performed with two primers, flanking the region where the G_4C_2 expansion is present. One of the two primers is labeled with the fluorophore FAM (6-carboxy-fluorescein) at the 5' end. If the pathological expansion is present (> 30 repeats), the amplification will not be possible, thus only one peak, corresponding to the wild type allele would be visible after capillary electrophoresis (Figure 10). For negative subjects two peaks will be present and the length of the non-pathological expansion (< 30 repeats) can be calculated with the formula: [(length of the amplicon (bp) – 129)/6]+2 (129 bp is the size of the amplicon containing two hexanucleotide repeats) (DeJesus-Hernandez et al. 2011). Since none of the subjects presented the expansion, repeated primer PCR (RP-PCR), able to determine the length of the pathological expansion, was not performed.

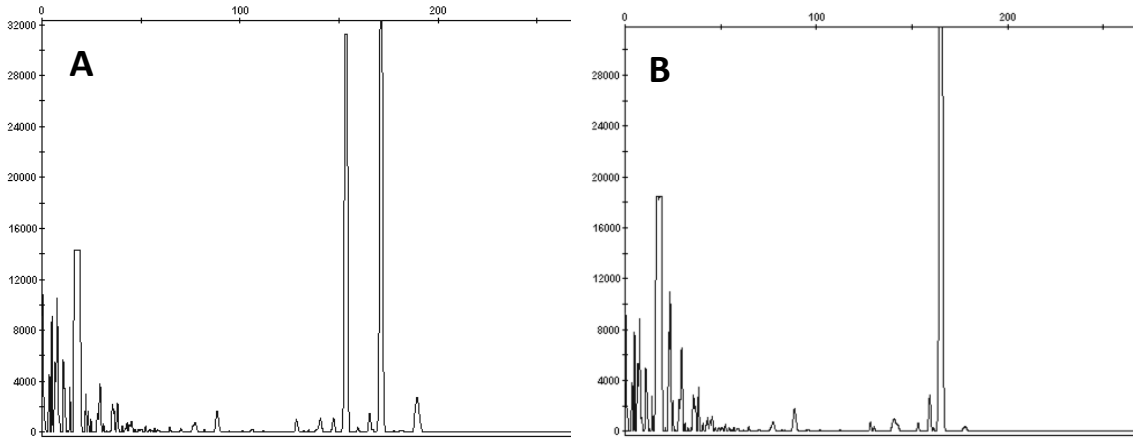


Fig. 10. Results of electrophoretic runs obtained through fragment length analysis: A) Heterozygous subject with two alleles within the normal range of expansion. B) Homozygous subject with an expansion within the normal range or heterozygous subject bearing a wild type allele and an expanded allele (not detectable).

Statistical analyses

Classical statistics:

Student's t-test was applied to identify differences in the analytical data between patients and controls. Correlations between the variables and clinical data, such as disease duration, age at blood sampling and age at onset, were evaluated with Pearson's and Spearman's correlation coefficient and linear regression, performed using IBM SPSS Statistics Base 23 and Analyse-it software, version 4.65.2.

Artificial Neural Networks:

Artificial neural network (ANN) analyses shift the paradigm of data analysis from forcing data into a pre-constituted scheme, into a bottom-up definition of the regulating processes, generated on the basis of the relations between data. This approach has been developed to analyze a complex system, that adapts itself at the environment during time. Thus, in this context, time is not a noise, but an evolutionary parameter. Health and diseases processes are based on complex networks of interacting elements (from genes to environment), that are the consequence of their regulatory dynamic processes (Figure 11). However, huge amount of data per subject hampered statistical tests. With this background it is evident that this approach applies perfectly to ALS. Indeed, it is really important to manage the complexity of this disease, from a statistical point of view, with tools able to handle this complexity of relations and data, rather than treating them with reductionist approaches, unable to disclose the hidden interactions that are supposed to predispose to the disease (Buscema et al. 2012).

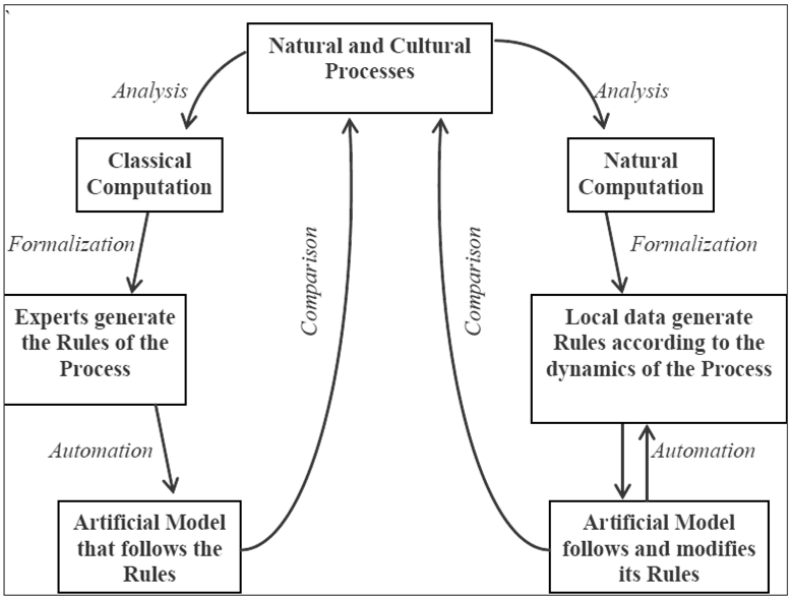


Fig. 11. Comparison between classical computation approach and bottom-up approach (Capecchi et al. 2010).

In this work, Auto Contractive Map (Auto-CM, based on ANN architecture) was used. This method allows to visualize the correlations among the variables by constructing a space where closeness reflects accurately their association. Indeed, closeness is visualized in a graph that

highlights only the relevant correlations and presents a global picture of the whole pattern of associations.

The Auto-CM software is organized with a three-layer architecture (Figure 12). Each layer contains an equal number of units, corresponding to the variables loaded by the user. In the input layer, the signal is captured from the environment, and in the hidden layer it is modulated inside the software. Through the output layer, Auto-CM feeds back on the basis of the *stimuli* received and processed. Connections between the input and the hidden layer are mono-dedicated, while those between the hidden layer and the output layer are at maximum gradient.

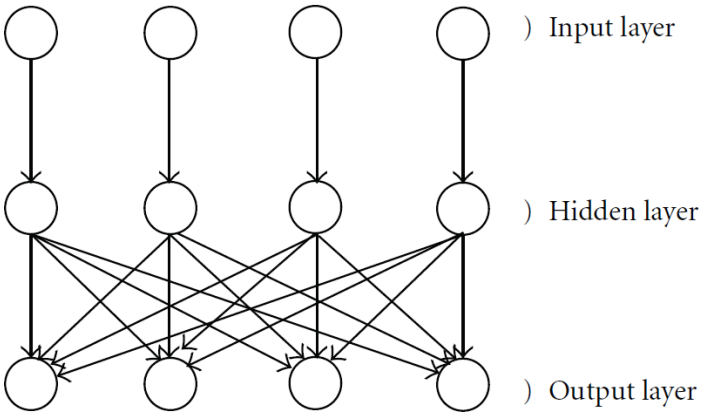


Fig. 12. An example of Auto-CM with four units (Buscema et al. 2012).

The learning algorithm of the software may be summarized in two transfer steps (from input to hidden layer and from hidden to output layer), and two steps where adaptation of the values of the connections between the layers occurs. In conclusion, the matrix of the weights of each variable is transformed into a matrix of distances among the nodes in the final output graph. Generally speaking, a graph is a mathematical abstraction consisting of a set of vertices (V), and a set of edges (E), connecting two vertices in the graph, to which it is possible to associate scalar values (in this case, the distance). The graph represented in the output of Auto-CM elaboration is the Minimum Spanning Tree (MST). It represents the energy minimization state of a structure, thus, in this situation, the most probable state that the system tends to assume (Buscema et al. 2012). Indeed, it is what we could call the framework of any dataset. In the building of the MST only the connections that are really necessary to keep the system coherent are selected. Consequently, all the links included in the MST are fundamental, but, on the contrary, not every “fundamental” link of the dataset needs to be in it, since every link that gives rise to a cycle is eliminated. Consequently, to better capture the intrinsic complexity of a dataset, it is necessary to add more links to the MST by creating a Maximally Regular Graph (MRG). The MRG adds to the MST the most important relationships between the variables, to identify the graph with the maximum number of regular micro-structures inside the system, by adding back to the original MST, one by one, the missing connections previously skipped during the computation of the MST itself (Buscema et al. 2012).

RESULTS AND DISCUSSION

POPULATION SELECTION

The study involved 7 patients (4 men and 3 women) and 5 controls (2 men and 3 women, age-matched with the patients – 68.7 ±11.9 years vs. 63.7 ± 6.3 years) all living in the same geographical area (Table 2 and 3). Among the 12 subjects, 8 were born and lived in the reference area, one patient moved there in 1970, another in 1988; one control moved in at the age of 12. During the study one patient reported to have had the onset of the disease four years before moving in the geographical area of interest. However, at the moment of the blood sampling he was living in the area for seven years. Analyses already performed were reconsidered in the light of this new information, but results did not change.

All patients were diagnosed with clinically defined sporadic ALS; in six of them onset was spinal, in one bulbar. The mean age at onset was 61.3 ± 7.8 years; the mean duration of disease, from onset to the time of blood collection, was 7.8 years (range = 2.1-19.1 years). All the patients are currently treated with Riluzole; two of them underwent PEG.

All subjects enrolled in the study signed an informed consent, according to the guidelines of the Niguarda Ca' Granda Hospital ethics committee. Simultaneously to the blood sampling and informed consent signing, all subjects underwent an interview concerning their eating habits, including the propensity at drinking bottled *vs* tap water.

ID	*Gender*	*Age at onset (y)*	*Site at onset*	*Duration of disease (y)*	*PEG*	*BMI at onset (m^2Kg^{-1})*	*Occupation*	*Smoke*
1 SU	M	48	LL	2.1	N	29.4	Shopkeeper	N
2 SU	M	57	Bulbar	3.0	Y	23.0	Manager	Y
3 SU	M	65	UL	3.2	N	28.1	Workman	N
4 SU	F	60	LL	19.1	N	22.9	Seamstress	N
5 SU	F	71	LL	12.1	N	27.9	Farmer	N
6 SU	M	69	LL	11.0	N	23.7	Manager	N
7 SU	F	59	LL	4.2	Y	29.3	Teacher	EX

Table 2: Minimum clinical data for ALS patients. LL: lower limbs, UL: upper limbs. Duration of disease is calculated as time passed from symptoms onset to blood sampling or PEG start.

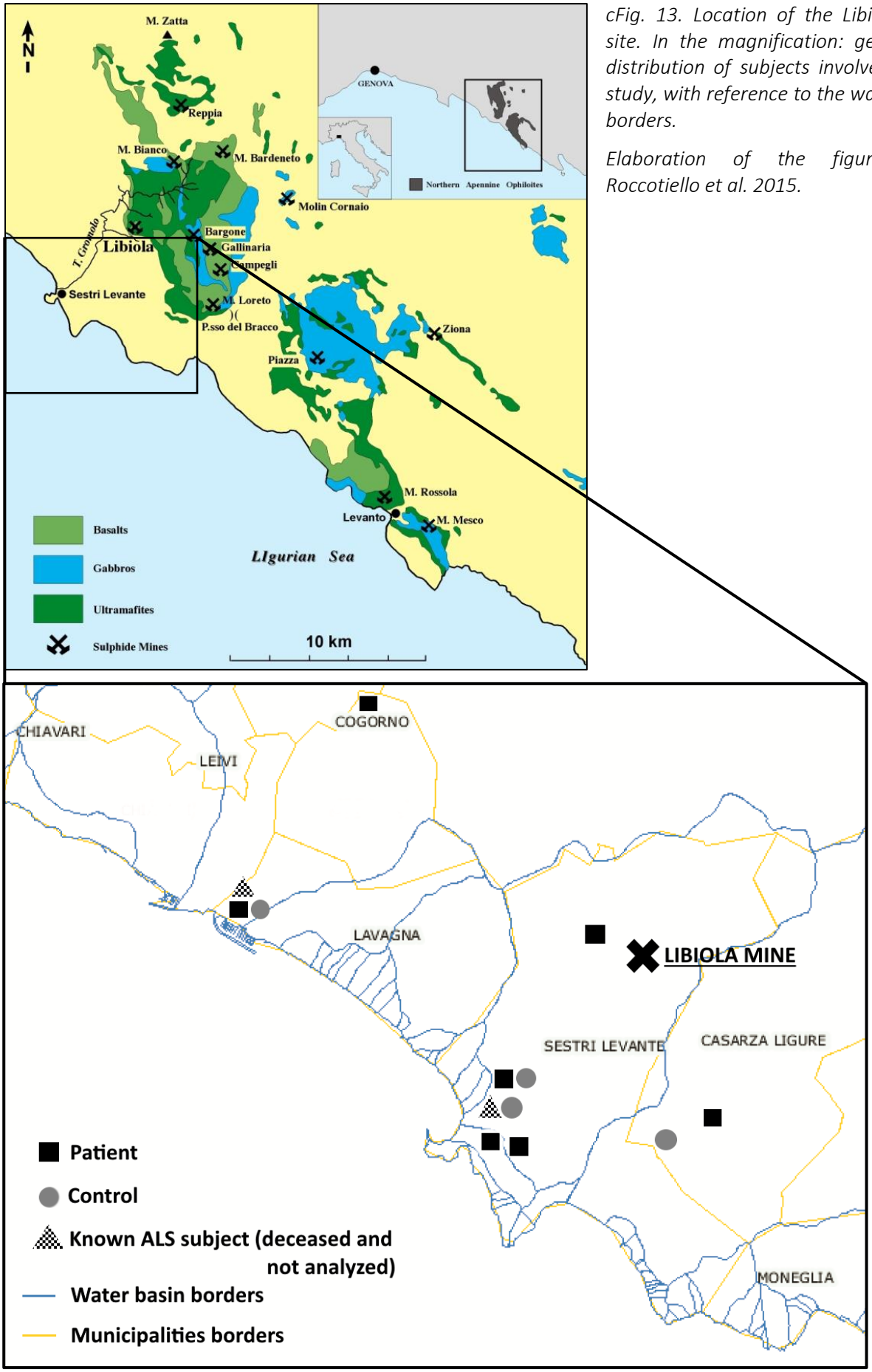
ID	Gender	BMI (m^2Kg^{-1})	Occupation	Smoke
244 N	F	20.8	Odontologist	N
245 N	F	22.3	Teacher	Y
246 N	F	25.7	Housewife	EX
247 N	M	28.1	Professor	N
248 N	M	31.1	Manager	EX

Table 3: Data for control subjects.

Geographical area overview

The study area includes four municipalities – Casarza, Lavagna, Cogorno and Sestri Levante – with a total population of about 36,000 individuals over an area of 83.5 km^2. It is located between two narrow mountain valleys and the Mediterranean seaside, in the Genoa district of the Liguria region in northwestern Italy. ALS point prevalence in the Liguria region is 7.85/100,000 (95% CI 6.54–9.36), (Scialò et al. 2016), higher than what reported for the general Italian population (Chiò et al. 2013). Authors of this study suggest that this occurrence could be related either to a better case ascertainment in a relatively small area (5420 km^2 with about 1,600,000 inhabitants) and/or to the demographic structure of Liguria, where about 28% of the population is over 65 years of age. Only on the basis of point prevalence reported for the Liguria region, in the area of interest we would expect to find 2.83 ALS cases, however we are aware of a markedly higher number of affected subjects.

In the same area is documented the presence of the Libiola Fe-Cu sulfide mine, exploited until 1962. During its activity the Libiola mine produced over 1 Mt of Fe-Cu sulfides (Marescotti et al. 2012). The area of the mine lies within the drainage basin of the Gromolo Creek and two of its main tributaries (Figure 13), collecting mine waters. The whole mining area is subjected to intense Acid Mine Drainage (AMD) processes, involving the five major waste rock and tailings dumps and the remaining exposed unmined portions of the ore veins (Carbone et al. 2005a; Carbone et al. 2005b). This phenomenon leads to the acidification of the circulating waters, that are reported to have a pH < 3. This acidity of streams circulating in the mine galleries and within the rock deposits, leads to the alteration of the mineral assemblage of the rocks itself. For this reason, several studies report a very bad quality of the waters flowing from the mine, with very high levels of potential toxic elements (Fe, Cu, Zn, Ni, As, Cr, V, Ti, Al) (Dinelli et al. 2001; Marini et al. 2003). Most of the metals detected in the mine tailings and in ore-accompanying rocks, strongly exceed the limits for industrial and residential sites imposed by the Italian law (Legislative Decree n. 152, 3 Aprile 2006).



cFig. 13. Location of the Libiola mine site. In the magnification: geographic distribution of subjects involved in the study, with reference to the water basin borders.

Elaboration of the figure from Roccotiello et al. 2015.

NUTRITION SURVEY

An exploratory questionnaire was submitted to the subjects involved in the study. All of them were instructed as for filling the survey. In particular, ALS patients were asked to recall information on nutrition habits in the time period before the onset of disease. Information about occupation, smoking habits, body mass index (BMI) and use of tap *vs* bottled water to drinking purposes were also collected.

The section regarding nutrition took account of the frequency intake of several foods: cookies, croissant, rusks, bread, crackers, pasta or rice, red meat, white meat, legumes, potatoes, fish, egg, cold cuts, fresh cheese, ripened cheese, vegetables, fruits, sweets, pizza (Table 4). The aim of the survey was, besides collecting information, to assess if there were differences in the diet of ALS subjects and healthy controls and/or provide information as for whether the consumption of one or more foods were associated to the disease status.

Auto-CM software was used to analyze the results. There were no evident differences in the nutrition habits of ALS subjects with respect to healthy controls. The MST built according to the answers reported in the questionnaire does not show any clustering of patients or controls (Figure 14 A). This leads to concluding that there is no significant combination of foods able to discriminate between the two groups, since all subjects represented in the output graph are mixed. Furthermore, the high complexity of interactions shown in the MRG highlights that the subjects involved in the study are highly interconnected, thus sharing common nutrition habits (Figure 14 B). In light of these results a homogeneous exogenous exposure originating from food consumption can be assumed for all the subjects involved in this study.

Despite the absence of differences able to discriminate the two groups, an elaboration with Auto-CM was performed, in order to evaluate which variables were more strongly associated with the disease status or health status. Variables best describing the disease status were: fruit, vegetables, white meat consumption and a lower BMI, whereas sweets, bread and croissant consumption together with a higher BMI were associated to the control group (Figure 15).

SUBJECT ID	BISCUITS	CROISSANT	RUSKS	BREAD	CRACKERS	PASTA/ RICE	RED MEAT	WHITE MEAT	LEGUMES	POTATOES	FISH	EGG	COLD CUTS	FRESH CHEESE	RIPENED CHEESE	VEGETABLE	FRUITS	SWEETS	PIZZA	TAP WATER	BOTTLED WATER
1 SU	1	0	0	1	0	0.67	0.33	0.33	0	0.33	0.33	0.33	0.67	0.33	0.33	1	1	0.33	0.33	Y	N
2 SU	0	0	0.67	1	0	1	0.67	0.67	0.33	0	0.33	0.33	0	0.33	0.33	0.67	1	0.33	0.33	Y	N
3 SU	0	0.33	0	1	0	1	0.33	0.67	0.33	0.67	0.33	0.33	1	1	1	0.67	0.67	1	0.33	Y	Y
4 SU	1	0	1	1	0	1	0	0.67	0.33	0.33	0.33	0.33	0.67	1	0	0.67	1	0.33	0.33	Y	N
5 SU	0.33	0	0	0.67	0.67	0.67	0.33	0.67	0.67	0.67	0.67	0.33	0.33	0.67	0.33	0.67	1	0.67	0.67	N	Y
6 SU	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1	N	Y
7 SU	0.33	0	0.33	1	0	1	0.67	0.67	0.33	0.33	0.33	0.67	0.67	1	1	0.67	1	0.67	0.33	Y	N
244 N	0	0	0.33	0.67	0.33	0.67	0.33	0.67	0.33	0	0.33	0.33	0.33	0.33	0.67	1	1	0.33	0	Y	N
245 N	0.67	0	0.67	0.67	0	0.67	0	0.33	0.33	0.33	0.33	0.33	0	0.33	0.33	0.67	0.33	0.67	0.33	Y	N
246 N	0.67	0.67	0.33	1	0	0.67	0.33	0.67	0.67	0.33	0.33	0.33	0.33	0	0.33	0.67	0.67	1	0.67	N	Y
247 N	0	0	0	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	0	Y	Y
248 N	0.67	0.33	0	1	0	0.67	0.67	0.33	0	0.33	0.33	0	0.33	0.67	0	0.33	0.33	0	0.33	N	Y

Table 4: Food frequency intake as resulted from the survey administered to subjects involved in the study. 0: never, 0.33: once a week, 0.67: two or three times a week, 1: every day. SU: ALS patients, N: healthy controls. Y: yes, N: no.

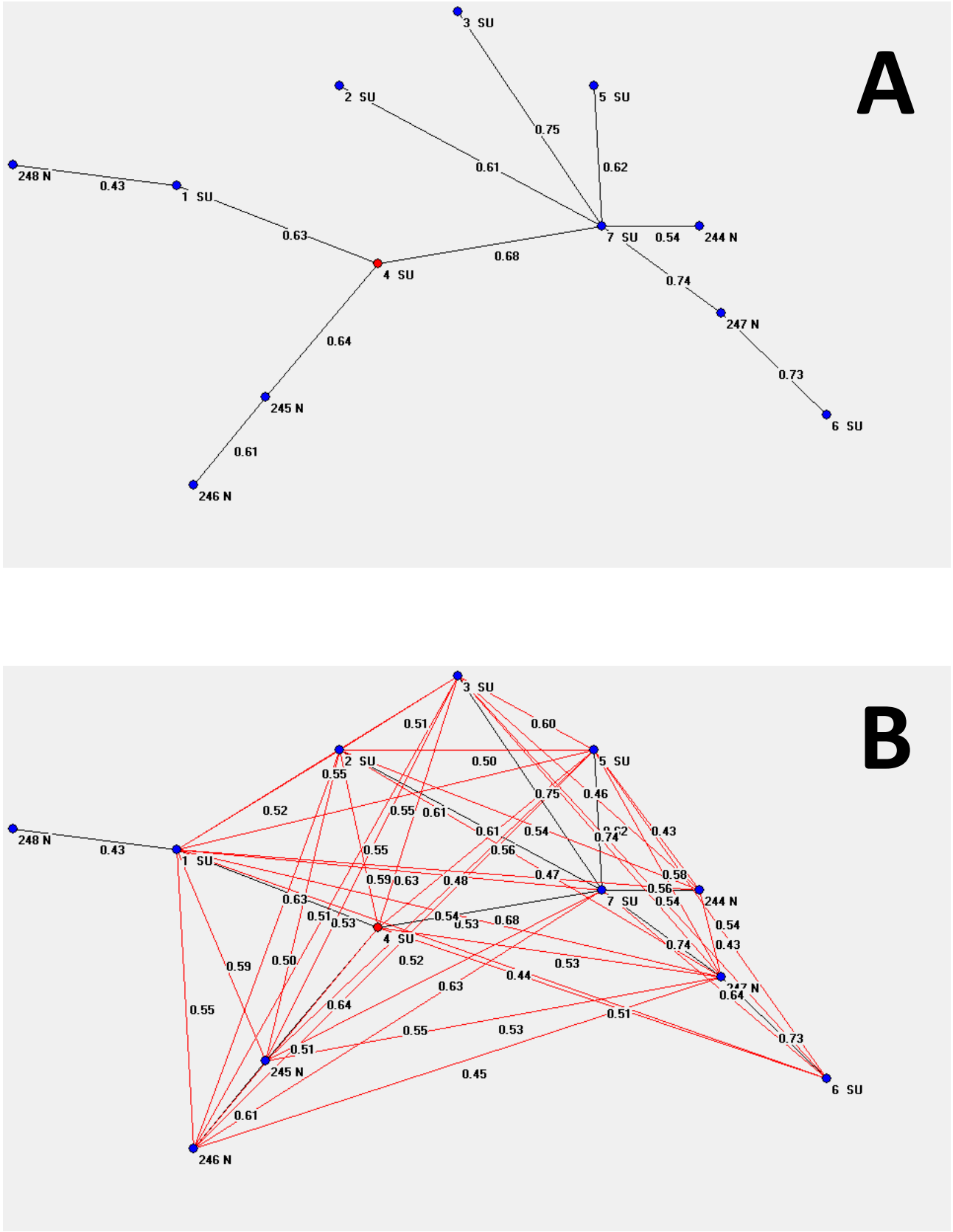


Fig. 14. Graphs showing the relations between patients and controls on the basis of food frequency consumption. Each node represents a subject (SU= ALS patient; N=healty control). Values on the arches of the graph refer to the strength of the association between two adjacent nodes. The range of this value is between 0 and 1. A) MST; B) MRG.

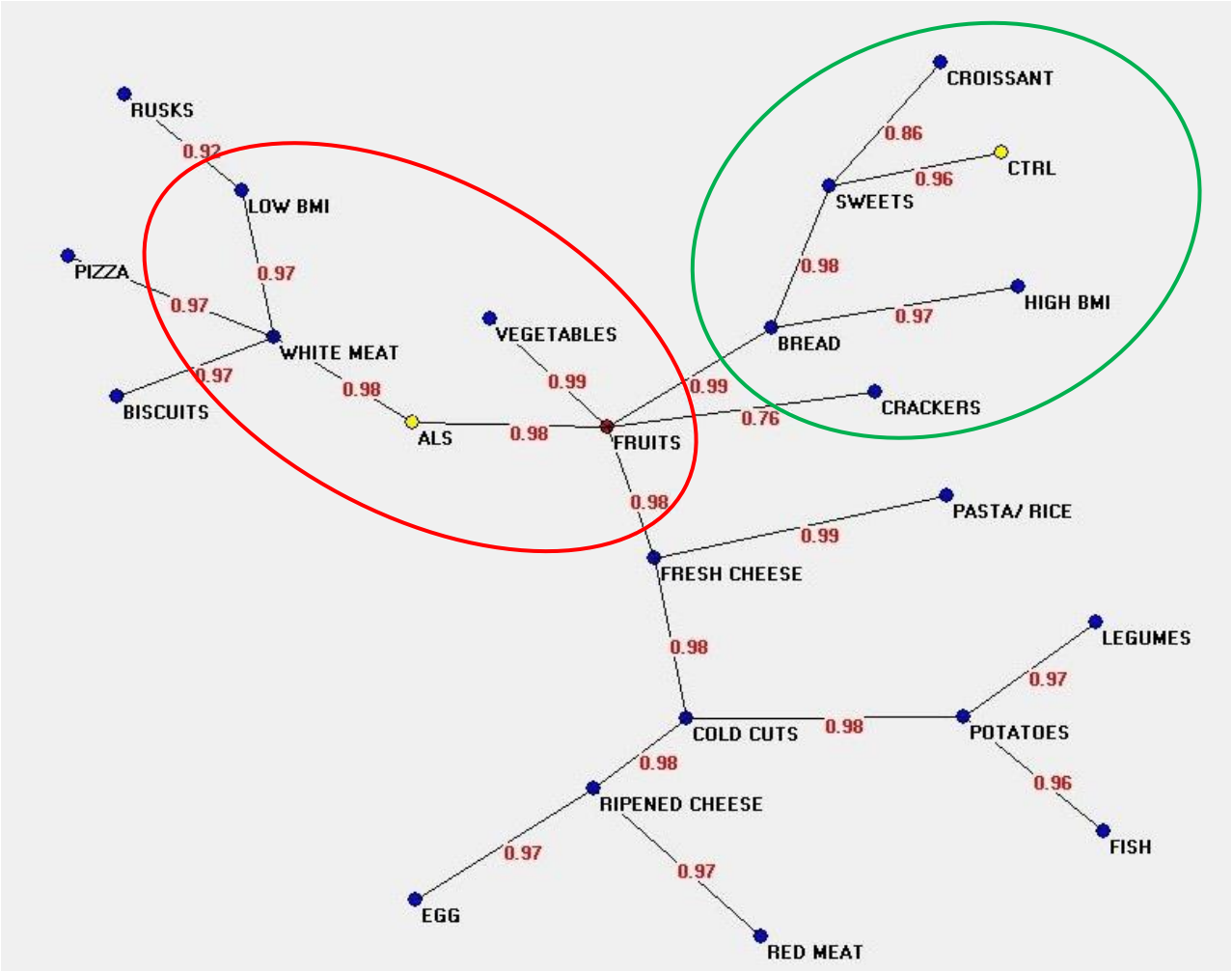


Fig. 15. Semantic connectivity map obtained with Auto-CM System. The Minimum Spanning Tree (MST) shows the connections between the variables obtained with the food frequency questionnaire. Values on the arches of the graph refer to the strength of the association between two adjacent nodes. The range of this value is between 0 and 1. Red circle identifies variables associated to the ALS group, green circle identifies those variables associated to controls.

Far from being conclusive, these results suggest that a higher BMI and more caloric dietary habits seem to be associated to health status with respect to ALS subjects. On the contrary a leaner physical shape and "healthier" nutrition habits seem to be associated with ALS. Some authors have described already that overweight and obese subjects show a reduced risk to develop ALS (O'Reilly et al. 2013), thus increased fat and cholesterol intake may represent a protective factor (Paganoni and Wills 2013). Conversely, high levels of physical fitness, thus lower BMI, have been associated to a lower survival in ALS (Bouteloup et al. 2009; Huisman et al. 2013). These indications have to be considered more as suggestions than conclusions, but results are encouraging further elaboration through ANNs.

Furthermore, while pointing out that studies on the quality of public drinking waters and soils were not performed in this study and the origin of fruits and vegetables in the subjects' diet was not investigated, it should be highlighted that a local origin of these food cannot be excluded. Also, locally sourced water could have been likely used for irrigation of crops and gardens and for washing fruits and vegetables prior to consumption. As already mentioned, several reviews and meta-analyses associated pesticides exposure to increased ALS risk (Sutedja et al. 2009; Kamel et al. 2012; Malek et al. 2012; Capozzella et al. 2014). Exposure to pesticides has been reported to occur not only in professional workers (Bozzoni et al. 2016) and could potentially represent the link between fruits and vegetables consumption and ALS, in this small group of subjects.

METALLOMICS

The determination of metal concentrations in serum firstly focused on a panel composed by the most relevant metals and trace elements, stemming from the reports of Roos and colleagues (Roos et al. 2006 and 2013). The first analysis carried out in our study evaluated concentrations of chromium (Cr), iron (Fe), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), cadmium (Cd) and lead (Pb). In a second time, the determination of manganese (Mn), aluminum (Al), mercury (Hg), tin (Sn), cobalt (Co), uranium (U), vanadium (V) and barium (Ba) concentrations was performed. Most of these elements were reported to be in high concentrations in the mining area waters. These determinations took place separately, since the concentrations of the elements evaluated in each batch of analyses differ of several order of magnitude, so it would have been technically impossible to perform all these analyses together. Table 5 reports the values of metals measured in serum of patients and controls.

Results show elevated levels of Mn, Al, Ni, Cr, Ba and V both in controls and in patients of our study groups in comparison with published reference values for the Italian population (Alimonti A, Bocca B, Mattei D 2010). In serum, only the As concentration was significantly different between the two groups, with As at lower levels in patients ($p < 0.01$). Mn levels also were lower in patients than in controls, but the difference did not reach statistical significance ($p = 0.10$). Conversely, Al and Se had higher concentrations in patients than in controls but, again, the difference failed to reach statistical significance.

Element	*Average Patients ± SD (µg/L)*	*Average Controls ± SD (µg/L)*	*p-value*	*Reference Values (µg/L)*
As	0.34 ± 0.03	0.44 ± 0.07	< 0.01	NA
Mn	1.33 ± 0.69	2.36 ± 1.12[a]	0.10	0.31-1.02
Se	100 ± 12	90 ± 8	0.12	56-105
Al	23.2 ± 5.4	17.2 ± 6.9	0.13	0.4-5.3
Hg	0.90 ± 0.80	1.62 ± 0.90	0.20	0.32-2.75
Pb	1.09 ± 0.41	0.83 ± 0.40	0.29	0.20-0.98
Ni	2.82 ± 0.35	3.00 ± 0.42	0.45	0.26-0.75
Sn	0.14 ± 0.03	0.17 ± 0.07	0.45	0.27-1.69
Co	0.49 ± 0.09	0.51 ± 0.03	0.65	0.06-0.42
Sr	38.4 ± 19.7	34.5 ± 7.2	0.69	23.0-61.5
U	0.03 ± 0.01	0.02 ± 0.01	0.75	NA
V	0.96 ± 0.11	0.94 ± 0.12	0.79	0.0-0.11
Fe	1,165 ± 521	1,225 ± 202	0.81	886-2,455
Ba	13.26 ± 4.87	12.75 ± 2.14	0.83	0.32-1.37
Cr	1.56 ± 0.18	1.54 ± 0.08	0.84	0.07-0.28
Zn	846 ± 151	835 ± 105	0.90	597-1,028
Cu	1,140 ± 216	1,142 ± 136	0.99	648-1,301

Table 5. Averages ± Standard Deviation of the measures of metals concentrations in serum. NA: Not Available. Reference values from ISTISAN (2010) (Alimonti A, Bocca B, Mattei D 2010). [a]One subject from controls not analyzed.

AutoCM was able to discriminate the group of patients from the control subjects on the basis of the concentration of metals in serum (Figure 16). Low concentrations of As had the strongest association with the ALS group. Overall, high concentrations of metals, in particular Se, Al and, with a lower closeness, Mn, Sn, Ni, Co and V were linked to the group of patients. Control group was best described by high concentrations of As and low concentrations of metals, among which Se and Sr were associated with controls more strongly. While analyzing our eating habit questionnaires, we also noticed that patients have higher propensity at drinking tap than controls (71% vs 60%). The Auto-CM software clusters this variable close to the disease status, with a stronger association with serum levels of As and Se.

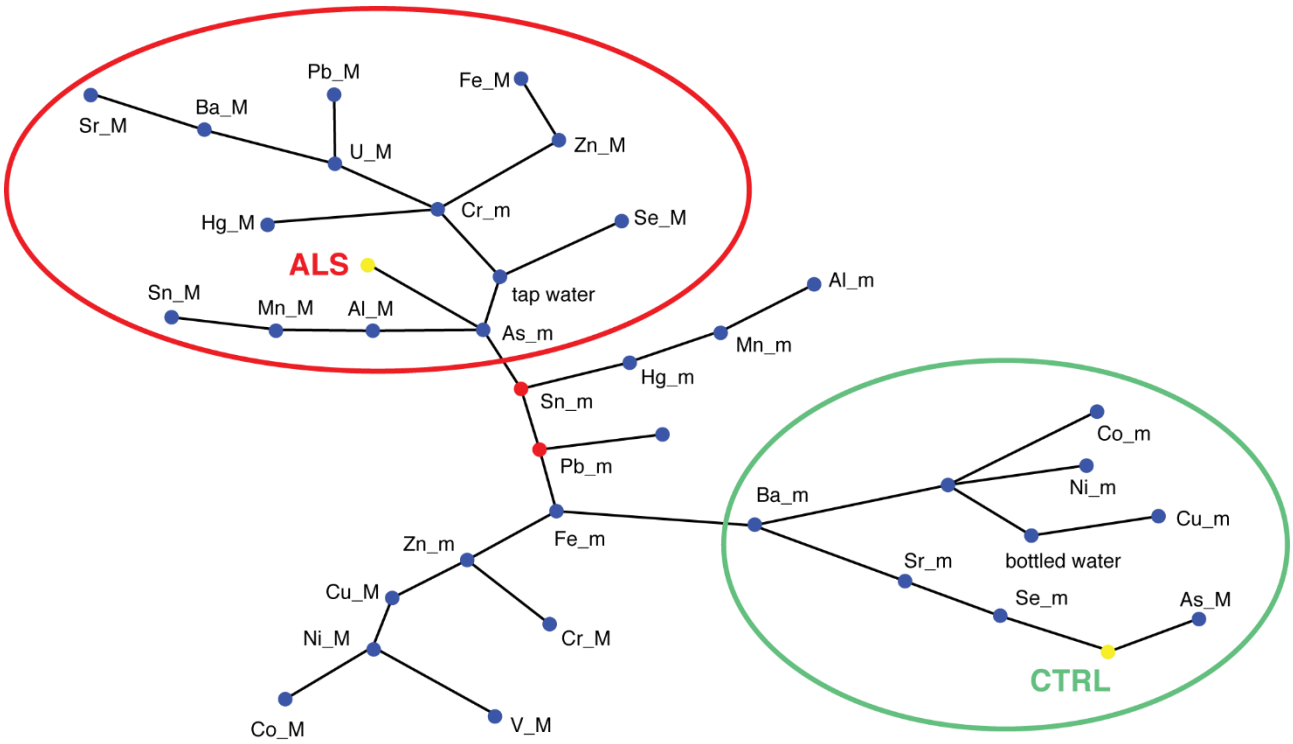


Fig. 16. Semantic connectivity map obtained with Auto-CM System. The Minimum Spanning Tree (MST) shows the connections among metals concentrations and water consumption. M: high levels of the metals; m: low levels of the metals. Strenght of the connections is always > 0.96.

Possible correlations between metal concentrations in serum and available clinical data were also evaluated. Again, the most interesting result regarded As, since its concentration in serum is in direct, linear correlation with the duration of disease, calculated as time passed from the onset of symptoms to blood sampling or PEG start (Pearson's $\rho = 0{,}974$; $p < 0.01$ – Spearman's $\rho_s = 0.857$; $p = 0.01$) (Figure 17 A). Furthermore, the increase in serum concentrations of As seems to be time dependent, independently of the disease status, and proceeds with the same rate both in patients and in controls (Figure 17 B). Se concentration, instead, was inversely correlated with age at onset (Pearson's $\rho = -0.804$; $p = 0.03$) (Figure 17 C) and - in a non-linear way - with disease duration (Spearman's $\rho_s = -0.821$; $p = 0.02$) In control subjects, Al (Figure 17 D) and Mn concentrations positively correlated with age ($R^2 = 0.925$ and $R^2 = 0.793$ respectively), but no similar correlation was detected in patients.

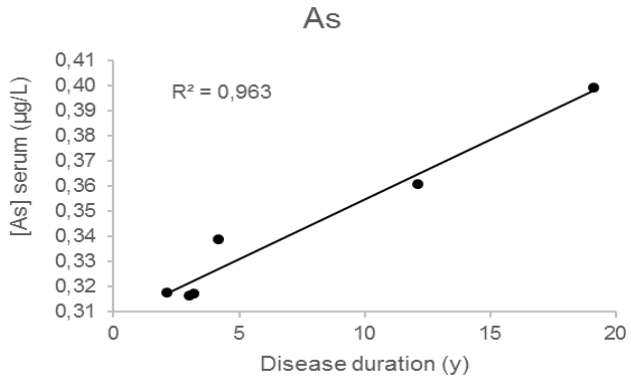


Fig. 17 A. Correlation of serum As concentration with disease duration

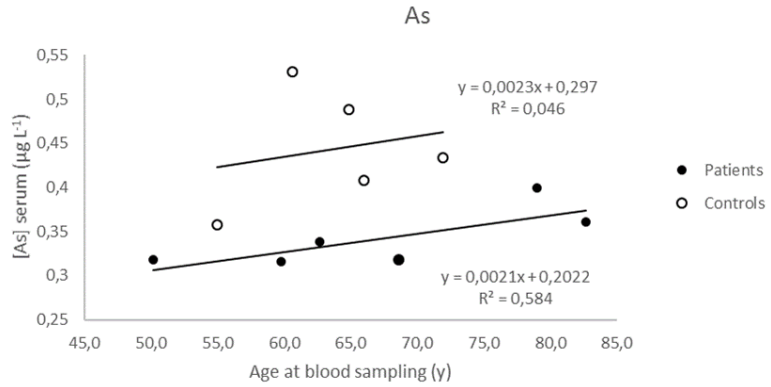


Fig. 17 B. Correlation of serum As concentration with age in patients and controls

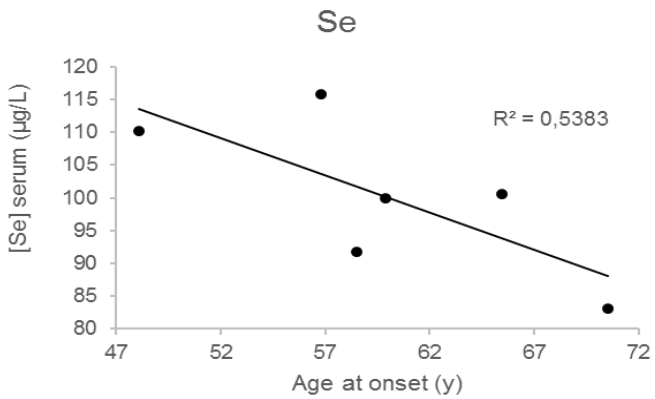


Fig. 17 C. Correlation of serum Se concentration with age at onset

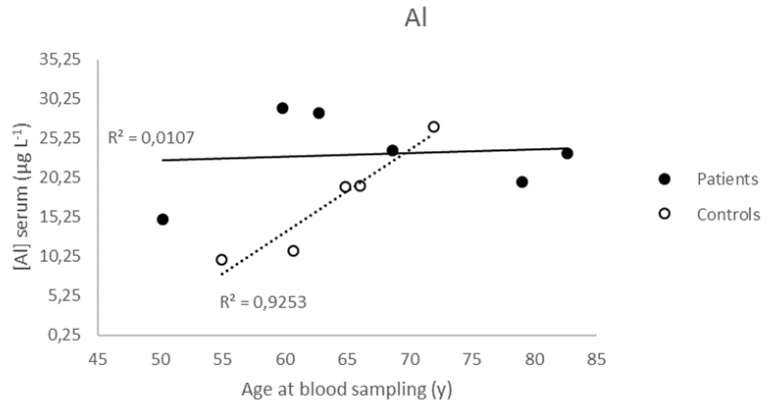


Fig. 17 D. Correlation of serum Al concentration with age in patients and controls

The most striking data in our study come from the association of lower levels of As with the disease status. This is at contrast with other reports analyzing metals concentration in body fluids of ALS patients, where generally an overload was described. In light of this result, was formulated the hypothesis that As could be sequestered somewhere and not released in circulation, thus altering its clearance.

The concentration of a smaller panel of metals, among those evaluated in serum, was evaluated in whole blood samples, to investigate a possible accumulation of trace element in blood cellular components. Table 6 reports the results of concentrations of metals in whole blood. Levels of Mn, Zn, Hg, Co and Cr were found higher both in patients and controls in comparison with reference values for the Italian population, Pb was higher than reference values only in the patients' group, but none of the metals analyzed presented significant differences between patients and controls. As and Cr concentrations in the whole blood of patients positively correlated with disease duration (for As: Pearson's $\rho = 0.847$; $p = 0.02$; Spearman's $\rho_s = 0.750$; $p = 0.05$; for Cr: Pearson's $\rho = 0.800$; $p = 0.03$ - Spearman's $\rho_s = 0.964$; $p < 0.01$).

Element	*Average Patients ± SD (µg/L)*	*Average Controls ± SD (µg/L)*	*p-value*	*Reference Values (µg/L)*
Co	2.00 ± 0.45	2.39 ± 0.39	0.16	0.03-0.24
Pb	94.0 ± 46.0	60.6 ± 25.1	0.18	12.8-79.5
Cr	19.03 ± 4.68	21.56 ± 2.07	0.29	0.12-1.07
Se	110 ± 18	121 ± 17	0.30	76-140
Zn	10,134 ± 999	9,492 ± 1,112	0.33	5,189-8,337
Mn	17.26 ± 3.39	18.54 ± 1.15	0.44	1.53-13.20
As	8.97 ± 6.90	10.70 ± 4.32	0.65	0.40-11.90
Hg	17.71 ± 6.81	18.61 ± 3.58	0.79	1.97-14.50
Cu	1,032 ± 157	1,031 ± 76	0.99	686-1,157

Table 6. Averages ± Standard Deviation of the measures of metals concentrations in whole blood. Reference values from ISTISAN (2010) (Alimonti A, Bocca B, Mattei D 2010).

Arsenic concentration, despite being lower in patients than in controls, was not statistically different. However, As concentrations in whole blood and serum show a good linear correlation (Figure 18). This could lead to the hypothesis that As species in serum are in equilibrium with those in whole blood. Thus, if an accumulation of this element occurs, it should be in an extra-circulatory district.

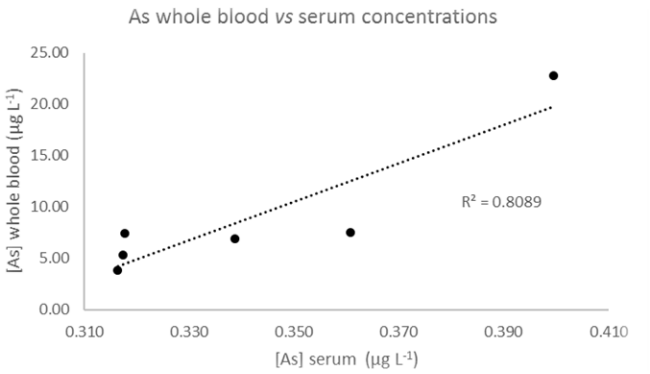


Fig. 18. Correlation between serum and whole blood As concentrations

To evaluate the relationships between blood and serum concentrations of metals, the ratio among the values measured in each matrix for each element was calculated. The most interesting result was related to Hg (Figure 19). It is evident the low variability in the concentrations' ratio among control subjects, at contrast with what observed for ALS patients.

The high ratio values are caused by a lower concentration of Hg in serum, whereas concentrations in whole blood are similar between patients and controls. This could be interpreted as a strong deregulation in the clearance of this element, again supporting the hypothesis of an accumulation of this element in an extra-circulatory district.

The common geographical origin among patients and controls allowed to investigate metal concentrations in serum and whole blood under comparable conditions of environmental exposure. A metal overload involving different elements was observed both in serum and whole blood, possibly reflecting the environmental pollution resulting from the presence of the mine. The possible influence of local sourcing of nutrients is circumstantially underscored by the observation that consumption of tap water was associated, through neural network analyses, to the ALS group (Figure 16). Although yet inconclusive, this finding calls for an in-depth investigation on the levels of metals in water in this area, also in consideration of the use of the same water for agricultural and food preparation purposes. Unfortunately, specific and

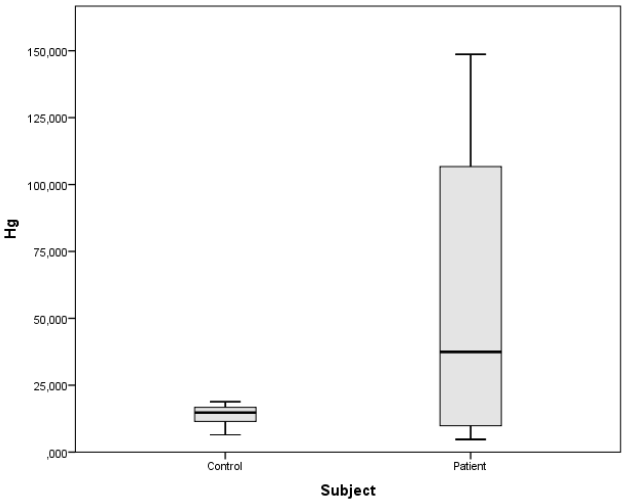


Fig. 19. Blood / serum ratio of Hg concentrations.

credible data on water quality in this area and of its content in metals relevant to the present study are not available yet.

The most relevant difference between patients and control subjects is related to As concentrations, with As being lower in patient's serum than in control's subjects. This result is quite surprising, since a pathological condition could be expected to be connected, if anything, to higher levels of circulating As. However, As levels strongly correlated with the duration of the disease, possibly with connection with the duration of the exposure to the environment. This could support the hypothesis of an accumulation of this element over time leading to a damage only in susceptible subjects. Serum concentration of As is positively correlated to the concentration of this element in whole blood, thus accumulation of As should occur in an extra-circulatory district. In fact, As can easily cross the Blood Brain Barrier; furthermore, peripheral neuropathological symptoms are common in subjects chronically exposed to As-contaminated drinking water (Vahidnia et al. 2007). This point deserves further investigation in this cohort of patients.

Arsenic can form both inorganic and organic compounds in the environment and in the human body, as either arsenite (As^{3+}) or arsenate (As^{5+}). Since absorbed arsenate is mostly reduced to arsenite in blood, the effect of the two forms appears to be very similar (Valko et al. 2005). Many studies confirmed the generation of free radicals during As metabolism in cells, and oxidative stress has been linked to the development of As-related diseases (Shi et al. 2004). On the other hand, As^{3+} induces the transcription of metallothionein-I (MT1) in mouse hepa1c1c7 cells through direct interaction with metal-activated transcription factor 1 (MTF1) (He and Ma 2009), and this could possibly result in a protective effect, in case of non-toxic levels of As overload.

The involvement of Se in ALS has been extensively investigated in a well-defined case series of residents of the Emilia-Romagna region (Vinceti et al. 2013). These Authors found an association of increased ALS risk with higher concentrations of selenite in cerebrospinal fluid (CSF), and of serum albumin-bound selenium. We did not investigate Se speciation in our samples, but concentrations of total Se were higher in the patient's group, with the highest values in subjects with the earliest onset.

Considering other neurotoxic elements, Al was found in higher concentration in ALS patients than in controls, whereas the opposite was true for Mn. In the control group, concentrations in serum of both metals are positively correlated with the age of subjects, possibly reflecting a time-

dependent non-pathological accumulation. This trend was not identified in the patient's group, maybe due to an alteration in the clearance of these two metal species.

The role of the analyzed metals in ALS is controversial and these results represent a starting point for an in-depth investigation on their possible involvement in the pathogenesis and in the course of the disease, as well as in looking for influence of metals with susceptibility disease factors. An impaired metal homeostasis could enhance oxidative stress in cells, leading to the production of toxic radicals, but can also lead to a redox-dependent competition of suitable metals for the binding sites of metal-containing proteins, such as those carrying iron-sulfur clusters and involved in an impressive variety of cellular events (Iametti et al. 1998; Bonomi et al. 1998; De Benedetti et al. 2016). This damaging effect could be exerted by all those metal species having affinity for thiols, whose toxicity in bacteria has been shown to be proportional to the affinity for sulfur (Nies 2003).

The role of iron-sulfur clusters biosynthetic machinery in ALS is still poorly explored. Upregulation of frataxin (Fxn) and of the human orthologue of the cluster-assembly scaffold protein (IscU), two of the proteins involved in carrying out or in regulating FeS biosynthesis, has been explained as a response of the cell to iron overload (Hadzhieva et al. 2014). However, further investigations are required to assess a possible relationship between metal imbalance and the many biosynthetic steps leading to functional metalloproteins, as well as the molecular basis of mid- to long-term effects of metal imbalance on the energy status and the redox balance of neuronal cells. Effects on other proteins involved in metal homeostasis within the cytoplasm or in specific cellular compartments (such as metallothioneins or ferritin (He and Ma 2009; Hashimoto et al. 2011; Hadzhieva et al. 2013)) and on proteins involved in controlling the intracellular redox potential and/or the concentration of active chemical species (such as peroxides and superoxides) also remain to be assessed.

In conclusion, these results suggest that single metal evaluation may underestimate the relevance of the role of metals in health risks. It is more likely that all these metals interact with one another with an additive or even a synergistic effect (Andrade et al. 2015). If nowadays it is possible to analyze simultaneously several trace elements in different tissues, it is still hard to interpret these results as a whole. The elaboration of these data with software based on machine learning processes and artificial neural networks could be extremely useful to gain a comprehensive view of the complex interactions eventually leading to disease. Also, these approaches allow to study necessarily small-sized clusters of patients located in a limited geographical area. This combination

could be of outstanding value to minimize as much as possible the differences among individuals and to investigate further the possible role of environment-related issues in ALS etiology.

If not for a few studies (Malaspina et al. 2002; Vinceti et al. 2013), other reports on metallomics in ALS patients have been performed on wide cohorts of subjects such as US National Registry of Veterans (Peters et al. 2016) or cases selected from the Irish ALS register over a time period of 18 years (Rooney et al. 2016). Although the approaches applied in these studies are extremely valuable due to the high number of subjects (allowing to obtain reliable models and statistical analyses), the environmental exposure is necessarily highly variable among patients and controls.

This work is an attempt to switch the approach to these kinds of studies to a better consideration of the common environmental exposure. The obvious problematic of the lower number of subjects eligible to be involved in these studies could be overcome by using new methods of statistical analysis, based on artificial neural networks. At the same time, studies involving a lower number of subjects allow deeper evaluations - when the aim is to dissect different aspects potentially involved in the disease - of factors that sometimes are impossible to consider together in the same cohort of subjects. In light of these considerations, it would be extremely valuable to perform such studies after the identification and description of localized clusters of ALS patients, as recently reported by Nicoletti et al. (Nicoletti et al. 2016) and previously by Uccelli and colleagues (Uccelli et al. 2007).

COMET ASSAY

The comet assay is a rapid and simple technique for the evaluation of DNA damage in all types of eukaryotic cells and tissues. This assay has been used in various human biomonitoring studies to investigate the effects of exposure to various types of pollutants and environmental contaminants (Kassie et al. 2000; Valverde and Rojas 2009).

Metal compounds have shown to interfere with cellular redox regulation by promoting the formation of reactive oxygen species (ROS) and promoting protein and DNA damage. For example, chromium is documented to interact directly with DNA, forming Cr-DNA adducts, DNA-protein and DNA-DNA crosslinks. Nickel and cadmium showed to inhibit DNA repair enzymes resulting in genomic instability and mutations, while arsenic is reported to alter DNA methylation patterns,

thereby affecting the expression of oncogenes and tumor suppressor genes (Stohs and Bagchi 1995; Beyersmann and Hartwig 2008). Some studies reported increased levels of oxidative damage to DNA in sporadic ALS patients (Fitzmaurice et al. 1996; Bogdanov et al. 2000). Others reported a correlation between high level of DNA damage and DNA lesions, and high levels of metals (Co, Ni, and Pb) in blood and/or urine (Werfel et al. 1998). Some authors hypothesized that the mechanisms underlying the genotoxicity produced by these metals are linked to ROS production (De Boeck et al. 2003; Kasprzak et al. 2003; Botta et al. 2006). Same evidences are reported for Sn (Dantas et al. 1999; Dantas et al. 2002).

The analysis of the levels of FPG-sensitive sites - as markers of endogenous DNA damage - performed on the whole blood did not show any significant difference between patient and control group, since the DNA in tail amounted to 8.3 ± 2.5 % in ALS patients *vs* 8.2 ± 3.1 % in control subjects ($p = 0.94$) (Table 7). Statistical analyses were performed using Pearson's and Spearman's test, and only serum Sn concentration positively correlated with endogenous DNA damage in ALS patients (Pearson's $\rho = 0.845$; $p = 0.03$) (Figure 20), even though Sn concentrations in serum were lower in these subjects than the reference values for the Italian population. No correlation was evident between percentage of damaged DNA and clinical parameter such as age at onset and time from disease onset.

Patients	***EB***	***FPG***	***% DNA in tail***	***Controls***	***EB***	***FPG***	***% DNA in tail***
1 SU	37.1	44.4	7.3	**244 N**	48.5	55.3	6.8
2 SU	40.8	46.5	5.7	**245 N**	63.0	67.0	4.0
3 SU	38.9	46.7	7.8	**246 N**	65.1	77.4	12.3
4 SU	50.4	59.2	8.8	**247 N**	75.4	84.3	8.9
5 SU	38.2	44.3	6.1	**248 N**	66.1	74.9	8.8
6 SU	48.6	61.8	13.2				
7 SU	33.1	42.3	9.2				
		Mean	**8.3**			***Mean***	**8.2**
		SD	**2.5**			***SD***	**3.1**

Table 7: Results of the comet assay. EB: control sample; FPG: sample treated with FPG enzyme.

In the present study, the levels of endogenous DNA damage did not differ between ALS and control group, possibly since both the groups of subjects showed overall comparable levels of metals in their blood. Moreover, the levels of DNA damage obtained in the present study were low and in line with those generally obtained in our laboratories in apparently healthy subjects or in subjects with CVD risk.

ALS indeed is a tissue-specific disease, affecting selectively the motor neurons, and the relevant oxidative insults, possibly responsible for the disease, are only those – if any – involving motor neurons. Comet assay performed at low doses of H_2O_2 in NSC34 cells, a line of immortalized motor neuronal cells, revealed that overexpression of either wild type SOD1 and G93A-SOD1 mutant resulted in protection from free radical damage in nuclei (Sau et al. 2007). However, results reported in this study, were obtained in PBMCs. Hence, the evidence is not conclusive about the connection between ALS and DNA oxidative stress. Indeed, a recent work reports that an altered intracellular localization of SOD1 in leukocytes from patients with SALS (Cereda et al. 2013), could lead to a higher peripheral protection in subjects affected by the disease, with effective contrast of the oxidative DNA damage down to physiological levels.

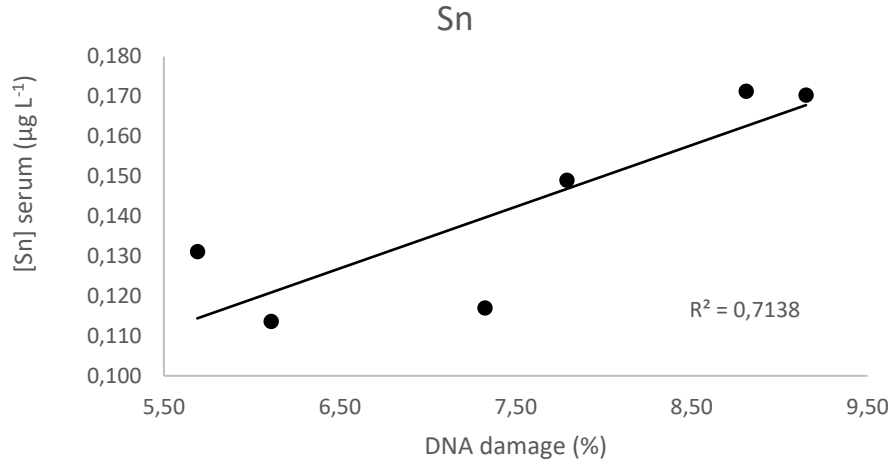


Fig 20: Correlation of Sn serum concentration with % of DNA damage in ALS patients.

PROTEOMICS

2DE analyses of the serum proteome in both controls and patients have been carried out under reducing as well as under non-reducing conditions. Table 8 lists the results for those proteins in which a significant difference in volume was observed under reducing conditions in one or more of their spots, corresponding to one or more of their PTM-species (Jungblut et al. 2008) or of their proteolytic fragments, in a patients vs controls comparison.

Lower levels of apolipoprotein A-I (APOA1), apolipoprotein A-II (APOA2) and transthyretin (TTR) were found in patients, whereas antithrombin-III (SERPINC1) was markedly increased. To highlight features associated with early vs late onset the comparison was then shifted to two subgroups in which patients were sorted depending on whether the onset of the disease had occurred before or after their being 60 years in age. With respect to controls, lower amounts of retinol-binding protein 4 was found in patients with an earlier (and more remote) onset, but not in patients with a late (and more recent) onset. Figure 21 presents 2DE maps obtained under reducing conditions, that allowed the identification of these proteins.

Protein	*Gene name*	*Uniprot accession #*	*All Patients*	*Onset ≤ 60 years (n = 4)*	*Onset > 60 Years (n = 3)*
apolipoprotein A-I	APOA1	P02647	-17 % *	=	-22 % **
transthyretin	TTR	P02766	-30 % *	-28 % **	-32 % **
antithrombin-III	SERPINC1	P01008	+71 % *	+71 % **	=
retinol-binding protein 4	RBP4	P02753	=	-25 % **	=
apolipoprotein A-II	APOA2	P02652	-30 % *	-29 % **	-33 % **

*Table 8. Differential abundance, % variation between controls and patients, of the serum proteome components evaluated with 2DE under reducing conditions and identified by MS. *p ≤0.05, **p ≤ 0.1*

In figure 22 is reported one representative gel among those performed in non-reducing conditions. This separation condition allowed the identification of several additional spots with a significantly different mean volume between patients and controls. In one case, these corresponded to species or fragments of a protein already identified, but others proteins were only evident under these conditions, as summarized in Table 9.

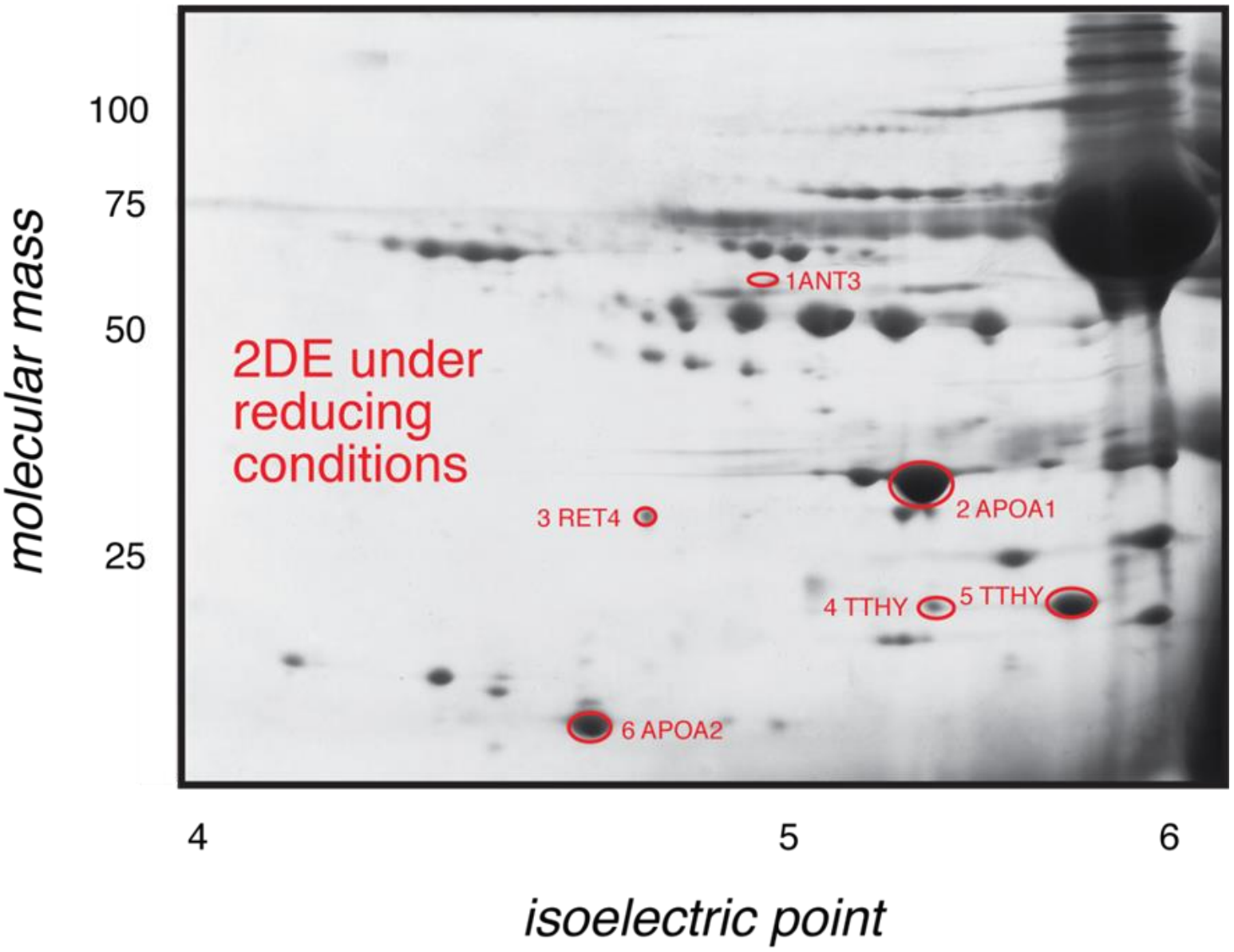


Fig. 21. Representative 2DE gel performed under reducing conditions

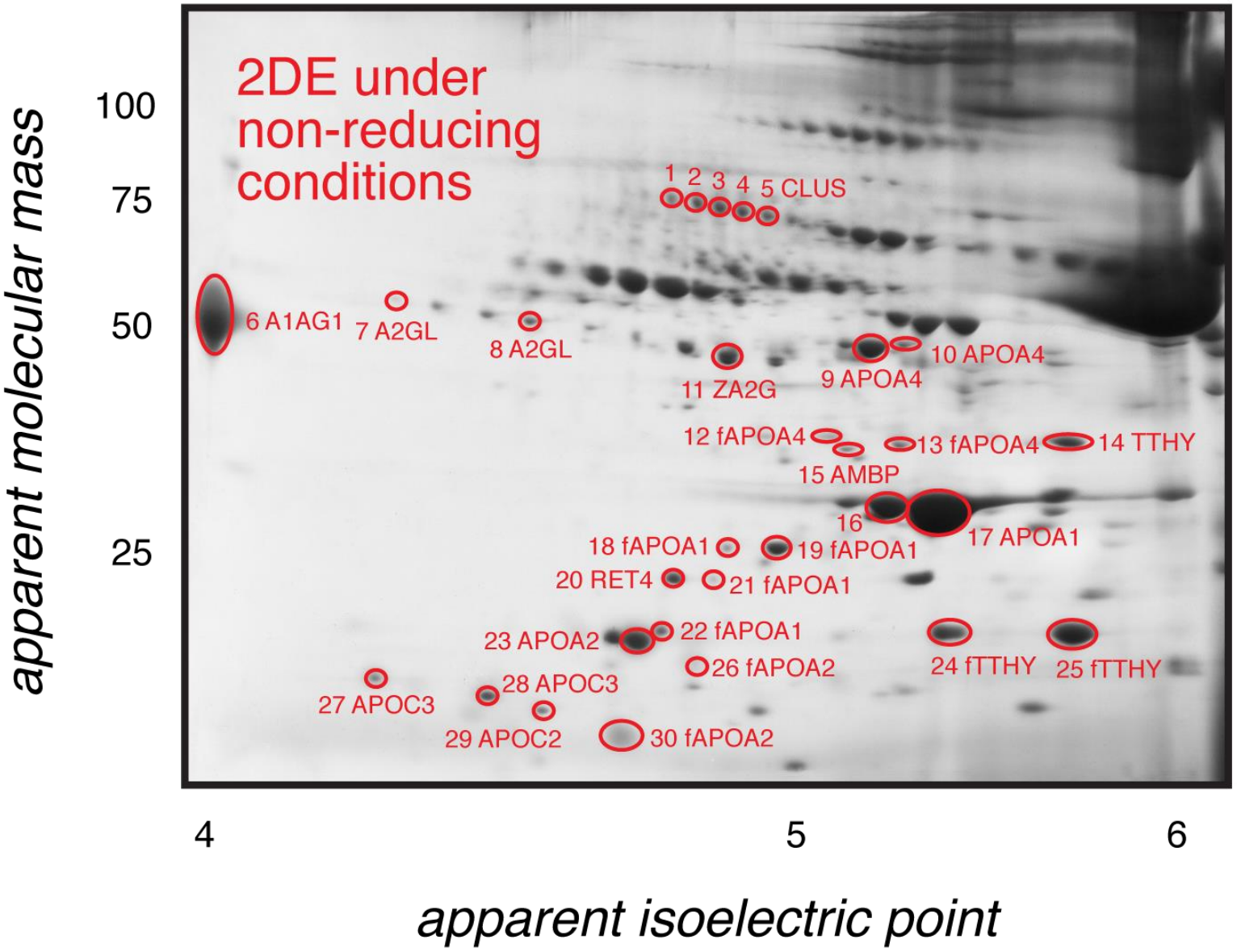


Fig. 22: Representative 2DE gel performed under non-reducing conditions

Protein	*Id*	*Uniprot accession #*	*Patients*	*Controls*	*P-value*	*% variation in patients vs controls*
apolipoprotein A-I	18_fAPOA1	P02647	0.07 ± 0.02	0.10 ± 0.02	0.06	-30%
apolipoprotein A-I	21_fAPOA1	P02647	0.06 ± 0.02	0.14 ± 0.06	0.02	-57%
apolipoprotein A-I	22_fAPOA1	P02647	0.12 ± 0.05	0.21 ± 0.06	0.03	-42%
apolipoprotein A-IV	6_APOA4	P06727	0.13 ± 0.05	0.19 ± 0.06	0.09	-31%
apolipoprotein A-IV	12_fAPOA4	P06727	0.08 ± 0.02	0.23 ± 0.06	< 0.001	-65%
protein AMBP	15_AMBP	P02760	0.08 ± 0.05	0.05 ± 0.01	0.02	+60%
clusterin	4_CLUS	P10909	0.45 ± 0.25	0	< 0.001	-
clusterin	SUM_CLUS	P10909	1.94 ± 0.74	1.22 ± 0.37	0.09	+60%

Table 9. Differential abundance between controls and patients of the serum proteome components evaluated with 2DE under non-reducing conditions and identified by MS. Relative abundances are expressed as % vol ± SD. Id: spot tag; SUM_CLUS: sum of the values relative to all clusterin species

The abundance of some proteins, calculated as the sum of the intensities of all the individual spots identified by MS was correlated with the duration of the disease (i.e., years from the time of onset to the time of blood sampling or PEG start). A positive correlation is present between disease duration and protein levels for alpha 1-acid glycoprotein (Pearson's ρ = 0.791, p = 0.03; Spearman's ρs = 0.857, p = 0.01) and leucine-rich alpha-2-glycoprotein (Spearman's ρs = 0.750, p = 0.05). Conversely, a negative correlation was found for apolipoprotein A-II (Pearson's ρ = -0.718, p = 0.07; Spearman's ρs =- 0.893, p = 0.007) and transthyretin (Pearson's ρ = -0.866, p = 0.01; Spearman's ρs =- 0.893, p = 0.007) as shown in figure 23. From these graphics is evident that any variation is non-linear, regardless of whether a given protein is increasing or decreasing. The most drastic evolution of the abundance of the proteins occurs in the early years of the disease, while in the long-lasting subjects the variation is less pronounced.

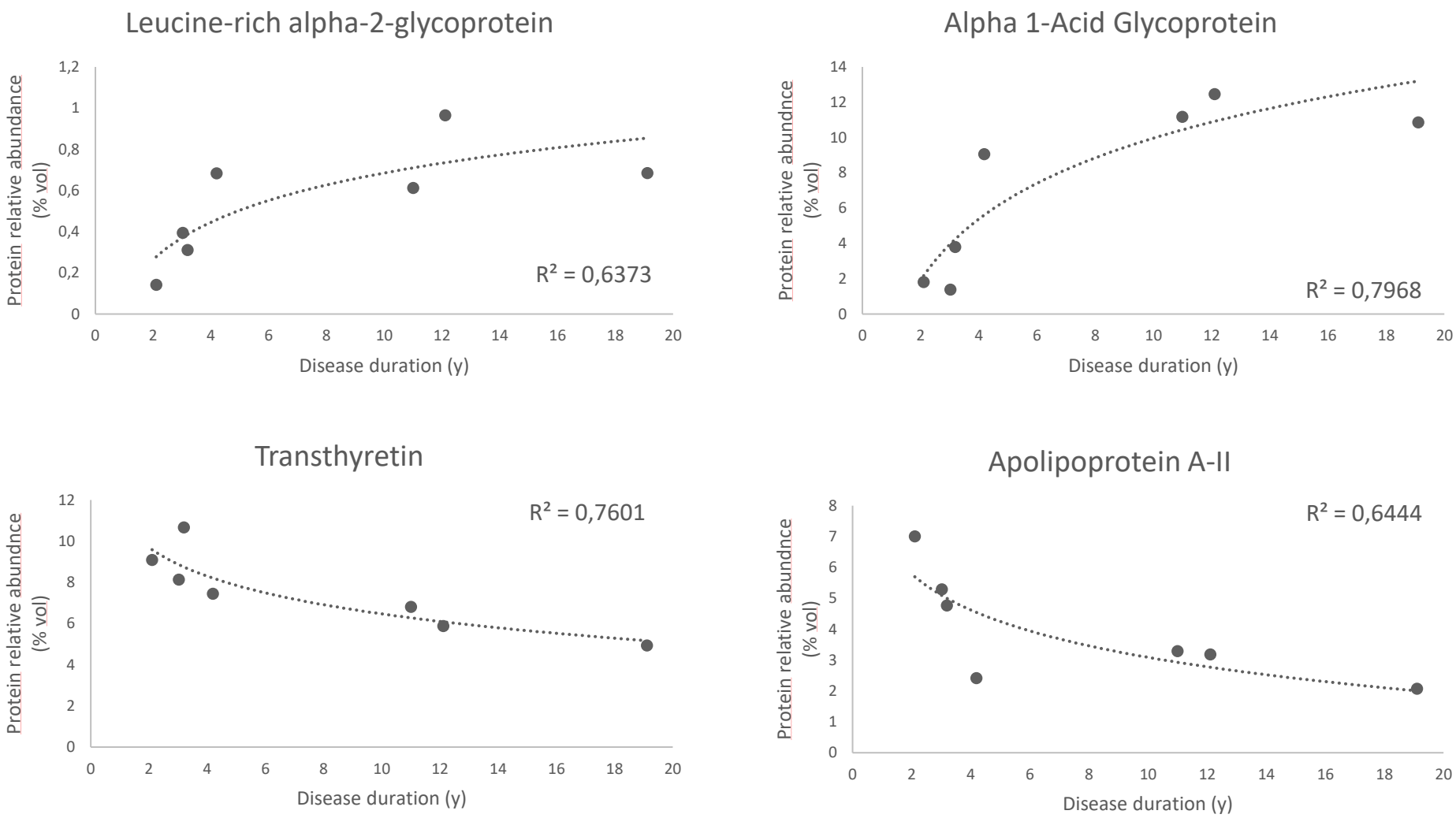


Fig. 23. Correlations of proteins relative abundance with disease duration. Non-linear trend is shown by the regression dotted line.

A dataset composed by the proteins found in different abundance through the 2DE approaches (APOA1, TTR, SERPINC1, RBP4, APOA2, APOA4, AMBP, CLUS, LRG1 and ORM1) was used as a query in the String Database (ver. 10.0), a database able to reconstruct known and predicted protein-protein interactions. The interactions include direct (physical) and indirect (functional) associations, assessed on the basis of computational prediction, knowledge transfer between organisms and interactions aggregated from other (primary) databases (Szklarczyk et al. 2015). In the output graph, nodes represent the proteins of interest, among which are obviously present those input by the user, their closest interactors (represented as colored balloons) and the secondary interactors (white balloons). Edges instead, represent specific and meaningful protein associations, that do not necessarily involve a physical binding between proteins, but rather reflect their contribution to a shared function. These interactions are derived from experimental evidences and curated databases, but can also be predicted from gene neighborhood, gene fusions and gene co-occurrence.

Other interactions can be assessed from text-mining, and from co-expression and protein homology data. All the proteins found in differential abundance in the 2DE experiments are highly interconnected, as shown in Figure 24. From this analysis is possible to define a cluster of proteins involved in lipid homeostasis (red circle), one involved in coagulation cascade (blue circle) and one involved in cellular apoptosis and DNA repair (yellow circle).

Most of these proteins are involved also in Acute Phase Response. Gene Ontology and KEGG Pathway analysis assessed an enrichment in proteins whose molecular function is related to cholesterol and lipid metabolism (Table 10). As highlighted by KEGG analysis there are also present proteins, strictly interacting with the entries, that are involved in neurodegeneration in general and ALS specificlly (APOE, PSEN1, BAX, BCL2 and BID). These results highlight the necessity to consider the proteins selected from 2DE analyses as a part of a complex network, as their differential abundance may be the reflection of a broad array of biological interactions. In the same time, this approach could be useful to identify other interactors potentially involved in ALS, not yet considered.

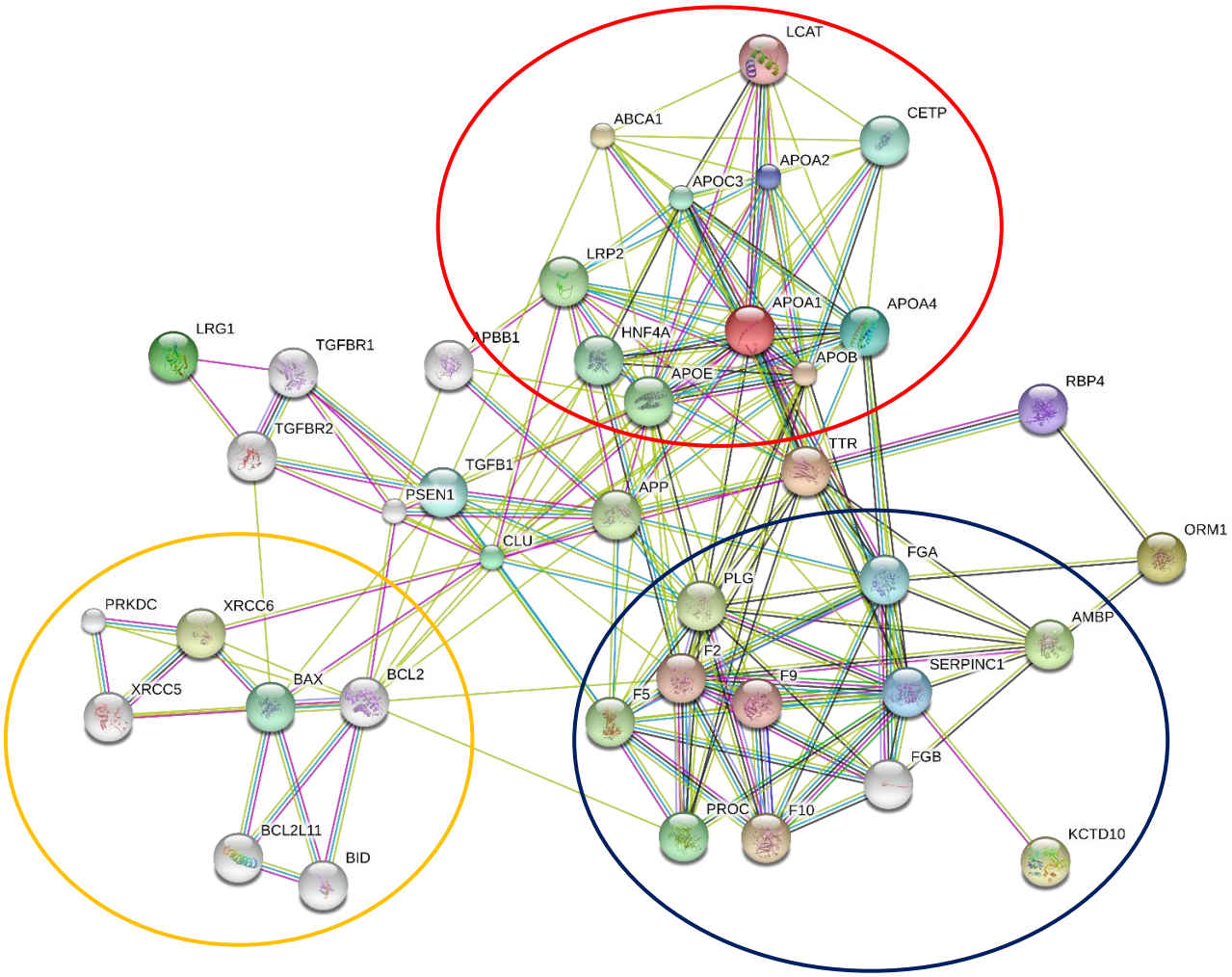


Fig. 24. Output network from STRING analyses. Detailed description is reported in the text. (http://string-db.org/).

Molecular Function (Gene Ontology)	***Observed gene count***	***FDR***	***Network matching proteins***
cholesterol transporter activity	7	2.92E-12	ABCA1, APOA1, APOA2, APOA4, APOB, APOE, CETP
cholesterol binding	7	1.04E-09	ABCA1, APOA1, APOA2, APOA4, APOC3, APOE, CETP
protein binding	29	2.93E-09	ABCA1, AMBP, APBB1, APOA1, APOA2, APOA4, APOB, APOC3, APOE, APP, BAX, BCL2, BID, CLU, F2, FGB, HNF4A, LCAT, LRP2, PLG, PRKDC, PSEN1, SERPINC1, TGFB1, TGFBR1, TGFBR2, TTR, XRCC5, XRCC6
lipid transporter activity	8	3.30E-09	ABCA1, APOA1, APOA2, APOA4, APOB, APOE, CETP, RBP4
alcohol binding	8	4.11E-09	ABCA1, APOA1, APOA2, APOA4, APOC3, APOE, CETP, RBP4
phosphatidylcholine-sterol O-acyltransferase activator activity	4	8.98E-08	APOA1, APOA2, APOA4, APOE
lipoprotein particle receptor binding	5	1.38E-07	APOA1, APOA2, APOB, APOC3, APOE
lipid binding	12	3.20E-07	ABCA1, APOA1, APOA2, APOA4, APOB, APOC3, APOE, BAX, CETP, F10, HNF4A, RBP4
receptor binding	14	1.58E-06	ABCA1, APOA1, APOA2, APOB, APOC3, APOE, APP, BID, F2, HNF4A, PLG, TGFB1, TGFBR1, TGFBR2
high-density lipoprotein particle receptor binding	3	2.19E-06	APOA1, APOA2, APOC3
phospholipid binding	9	2.63E-06	ABCA1, APOA1, APOA2, APOA4, APOB, APOC3, APOE, CETP, F10
KEGG pathway description	***Observed gene count***	***FDR***	***Network matching proteins***
Complement and coagulation cascades	9	2.07E-12	F10, F2, F5, F9, FGA, FGB, PLG, PROC, SERPINC1
Fat digestion and absorption	4	0.000141	ABCA1, APOA1, APOA4, APOB
Non-homologous end-joining	3	0.000187	PRKDC, XRCC5, XRCC6
Colorectal cancer	4	0.00033	BAX, BCL2, TGFB1, TGFBR2
Alzheimer s disease	5	0.000781	APBB1, APOE, APP, BID, PSEN1
Vitamin digestion and absorption	3	0.000781	APOA1, APOA4, APOB
Amyotrophic lateral sclerosis (ALS)	3	0.00522	BAX, BCL2, BID
Non-alcoholic fatty liver disease (NAFLD)	4	0.00686	BAX, BCL2L11, BID, TGFB1
Tuberculosis	4	0.00956	BAX, BCL2, BID, TGFB1
PPAR signaling pathway	3	0.00956	APOA1, APOA2, APOC3
Pathways in cancer	5	0.00995	BAX, BCL2, BID, TGFB1, TGFBR2
Apoptosis	3	0.0141	BAX, BCL2, BID
Neurotrophin signaling pathway	3	0.0324	BAX, BCL2, PSEN1
FoxO signaling pathway	3	0.034	BCL2L11, TGFB1, TGFBR2
Hepatitis B	3	0.0483	BAX, BCL2, TGFB1

Table 10. Molecular function description and pathways in which proteins submitted to the software and their interactors are involved. FDR: False Discovery Rate.

All the results obtained from 2DE performed in non-reducing conditions were then analyzed with AutoCM software (Semeion 2009-2012 ver 8.0). Use of non-reducing condition was preferred, since it allowed the identification of a higher number of proteins. The software was able to group all the species and/or fragments identified for each protein by relying only on the relative intensity of individual spots. Using the % volume data, AutoCM was able to discriminate the group of ALS patients from the group of healthy controls (Figure 25).

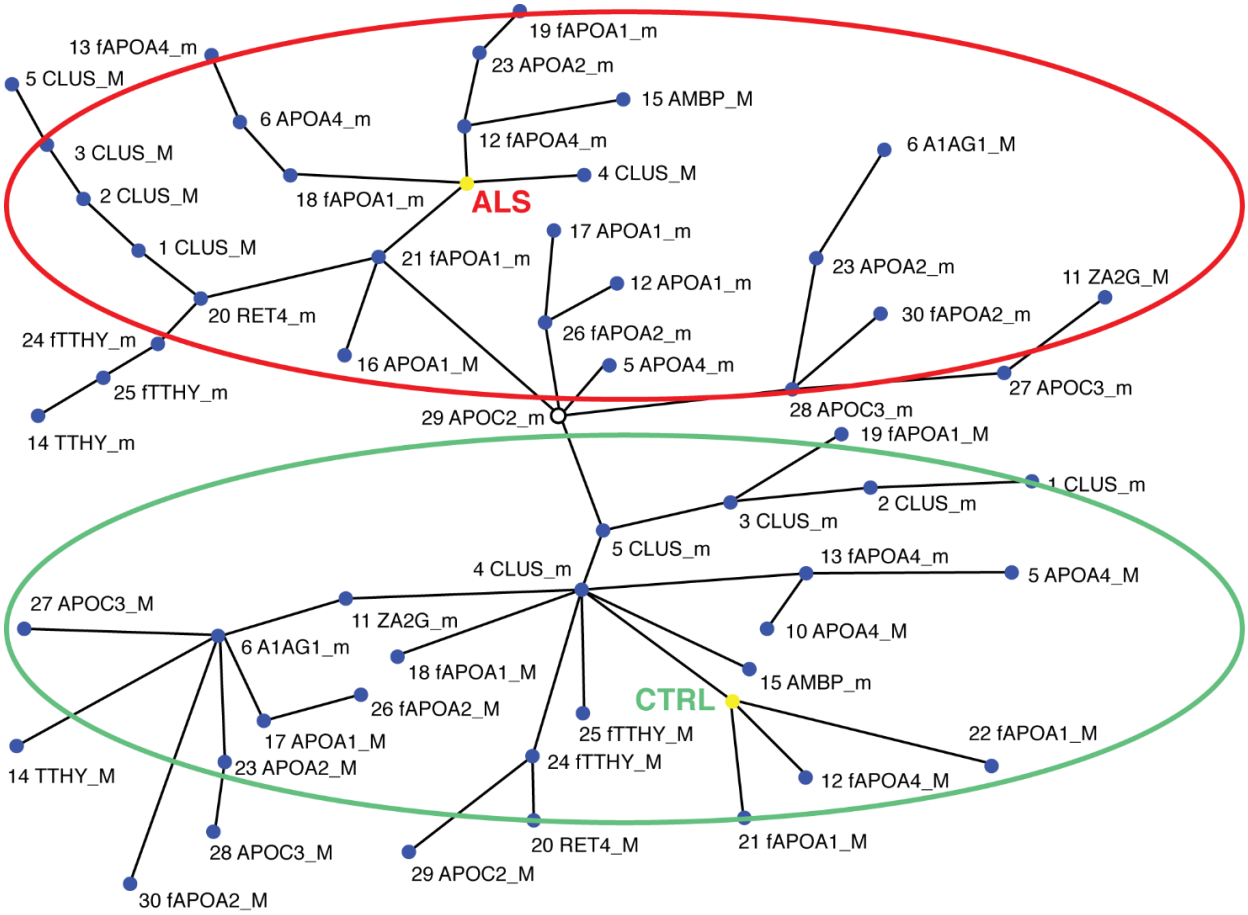


Fig. 25. Semantic connectivity map obtained with Auto-CM System. The Minimum Spanning Tree (MST) shows the connections between species and/or fragments of the peptides selected with MS. Values on the arches are > 0.96 for all the connections. These refer to the strength of the association between two adjacent nodes. The range of this value is between 0 and 1.

The closest nodes, i.e. those most strongly associated to ALS, correspond to low levels of various fragments of apolipoprotein A-I and A-IV, and to high levels of protein AMBP, zinc-alpha-2-glycoprotein and one clusterin species, that was found only in six out of seven patients, but never in controls. Nodes featuring a looser association, but still clustering in the ALS group, correspond to low levels of apolipoprotein A-II, apolipoprotein C-II and C-III, retinol-binding protein 4, and transthyretin, in agreement with results obtained from 2DE performed in reducing conditions. High

levels of alpha-1-acid glycoprotein 1 and of the other clusterin species are also associated with the ALS group.

A confirm of the potential of this approach is attributable to the ability of the software to put in close association the different fragments or species of the same protein, as particularly evident in the case of clusterin, where the train of species detected in the gel is almost perfectly reproduced by the software, especially in the healthy subject group. The same can be stated for the transthyretin fragments, all closely associated each other. Furthermore, the most strongly associated protein to the transthyretin group is retinol binding protein 4, that in bloodstream, interacts with transthyretin itself, preventing its loss by filtration through the kidney glomeruli.

All proteins for which a significantly different abundance in ALS patients *vs* controls was observed are involved in the Acute Phase Response (APR), a systemic reaction to a wide variety of *stimuli* able to cause tissue damage. Indeed, the differential abundance detected for several components of the serum proteome could be related to the compromised general status of the patients. During APR, a massive alteration occurs in the concentration of plasma/serum proteins, some of them resulting in increased and some other in decreased circulating levels. Indeed, all the proteins found in lower concentration in ALS patients than in controls are known as negative acute phase reactants. An alteration in APR proteins in ALS patients was described in a recently published proteomic study performed on Cerebrospinal Fluid (CSF) (Chen et al. 2016).

Some of the proteins identified in this study are involved in lipid homeostasis, and neural network analysis associated low levels of APOA1, APOA2, APOA4, APOC2 and APOC3 with the ALS group. This is consistent with a proposed metabolic shift towards an increased peripheral use of lipids in ALS (Fergani et al. 2007) and with the evidence of hypolipidemia in presymptomatic murine ALS models (Kim et al. 2011). Another study, where ANNs were applied to unravel the hidden connections between the variables on sporadic ALS, highlighted that Single Nucleotide Polymorphisms (SNPs) in genes involved in lipid pathways were associated to the disease, among which *APOA4* was present (Buscema et al. 2012).

Many of the spots found differentially expressed correspond to APOA1 degradation products (proteolytic fragments). Apolipoprotein I is degraded *in vivo* mainly by Matrix Metalloproteases (MMPs) whose activation is driven by the oxidation of the conserved cysteine in the propeptide sequence (Van Wart and Birkedal-Hansen 1990; Eberini et al. 2007). Evaluation of the influence of metals on MMPs activity (including the possible replacement of the original metal cofactor with

other species) could be an interesting follow up of this work, in light of the hypothesis discussed above of a potential heavy metals pollution to soil and water.

Enzymes like plasmin, thrombin, and kallikrein, involved in the coagulation/fibrinolytic pathway, are able to degrade APOA1 to similar proteolytic fragments (Kunitake et al. 1990). One of their specific plasma inhibitor, antithrombin III, as well as another protease inhibitor (protein AMBP), are present at higher concentration in ALS subjects, particularly in those with disease onset before 60 years of age. This could be a likely explanation for the low abundance of APOA1 proteolytic fragments observed here.

Conversely, levels of clusterin were higher in serum samples from patients than from controls. In bloodstream clusterin is a component of the lipid-poor subclass of lipoproteins, the high density lipoproteins (HDL), which also contain APOA1 and paraoxonase (Jenne et al. 1991). Clusterin acts as a molecular chaperone, but it is also involved in apoptosis and, of course, in lipid transport (Koltai 2014), thus providing an interesting connection between lipid and protein homeostasis. Unfolded Protein Response (UPR) is triggered in ALS pathology, leading to the induction of chaperones to increase protein folding/refolding capacity (Kanekura et al. 2009). Furthermore, high levels of clusterin have been associated to cognitive decline in Alzheimer's Disease (AD). Specifically, a Single Nucleotide Polymorphism (SNP) within the clusterin gene has been described as a risk factor for AD in two independent genome-wide association studies (Harold et al. 2009; Lambert et al. 2009), thus providing a link to neurodegeneration. In a study on healthy Korean adults, Won et al. (2014) proposed that circulating levels of clusterin might be related to the pro-inflammatory state associated with increasing adiposity, and that it could represent a potential biomarker of inflammation-related human disease(s). Interestingly, in this study, higher levels of clusterin and lower levels of apolipoprotein A-I were detected, even though in the bloodstream they are associated within the supramolecular assembly of HDL particles, calling for further and deeper characterization. Finally, a link with the metals analyses previously reported is provided by a study stating that selenium-enriched yeast administration to healthy subjects, leads to increased circulating levels of serum clusterin isoform 1 (Sinha et al. 2010). In the metals analyses described previously a higher concentration of selenium was associated with the ALS group. On this basis, it seems worth evaluating further the link between metals concentration and serum proteins in ALS.

Another protein associated at high levels to the ALS group by the Auto-CM software is zinc-alpha-2-glycoprotein (ZA2G). Increased concentrations of this protein have already been described in ALS after a proteomic analysis on CSF (Brettschneider et al. 2008). Interestingly, ZA2G was also

reported to be affected in the CSF proteome of patients with frontotemporal dementia (FTD), a pathology that overlaps with ALS in some patients (Hansson et al. 2004). The function of this protein is related to the stimulation of lipolysis and the reduction of body fats (Vanni et al. 2009), which circumstantially support the involvement of proteins related to lipid homeostasis.

ALS biomarkers are badly needed to help diagnosis, to monitor the progression of the disease, and to define suitable endpoints for clinical trials. Concentration of alpha 1-acid glycoprotein or oromucoid (ORM1) and of leucine-rich alpha-2-glycoprotein (LRG1), while not significantly differs overall between patients and controls, steadily increases during the progression of the disease, and higher concentrations have been measured in patients with a longer disease course than in those with a shorter one. ORM1 is one of the major acute phase proteins in humans (Fournier et al. 2000) and it turned out to be one of the four circulating biomarkers able to predict the risk of all-cause mortality, in a study performed on participants from Estonian Biobank (Fischer et al. 2014). LRG1 is involved in protein-protein interaction, signal transduction, and cell adhesion and development (O'Donnell et al. 2002).

Despite not being conclusive, our data advocate focusing future investigation on the evolution of the concentration of these proteins with time. Apolipoprotein A-II and transthyretin, on the contrary, keep decreasing during the course of the disease. In view of the opposite trends, the evolution of a panel of such biomarkers and of their combinations could turn out to be especially effective in monitoring the disease.

In conclusion, 2DE technology still reveals its potential in proteomic studies. It is nowadays a well-known and very valuable approach to assure resolution and pattern recognition in complex matrices. Furthermore, it provides a very wide spectrum of customizable solutions, with minimal changes in the work pipeline, according to user requirements.

The culprit of proteomic studies is often data managing and interpretation of results. This study also reveals that data mining through software based on ANN appears very promising in dissecting the complexity of a multifactorial disorder such as ALS. Specifically, this approach may help to unravel the hidden interactions among variables that would be almost impossible to analyze with conventional statistical methods. Furthermore, this approach evolved specifically to manage situations where subjects are very low in number and several variables are at work, as is the case here, but, more in general, as is the case of the study of rare diseases. It is reassuring that most of the results obtained in this study confirm, or are in general agreement, with literature evidences

obtained with different approaches and on much larger cohorts of ALS patients; analyzing well defined cohorts of human patients, despite presenting the problem of a low number of subjects, could be a reliable model to study ALS. This approach would allow to evaluate different aspects of the disease all together, in order to have a more comprehensive view of the disease in the same group of subjects.

After extensive literature research, this appears to be the first report of serum proteome in subjects affected by sporadic ALS, obtained with 2DE. Despite its complexity, serum is the most accessible biological tissue, both in clinical practice and for basic research. The search for multiple biomarkers to help physicians in diagnosis would lead to a better care of patients; along with this practical implication, this would also provide useful insights into disease mechanisms. Indeed, at present, the leading field in ALS research is based on genomic studies. However, this approach is limited because genetics explains only a small percentage of ALS cases, and among these cases, very rarely a specific mutation is able to provide information on the onset and the course of disease, given the complex interactions that are supposed to drive the evolution of ALS. Evaluating the protein expression, that is the result of genetic background and post-transcriptional and post-translational events, together with protein transport, secretion and degradation, would be the key to discover their role in pathological conditions.

Most of the studies performed up to now, that led to define geographical clusters of ALS, were limited to describing this phenomenon only from the epidemiological point of view (Veiga-Cabo et al. 1997; Uccelli et al. 2007; Sabel et al. 2009; Alonso et al. 2011; Boumédiène et al. 2011; Keren et al. 2014; Henry et al. 2015). The study of geographical clusters, such as those found in Guam or Kii Peninsula, has been successful in the past years, to get more insights into the pathology of ALS. Basic research performed on ALS patients originating from a small geographical area, as described here, has the potential to be valuable for a systematic evaluation of various aspects of the disease, integrating different methods of analysis, in subjects with a common environmental background.

GENETICS

A screening for the most frequently mutated genes in ALS was performed in order to assess if the higher prevalence of ALS, detected in the study area, could be attributable to a genetic mutation, since it is often the cause for the appearance of a disease cluster, within a family or a geographical area.

As generally approved in clinical practice, the genetic test was performed overall *SOD1* gene, on exon 6 of *TARDBP* gene and on exons 5, 6, 13, 14 and 15 of *FUS* gene, the latter, considered mutational hotspots. Exons and intron-exon boundaries were PCR amplified and sequenced. None of the sequenced genes revealed mutations nor SNPs. As detailed under Methods, the presence of a *C9orf72* hexanucleotide expansion was evaluated, but results were negative for all ALS patients. Even if the influence of genetic factors in the determination of this cluster of patients cannot be totally excluded - given the always possible presence of rare variants - the absence of alterations in the most frequently mutated genes and the absence of a positive family history for neurodegenerative diseases led us to focus on other factors potentially involved in the disease.

As most proteomic findings point to an altered lipid metabolism, *APOE* genotype was evaluated in subjects involved in the study. Literature data indicate Apolipoprotein E (ApoE) as a link between lipids homeostasis and neurodegeneration, in particular in Alzheimer disease (AD) and - more controversially - in ALS. Furthermore, the evidence of an association of ApoE with differentially abundant proteins detected through proteomic studies, emerged also from the STRING analysis of the results described above. ApoE comprises a class of apolipoproteins essential for the normal catabolism of triglyceride-rich lipoprotein constituents. On the basis of the combination of polymorphisms in two positions, it may exist in three isoforms: ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158), and ApoE4 (Arg112, Arg158) (Ghebranious et al. 2005). The APOE*3 allele is the most common and does not seem to influence the risk of developing AD; the APOE*2 allele appears to lower it, whereas the APOE*4 allele has been associated to an increased risk for AD and other neurodegenerative conditions such as ALS (Corder et al. 1993; Praline et al. 2011).

Table 11 compares genotypes and allelic frequencies of *APOE* in the group of patients, in the healthy controls and in the whole population of Caucasian ancestry, as reported in the Alzheimer Research Forum website (http://www.alzgene.org/meta.asp?geneID=83).

Population	***Allele***			***Genotype***					
	2	**3**	**4**	**2/2**	**2/3**	**3/3**	**2/4**	**3/4**	**4/4**
Caucasian	0.08	0.78	0.14	0.01	0.12	0.61	0.02	0.22	0.02
Controls	0.10	0.80	0.10	0.00	0.20	0.60	0.00	0.20	0.00
ALS	0.00	0.71	0.29	0.00	0.00	0.43	0.00	0.57	0.00

Table 11. Frequencies of ApoE alleles and genotypes in the study populations and in the general Caucasian population

Despite the low number of control subjects, the results obtained from the cohort analyzed are concordant with literature data about the frequency of the alleles and of the genotypes in the healthy Caucasian population. Conversely, the allelic frequency for the APOE*4 allele is 3-fold higher as well as APOE*3/APOE*4 genotype in the patients' group than in control's. This finding leads to hypothesize that the APOE*4 allele might act as a genetic susceptibility factor or disease modifier in this group of SALS patients. However, the low number of subjects considered in this study must always be considered as a limit when raising such hypotheses.

Another disease associated factor, linking lipid metabolism and protection from environmental stressors, is represented by the paraoxonase gene cluster, localized on chromosome 7q21.3 and containing *PON1* and *PON2* genes. The PON1 protein is secreted by the liver, is associated with high-density lipoproteins, (HDL) and it prevents peroxidation of LDL through its antioxidant capacity. It is also able to detoxify organophosphate insecticides, pesticides and nerve gases (Li et al. 2003). PON2 is an intracellular protein, ubiquitously expressed with the function to protect cells from oxidative damage. Cells overexpressing PON2 are less prone to oxidize LDL and show considerably less intracellular oxidative stress when exposed to either H_2O_2 or oxidized phospholipid (Ng et al. 2001).

Single Nucleotide Polymorphisms (SNPs) have been identified in these genes as associated to the enzyme activity, plasma concentrations and lipoprotein metabolism. In detail, PON1 activity in paraoxon and chlorpyrifos oxon hydrolysis is reported to be enhanced by a Glutamine (Q) to

Arginine (R) substitution in position 192 (rs662), while the presence of a methionine (M) instead of a leucine (L) in position 55 (rs854560) is thought to be associated to lower serum concentration of this enzyme and to higher protective capacity towards LDL oxidation (Humbert et al. 1993; Zafiropoulos et al. 2010). The substitution of a Serine (S) to a Cysteine (C) in position 311 in PON2 (rs6954345) has been associated to variations in its plasmatic concentrations and to lipoproteins metabolism (Li et al. 2003). Homozygous subjects for the Serine substitution at position 311 have significantly higher APOB concentration, total plasma, and LDL cholesterol, conversely they have the lowest amount of HDL and APOA1, than subjects with the other two genotypes (Boright et al. 1998).

Table 12 reports allele and genotype frequencies of the analyzed SNPs in patients and healthy controls and compares them to the frequencies detected in the European population in the 1000 Genomes Project (Auton et al. 2015). Allelic frequencies in control group do not differ from those reported for the European population, except for PON2 S311C, where a higher frequency of the serine-containing isoform was detected in the control group.

Population	***PON1 L55M***					***PON1 Q192R***					***PON2 S311C***				
	Allele		***Genotype***			***Allele***		***Genotype***			***Allele***		***Genotype***		
	L	**M**	**LL**	**LM**	**MM**	**Q**	**R**	**QQ**	**QR**	**RR**	**S**	**C**	**SS**	**SC**	**CC**
ALS	0.71	0.29	0.57	0.29	0.14	0.79	0.21	0.57	0.43	0	0.79	0.21	0.57	0.43	0
Controls	0.60	0.40	0.60	0	0.40	0.80	0.20	0.60	0.40	0	0.90	0.10	0.80	0.20	0
EUR (Chr=1006)	0.64	0.36				0.71	0.29				0.75	0.25			

Table 12. Frequencies of PON alleles and genotypes in the study populations and in the European population

When comparing ALS and control group from the study population, PON1 Q192R genotype and allelic distribution were present in the same proportion. Genotype frequencies relative to PON1 L55M polymorphism reveal an almost 3-fold higher frequency of the homozygous MM, while no heterozygous subjects were detected in controls. Regarding PON2 S311C allelic frequencies, the cysteine isoform was two times more frequently detected in ALS patients, the same for the heterozygous state. The low number of subjects in this analysis made impossible the application of statistical tests in order to evaluate if differences are significant. However, the higher frequency of

the homozygous PON2 S311C in controls, could be associated to a protecting effect, supported by the fact that this genotype is associated to hyperlipidemia, reported, as already discussed, to be protective towards ALS. A similar effect could be hypothesized, in this cohort of subjects, also thanks to the finding of an apparently higher frequency of PON1 M55M genotype, associated to a higher protection from LDL oxidation.

Several studies tried to establish an association of PON cluster with SALS, but previous meta-analyses failed to detect any association between the analyzed SNPs and the disease (Wills et al. 2009). Data reported here are consistent with these findings, so that it still remains difficult to ascertain if PON genes could have a role in the disease. As different mutations in PON genes have been reported in ALS patients, their involvement cannot be excluded, with a pathogenic mechanism likely to reflect some properties shared by mutant PON proteins (Ticozzi et al. 2010). Further support to PON involvement in ALS is provided by their own role in protection from oxidation. In ALS indeed, motor neurons are exposed to abnormal levels of oxidative stresses such as lipid peroxidation in a chronic condition (Shibata et al. 2001). PON1 indeed, may have a role in protecting cellular membranes facing these conditions of exposure to oxidative agents and free radicals (Rodrigo et al. 2001).

Considering these data, the study performed in this cohort of patients, thus not providing evidences in the involvement of PON polymorphisms in ALS, provides some connections valuable to be considered. First, the alterations in lipid transport proteins detected with 2DE may alter the assembly of the supramolecular complexes, in which PON1 is involved. Changes in HDL size and shape are able to strongly affect the binding affinity and stability of PON1 resulting in impaired antioxidative capacity (Durrington et al. 2001). A specific PON genotype can affect the lipid metabolism through some still unknown mechanisms and, *vice versa*, lipid homeostasis alterations may have different detrimental effects on the organism because of a given PON genotype.

Secondly, it is reported that some elements, such as barium, lanthanum, copper, zinc, and mercurials are able to inhibit PON1 activity in rat or human liver (Gonzalvo et al. 1997). In the light of the high levels of metals reported in the area of this study, it would be interesting in the future, to evaluate the activity of PON enzymes and to correlate it with levels of metals detected in subjects involved in the study.

Lastly, in literature PON2 S311C and APOE*4 polymorphisms are described to have interactive effects on the development of AD related dementia (Janka et al. 2002). ApoE and PON1 L55M

polymorphisms show relevant interactions in establishing the risk for Coronary Artery Disease, demonstrating effectively functional interaction (Murphy et al. 2002), that may be relevant also in ALS.

NUTRITIONAL INTERVENTIONS IN ALS

The lack of an effective pharmacological treatment and the long time and costs required for the testing of safety and efficacy of new drugs (comprising stem cells and gene therapy), led researchers and patients itself to look for alternative ways to manage the disease. Nutritional interventions are actually in use with the aim of replacing normal feeding since it becomes impossible with the progression of the disease.

In the light of the results reported in this study, with the support of literature data, an approach pointed to modulate lipid homeostasis and/or manage metals impairment, through a properly designed nutritional intervention, could be a low-cost and non-pharmacological promising way to manage the disease in the short time.

Enteral nutrition by percutaneous endoscopic gastrostomy (PEG) or radiologically inserted gastrostomy (RIG) is generally recommended once patients have lost 10% of their premorbid weight (Greenwood 2013). For patients who need to be fed via a gastrostomy tube, no nutritional guidelines or consensus regarding recommendations for enteral nutrition are available. Dietary supplements are frequently used, but this intervention is usually based on anecdotal evidences in the hope of potential efficacy, in the absence of evidence-based research (Braun and Osecheck 2012).

Malnutrition is a common feature and is an independent negative prognostic factor for survival in ALS due to the deterioration of functional disabilities such as difficulties in mastication, oral transit and dysphagia. Furthermore, ALS patients, despite the progression of the disease leads to paralysis, experience a paradoxical abnormal increase in energy expenditure (hypermetabolism). Consequences of an inadequate dietary intake lead to the exacerbation of catabolism, muscle atrophy and weakness of the immune system (Cameron and Rosenfeld 2002). In consequence, appropriate nutritional intake seems to have the potential to modify the development of the disease.

Findings of the study here presented point to an alteration in the lipid profile in ALS patients, which is consistent with literature data. In fact, experiments performed on SOD1 transgenic mice show that mutant strains have a lower BMI than littermates, due to impaired lipid storage in white adipose tissue (Dupuis et al. 2004). Low lipid storage can be found before the animals develop motor signs and appears to be related to elevated energetic demand with recruitment of lipid body stores. When fed with a rich fat diet animals lived longer than those fed with normal diet (Hamadeh et al. 2005).

Resting energy expenditure (REE) is reported to be increased during the disease and leads to the depletion of energy stores, in particular, lipid reserves. Thus, hyperlipidemia and high BMI have been proposed as factors potentially able to modulate survival in ALS (Dupuis et al. 2008; Paganoni et al. 2011). Furthermore, cholesterol and phospholipids, in particular low-density lipoproteins, are essential to maintain a healthy axonal membrane and for peripheral nerve membrane injury repair (Posse de Chaves et al. 1997).

Considering these results, supported by data presented in this work, some clinical trials are currently being performed to evaluate the impact of nutritional intervention in ALS. At present an ongoing trial (NCT02152449) is aimed at evaluating the efficacy of a systematical and early oral nutritional supplementation, in order to allow patients to maintain proper nutritional status, and its impact on neurological functions. Such an intervention could delay the progression of the disease if the metabolic disorders in ALS are not only the result of progression of the disease, but are implicated in its course and outcome.

Another study, based on a dietary intervention, aimed at evaluating tolerability and safety of increasing bodyweight in ALS patients already receiving PEG and evaluated if it could improve survival (NCT00983983). Patients were split in three study arms: calorie replacement using an isocaloric diet with a goal of weight stability (control), a high-carbohydrate hypercaloric diet (HC/HC) and a hypercaloric diet high in fat calories (HF/HC). Hypercaloric diets were aimed at increasing body weight by giving about 125% of estimated energy requirements for 4 months. The study intervention resulted safe and tolerable since patients who received a HC/HC tube-feeding formula were less likely to have serious adverse events, including death, during the 5-month follow-up than were controls. The study results supported hypercaloric enteral nutrition as a new and potentially robust nonpharmacological intervention in ALS (Wills et al. 2014).

The mentioned study was not able to demonstrate that a hypercaloric diet high in fat calories could provide benefits in ALS patients, but it resulted instead in weight loss. Authors propose that

it could be related to the higher rates of gastrointestinal side-effects compared with the HC/HC diet even if previous studies using this feeding formula had not reported weight loss as a side-effect (Wills et al. 2014).

Several evidences point to a potentially beneficial effect of a high fat diet in managing ALS. First of all, there is epidemiological evidence that increased dietary fat intake may reduce the risk of developing the disease (Okamoto et al. 2007; Veldink et al. 2007; Morozova et al. 2008). Furthermore, a ketogenic diet (60% fat, 20% carbohydrate, 20% protein) in mutant mouse model demonstrated an improvement in motor functions and an increase in ATP production from mitochondria purified from spinal cord when treated with β-hydroxybutyrate; a supplementation of a ketone bodies precursor (caprylic acid) improved mitochondrial function and motor neuron count in ALS mouse model (Zhao et al. 2006; Zhao et al. 2012). The probable involvement of mitochondria in ALS makes the ketogenic diet (KD) a promising tool for the treatment of the disease; furthermore, it is an actual tool to manage pharmacoresistent epilepsy. Ketone bodies indeed, are able to act on mitochondrial function by restoring, for example, complex I function (Tieu et al. 2003). Ari and colleagues compared standard and ketogenic diets, both with and without adding key supplements from the Deanna Protocol (DP) in SOD1 mutant mice (Ari et al. 2014). The DP is comprised primarily of arginine alpha-ketoglutarate (AAKG), and includes several other agents that were reported to preserve metabolic function and prevent glutamate excitotoxicity. Their data demonstrated significant improvements and prolonged longevity in animals fed both the supplemented diets compared with the non-supplemented diets (Bedlack et al. 2015). Thus despite having a promising potential, in the lack of clinical trials at this time, ALSUntangled group does not recommend the Deanna Protocol to patients with ALS (ALSUntangled Group 2013).

However, all these data must still be translated in human patients. Only Silva et al. tested oral supplementation with milk whey proteins and modified starch in a small study of 16 subjects treated for 4 months; they demonstrated modest weight gain in the supplement arm while the controls continued to lose weight. Intriguingly, the supplement arm appeared to have a slower rate of decline in the Amyotrophic Lateral Sclerosis Functional Rating Scale–Revised (Silva et al. 2010). At present only one trial (NCT01016522) was performed to assess safety and tolerability of KD in ALS patients, but results are not yet published.

Given the complexity of the disease a multi-targeting approach could be extremely beneficial. At present, studies on the administration of a high Calorie Energy supplemented Diet (CED) together with low doses of the novel multifunctional monoamine oxidase (MAO)

inhibitor/iron-chelating compound (M30) are being performed in the SOD1-G93A transgenic mouse model, showing promising results. The treatment increased mutant mice survival and produced additive neuroprotective effects on motor performance, together with the preservation of neuromuscular junction and myofibril regular morphology. Furthermore, it upregulated the mitochondrial biogenesis master regulators, metabolic genes and proteins such as PPARγ, UCP1/3, NRF1/2, Tfam, and ERRα in gastrocnemius muscle. Effects on gene regulation have been showed also in the frontal cortex with elevated levels of Bcl-2 and neurotrophic factors (Golko-Perez et al. 2016a; Golko-Perez et al. 2016b; Golko-Perez et al. 2016c). This joint approach that targets two different disease mechanisms could enhance the neuroprotective effects and is strongly supported by the results of this work.

An emerging nutritional issue relating to ALS specifically and neuromuscular diseases in general is the use of nutraceuticals and functional foods. In relation to metal homeostasis management, this could be a promising approach instead of the administration of a chelating therapy, commonly used in the case of metal poisoning and that has the potential to be harmful since it is not intended for long-term use.

An interesting alternative approach proposed by Bisanz et al., is the development of a probiotic supplemented yogurt with food-grade microbes, such as lactic acid bacteria (in particular *Lactobacillus rhamnosus* strain GR-1), known to have an affinity for many toxic metals and pollutants (Pb and Cd *in vitro* and Hg, As and pesticides *in vivo*), probably due to a mechanism of passive sequestration, but also thanks to specific pathways for detoxification. In a pilot study, the Authors, demonstrated the value of long-term probiotic based intervention to counter arsenic and mercury in pregnant women environmentally and dietary exposed to high levels of heavy metals (Bisanz et al. 2014).

Song et al. instead, developed a yogurt enriched with galacto-oligosaccharides (GOS), a prebiotic able to improve the absorption and synthesis of B Vitamins. Its administration to a mouse model of ALS led to a delay in symptoms onset and a prolonged lifespan, it attenuated motor neuron loss and improved the muscular atrophy and myocyte mitochondrial activity; the treatment suppressed the activation of astrocytes and microglia by regulating several inflammatory- and apoptosis-related factors. (Song et al. 2013). The development of functional enriched yogurts seems to be promising and have the potential to be easily administered to ALS patients at every stage of disease, given its soft texture.

Among alternative approaches aimed at managing the disease, great attention has been given to natural food constituents and herbal compounds, as excellently reviewed by Zhang et al. (Zhang et al. 2014). Among these compounds, catechins (epigallocatechin, epicatechin, and epicatichin-3-gallate) are the most interesting polyphenolic flavonoids with antioxidant, metal chelating and protective properties toward glutamatergic excitotoxicity; they are able to cross the blood-brain barrier, to be incorporated in brain tissues and to modulate mitochondrial responses to oxidative insults. Catechins are found in high concentrations in some plants, fruits, and vegetables and are considered the healthy constituents of green tea, blue berries, cocoa, prune juice, red wine, and *Ginkgo biloba* (Bedlack et al. 2015). Studies in Parkinson's and Alzheimer's disease showed promising results but, none have been completed in ALS (Chan et al. 2016). However, experiments in ALS mouse model support the neuroprotective potential of catechins. In vitro studies showed that epicatichin-3-gallate reduced hyperexcitability in SOD1 motor neurons and had a rescue effect in motor neurons exposed to H_2O_2 (Xu et al. 2006). Further investigations in the ALS mouse model, showed that presymptomatic oral administration of epicatichin-3-gallate significantly delayed the onset of disease and extended the life span. In addition, the treated mice had an increased number of motor neurons, diminished microglial activation and a better inflammatory profile (Koh et al. 2006; Yu et al. 2010).

These results support the assumption that the use of combined dietary interventions to treat ALS have the potential to be a new, simple, low-cost and low-risk approach to impact disease progression. Furthermore, it will also contribute to the better understanding of disease mechanisms. It is possible that different diet interventions could have additive effects in ALS, and combined interventions should be tested together in Phase III trials.

CONCLUSIONS

- A multidisciplinary approach to study a limited cohort of sporadic Amyotrophic Lateral Sclerosis patients is presented in this work, in contrast with the trendy studies where wide cohorts of heterogeneous subjects are pooled together. The detection of clusters of ALS patients is often limited to an epidemiological description; here instead, the common environmental exposure and the limited number of subjects are exploited to dissect different aspects of the disease. Multidisciplinary studies on small cohorts of ALS patients are, at present, very rare. It would be of outstanding value to integrate different fields of knowledge, to create a picture of the several factors involved in the disease etiology and course, complete as much as possible. This approach resulted valuable since results reported are concordant with published data obtained in other studies.
- In this study is reported the first description of the serum proteome of ALS patients with 2DE, that allowed to identify a great number of proteins as potentially associated to the disease, not only with the aim of deeply investigating the factors involved in the disease, but also in order to assess potential biomarkers for disease progression.
- While the involvement of APR proteins is probably secondary to the compromised status of patients, the described deregulation of lipid homeostasis proteins seems to be more directly linked in modulating the disease progression, as supported from literature data.
- From the results obtained in this study, some metals appear to be involved in the disease, possibly secondary to the environmental influence, in an area restricted and close to a mine. Future assessments on soil and water pollution are strongly recommended. The investigation of ALS prevalence in other cohorts of subjects living in similar conditions would be very useful in order to confirm results and expand this research and to elucidate the effective role of metals in ALS.
- Great amount of data is, nowadays, a limit in understanding results of experiments performed with more and more sophisticated technologies, especially in complex diseases such as ALS. The application of new statistical analyses, based on machine learning, where data are the basis of the creation of models to interpret interactions among variables, would be the key to translate raw data into understandable models.

- The lack of an effective pharmacological treatment in ALS leads to rapidly looking for alternative approaches to modulate this devastating disease. To this purpose, a nutritional intervention, with the development of specific nutritional formulas, would be an effective tool to intervene on the patients' lipid profile and to contrast potential metal's homeostasis impairment. Thus, development of such formulations is strongly supported from the evidences raised from this study, together with its application in clinical trials.

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PRODUCTS

ABSTRACTS

- *XIX Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology – 24-26 September 2014*

  Micronutrients in a neurodegenerative disorder such as amyotrophic lateral sclerosis
  Stefano De Benedetti
  DeFENS - Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy
  This PhD thesis research project is aimed at investigating the role of micronutrients in Amyotrophic Lateral Scelrosis. Their levels will be correlated with the disease status to evaluate how micronutrients and their regulatory pathways could influence the manifestation and the difference of clinical signs of disease in pre-symptomatic subjects thus explaining the wide phenotypic spectrum of disease. This will be performed through different approaches: clinical, biochemical and genetic in order to obtain a nutritional outcome. This could help to elucidate the role of micronutrients and its metabolism in the pathogenesis and/or in the progression of ALS.

- *IUBMB Symposium FeS 2015 - Iron Sulfur Cluster Biogenesis and Regulation - 23-26 June 2015*

  Metals analysis in a small cohort of ALS patients originating from a restricted geographical area: preliminary data
  S. De Benedetti[1]*, G. Lucchini[2], A. Marocchi[4], S. Penco[4], S. Iametti[1], E. Gianazza[3], F. Bonomi[1]
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  Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder characterized by selective degeneration of both upper and lower motor neurons in the brain, brainstem, and spinal cord. This results in paralysis due to muscle weakness and atrophy, leading to death in 3-5 years since the first manifestations of symptoms. Genetic and environmental factors are involved in the pathogenesis of the disease and metals metabolism have been linked to ALS. Transition metal induced toxicity (including iron toxicity) has been proposed to be involved in ALS and higher concentrations of metals and proteins that regulate metal homeostasis have been described in ALS patients. Thiophylic metals have been shown to be capable of displacing iron from reduced FeS proteins, that thus may represent a yet underexplored target in this frame. We evaluated the concentration of different metals in both serum and urine by ICP-MS, in a cohort of subjects with diagnosis of ALS, originating from a restricted geographical area where the incidence of ALS is above average. Principal Component Analysis (PCA) showed higher concentrations of metals in ALS subjects, especially in urine. The only exception is for As, where lower levels have been found in ALS patients, particularly in serum samples. Artificial Neural Networks analysis was able to discriminate between subjects and controls, linking to the disease lower levels of As and higher levels of Ni. These data give an insight to the status of metals in these patients that, despite being few subjects, they share a common environmental exposure.

- *XX Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology – 23-25 September 2015*

Metals and proteomics of the biological fluids in ALS patients: Preliminary data

Stefano De Benedetti
DeFENS - Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy

Biochemical analyses described in the PhD dissertation project were performed on a small cohort of subjects affected by sporadic ALS, all originating from a restricted geographical area. In detail, serum and urine levels of a customized panel of metals were measured; proteomic analyses were performed on urine by SDS-PAGE and on serum by 2D-Electrophoresis. Data collected through a survey on lifestyle and nutrition were analyzed by Artificial Neural Networks.

- *XVIII CONGRESSO NAZIONALE SIGU – Società Italiana di Genetica Umana – 22-23 October 2015*

Valutazione delle concentrazioni di metalli in pazienti affetti da Sclerosi Laterale Amiotrofica geneticamente caratterizzati

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La Sclerosi Laterale Amiotrofica (SLA) è una malattia neurodegenerativa rara caratterizzata da paralisi muscolare progressiva conseguente alla degenerazione dei motoneuroni nella corteccia motoria primitiva, nei tratti corticospinali, nel tronco cerebrale e nel midollo spinale.

La SLA ha un'incidenza di 2.1 casi ogni 100˙000 individui all'anno e una prevalenza stimata di 5.4/100.000 casi, principalmente sporadica; le forme familiari sono circa il 5-10% del totale. Le cause della malattia sono al momento ignote. L'ipotesi attualmente accreditata è che si tratti di una patologia multifattoriale, per cui vari fattori ambientali concorrerebbero a determinare i sintomi della malattia in individui con una suscettibilità genetica di base, verosimilmente poligenica. Fino a ora sono stati individuati numerosi geni causativi tra cui quelli di maggiore rilevanza sono attualmente SOD1, FUS, TARDBP e C9ORF72. E' stato proposto che la tossicità indotta dai metalli di transizione possa essere coinvolta nella patologia e alte concentrazioni di metalli e proteine che regolano la loro omeostasi sono state descritte nei pazienti con SLA. In questo studio, è stato preso in esame un piccolo gruppo di pazienti (n=7) con diagnosi di SLA, provenienti da una ristretta area geografica della Liguria, che quindi condividono la stessa esposizione a fattori ambientali. I principali geni coinvolti nella malattia sono stati analizzati su DNA estratto da sangue periferico mediante sequenziamento diretto (SOD1, FUS, TARDBP) e Repeated Primer PCR (C9ORF72). Tutti i pazienti sono risultati negativi all'analisi genetica. Il siero dei pazienti è stato sottoposto all'analisi con ICP-MS per valutare le concentrazioni di diversi metalli (Fe, Zn, Se, Sr, Cd, Pb, Ni, Cu, Cr, As). I livelli di Ni e Pb sono risultati più alti nei pazienti rispetto a un gruppo di controlli sani (n=5) confrontabili per età e provenienti dalla stessa area geografica, viceversa per i livelli di As. Attualmente sono in corso sui sieri analisi di metalli più rari (Mn, Al, Co, V, U, Mo, Ag, Sn) e indagini di proteomica con Elettroforesi 2-D. Questo approccio permetterà di valutare l'influenza di uno dei fattori ambientali il cui coinvolgimento nella SLA è da lungo ipotizzato.

- *Riunione dei giovani biochimici dell'area Milanese – 20-22 March 2016*

Metallomics and proteomics of the biological fluids in ALS patients: preliminary data

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Introduction. Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder characterized by selective degeneration of both upper and lower motor neurons in the brain, brainstem, and spinal cord. This results in paralysis due to muscle weakness and atrophy, leading to death in 3-5 years. Genetic and environmental factors are involved in the pathogenesis of the disease and metals metabolism have been linked to ALS.

Methods. The study enrolled 7 patients and 5 controls (age matched, living in the same geographical area). For metal quantitation, samples were analyzed by ICP-MS. For proteomic analyses, immobilized pH gradient covered the 4-10 pH range. Image analyses were carried out with Image Master Software. Statistical analyses were carried out with Student's t-test.

Results. Among the 20 metals analyzed, As concentration resulted significantly lower in patients than in controls (p=0.007); Hg also was found in lower concentration in patients, but with a lower statistical significance (p=0.13). Higher concentration of Al in patients was detected (p=0.08). In this study, we were not able to confirm the higher concentrations of Ni and Pb in patients previously described in a smaller cohort. Our proteomics data show that APOA2 is decreased by 30% in patients with respect to controls. Furthermore, AHSG and SAP showed a significant decrease in patients with a story of more than 10 years of disease.

Conclusions. Impaired metal homeostasis, attributable to environmental exposure, could lead to mineral overload. Besides promoting oxidative stress, metals can compete for the binding sites of metal-containing proteins, such as those containing iron-sulfur clusters[3]. At present, no literature data link APOA2 to ALS, but the fact that its mRNA is processed by TDP43, provides a possible connection with the disease. The proteins differentially expressed belong to the group of Acute Phase Reaction proteins, possibly linking ALS to a chronic inflammation status.

- *ENCALS Meeting 2016 – 19-21 May 2016*

Metal and proteomic analysis of sporadic ALS patients with common geographical origin

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Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder characterized by selective degeneration of both upper and lower motor neurons in the brain, brainstem, and spinal cord. This results in paralysis due to muscle weakness and atrophy, leading to death in 3-5 years. Genetic and environmental factors are involved in the pathogenesis of the disease and metals metabolism have been linked to ALS. This study enrolled seven patients and five controls (age matched, living in the same geographical area). For metal quantitation, samples of serum were analyzed by ICP-MS. For proteomic analyses, immobilized pH gradient covered the 4-10 and 3- 7 pH range. Statistical analyses were carried out with Student's t-test and Artificial Neural Networks. Among the metals analyzed, As concentration resulted significantly lower in patients than in controls (p=0.007); Hg too was found in lower concentration in patients, but with a lower statistical significance (p=0.13). Higher concentration of Al in patients was detected (p=0.08). In this study, we were not able to confirm the higher concentrations of

Ni and Pb in patients previously described in a smaller cohort. Our proteomics data show that APOA2 is decreased by 30% in patients with respect to controls. Furthermore, AHSG and SAP showed a significant decrease in patients with a story of more than 10 years of disease. Impaired metal homeostasis, attributable to environmental exposure, could lead to mineral overload. Besides promoting oxidative stress, metals can compete for the binding sites of metalcontaining proteins, such as those containing iron-sulfur clusters. At present, no literature data link APOA2 to ALS, but the fact that its mRNA is processed by TDP43, provides a possible connection with the disease. The proteins differentially expressed belong to the group of Acute Phase Reaction proteins, possibly linking ALS to a chronic inflammation status. Further experiments are still ongoing.

- *FEBS/IUBMB Advanced Lecture Course "Molecular basis of human diseases: 50 years anniversary of Spetses summer schools" – 27th May-1st June 2016*

Serum metal concentrations and proteomics of ALS patients originating from a small geographical area

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Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder characterized by selective degeneration of both upper and lower motor neurons in the brain, brainstem, and spinal cord. This results in paralysis due to muscle weakness and atrophy, leading to death in 3-5 years. Genetic and environmental factors are involved in the pathogenesis of the disease and metals metabolism have been linked to ALS. This study enrolled seven patients and five controls (age matched, living in the same geographical area). For metal quantitation, samples of serum were analyzed by ICP-MS. For proteomic analyses, immobilized pH gradient covered the 4-10 and 3-7 pH range. Statistical analyses were carried out with Student's t-test and Artificial Neural Networks. Among the 20 metals analyzed, As concentration resulted significantly lower in patients than in controls (p=0.007); Hg too was found in lower concentration in patients, but with a lower statistical significance (p=0.13). Higher concentration of Al in patients was detected (p=0.08). In this study, we were not able to confirm the higher concentrations of Ni and Pb in patients previously described in a smaller cohort[3]. Our proteomics data show that APOA2 is decreased by 30% in patients with respect to controls. Furthermore, AHSG and SAP showed a significant decrease in patients with a story of more than 10 years of disease. Impaired metal homeostasis, attributable to environmental exposure, could lead to mineral overload. Besides promoting oxidative stress, metals can compete for the binding sites of metal-containing proteins, such as those containing iron-sulfur clusters. At present, no literature data link APOA2 to ALS, but the fact that its mRNA is processed by TDP43, provides a possible connection with the disease. The proteins differentially expressed belong to the group of Acute Phase Reaction proteins, possibly linking ALS to a chronic inflammation status. Further experiments are still ongoing.

- *XXI Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology – 14-16 September 2016*

Sporadic Amyotrophic Lateral Sclerosis in patients with common geographical origin: a multidisciplinary study.

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Amyotrophic Lateral Sclerosis, a fatal neurodegenerative disorder, is object of intensive research since the causes are still unknown and a treatment still lacks. Thanks to the recent technological improvements, the current trend of studies on this pathology points toward the analysis of huge groups of patients, in the search of new causative genes or diagnostic biomarkers. Being a rare disease however, to recruit a big number of patients, is necessary to analyze simultaneously subjects originating from completely different areas, thus losing the chance to evaluate patients with a common environmental exposure. This PhD project is aimed to study a small cohort of ALS subjects originating

from a restricted geographical area, with a multidisciplinary approach spanning from genetics to proteomics, to nutrition.

- *XIV Congresso FISV – Federazione Italiana Scienze della Vita – 20-23 September 2016*

A multidisciplinary approach to study Sporadic Amyotrophic Lateral Sclerosis in patients with common geographical origin.

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Amyotrophic Lateral Sclerosis (ALS), a fatal neurodegenerative disorder, is object of intensive research as the causes are still unknown and a treatment not available yet. This project is aimed to study, with a multidisciplinary approach, a small cohort of ALS subjects with a common environmental exposure. For metal quantitation, samples of serum and whole blood were analyzed by ICP-MS. For proteomic analyses, immobilized pH gradient covered the 4-10 and 3-7 pH range.
Arsenic concentration resulted significantly lower in patients than in controls. Also, Mn and Hg showed lower levels in patients. Levels of plasma APOA2 protein resulted decreased in patients with respect to controls, whereas SAMP showed a significant decrease only in the late onset group. APOA1 and TTHY also were decreased, the latter in late-onset patients. RET4 was decreased only in the early-onset group. When evaluating APOE genotype we found a 3-fold increase in the frequency of E3/E4 genotype in the patient's group. DNA oxidative stress has been evaluated through a Comet Assay. The multidisciplinary approach applied in this study allowed to dissect different aspects of ALS, often are evaluated separately and in heterogeneous cohorts of patients.

- *27th International Symposium on ALS/MND – 6-9 December 2016*

A multidisciplinary study of sALS in patients originating from a restricted geographical area.

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Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder characterized by selective degeneration of both upper and lower motor neurons in the brain, brainstem, and spinal cord. This results in paralysis due to muscle weakness and atrophy, leading to death in 3-5 years. Genetic and environmental factors are involved in the pathogenesis of this disease and metals metabolism has been linked to ALS. Proteomic studies are currently being performed to search for possible biomarkers. Here we present a study aimed at investigating different aspects of the disease, based on a multidisciplinary approach. The cohort of ALS patients that we analyzed includes seven patients, all originating from a common, restricted, geographical area and five matched controls. Environmental exposure is the same for all these subjects. SOD1, FUS, TDP43, C9ORF72 and APOE genotypes were evaluated. For metal quantitation, samples of serum and whole blood were analyzed by ICP-MS. For proteomic analyses, immobilized pH gradient covered the 4-10 and 3-7 pH range both in reducing and non-reducing conditions. Levels of DNA oxidation were evaluated by a comet assay. Statistical analyses were carried out with Student's t-test and Artificial Neural Networks. Among the metals analyzed in serum, As concentration resulted significantly lower in patients than in controls ($p=0.007$); Mn and Hg showed lower levels in patients. Auto-CM analysis linked closely high concentrations of Al and Se to the ALS group. Levels of metals in whole blood have been correlated with levels in serum. Our proteomics data show that some proteins related to Acute Phase Response (APR) and lipid homeostasis are decreased in patients (APOA1, APOA2, TTR, RET4 and SAP) while only ANT3 results increased. For some of these proteins we can describe a drastic reduction in the first 5 years of disease. Apoε4 allele is more represented in the patient's group than in controls'. Impaired metal homeostasis, attributable to

environmental exposure, could lead to mineral overload. Waters of the creek of the narrow valley where these subjects are located, are reported to be strongly polluted due to Acid Mine Drainage. Besides promoting oxidative stress, metals can compete for the binding sites of metal-containing proteins, such as those containing iron-sulfur clusters. The different expression of the APR proteins reported could be a reflection of the disease status of the subjects analyzed, possibly linking ALS to a chronic inflammation status. Enrichment in Apoε4 allele frequency in patients may provide a link between neurodegeneration and lipid metabolism disturbances. It is important to highlight the fact that all the proteins found differentially expressed in our study have already been described in other studies. This strengthens our methodological approach, based on a small number of patients but with a common environmental exposure.

PAPERS

- De Benedetti S, Lucchini G, Marocchi A, et al (2016) Serum metal evaluation in a small cohort of Amyotrophic Lateral Sclerosis patients reveals high levels of thiophylic species. Peptidomics 2:29–34. doi: 10.1515/ped-2015-0004

- De Benedetti S, Gianazza E, Banfi C, Marocchi A, Lunetta C, Penco S, Bonomi F, Iametti S. Serum proteome in a sporadic amyotrophic lateral sclerosis geographical cluster. Submitted

- De Benedetti S, Lucchini G, Del Bo' C, Deon V, Marocchi A, Penco S Lunetta C, Gianazza E, Bonomi F, Iametti S. Blood trace metals in a sporadic amyotrophic lateral sclerosis geographical cluster. Submitted

POSTERS

Micronutrients in a Neurodegenerative Disorder such as Amyotrophic Lateral Sclerosis

State of the art

Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder with an incidence of about 1/100.000 case per year. This progressive disease is characterized by a selective degeneration of both upper and lower motor neurons in the brain, brainstem, and spinal cord, resulting in paralysis due to muscle weakness and atrophy, leading to death in 3-5 years since the first manifestations of symptoms (Mitchell et al., 2007) (Figure 1). In a small percentage of the cases, dementia is observed.

Genetic and environmental factors are involved in the pathogenesis of this complex disease. In familial forms, mutations in several genes have been found segregating with the pathology in dominant, recessive and X-Linked pattern, giving to ALS the characteristic of an oligogenic disease (Table 1).

Neurodegenerative disorders such as ALS have been linked to iron and metals metabolism in different studies through the years (Crichton et al., 2006; Hadzhieva et al., 2013). Transition metal induced toxicity has been proposed to be involved in ALS (Carrì et al., 2003) and higher concentrations of metals and proteins that regulate metal homeostasis have been described in ALS patients (Roos et al., 2012). However, it is difficult to establish the real role of metals in ALS aetiology and progression since, when the pathology is diagnosed, all the motorneurons are already dead. In this context when we analyze micronutrients we don't know if we are measuring something that is partial cause of disease or if we are observing the endpoint of an unknown process started years before. Moreover the iron content of formulas usually employed in ALS Home Enteral Nutrition is significantly higher than the recommended daily intake for healthy individuals.
This PhD project aims to disclose the role of micronutrients in ALS in a cohort of patients bearing all the same mutation in TARDBP gene. This mutation leads to heterogeneous phenotypes (typical ALS, FTD, ALS and FTD, no symptoms) even in subjects with comparable ages. These subjects have a common geographical origin in a geographical restricted area (Acese - Catania), they share common diet, water, and exposure to environmental factors.
The analysis of micronutrients' concentrations, the evaluation of proteins that are involved in their homeostasis, and the analysis of the genes coding for these proteins - in different subjects with an heterogeneous phenotype - could help to clarify the effective role of micronutrients in ALS. Recent studies also indicate that the properties of epigallocatechin gallate may account for its neuroprotective capacity (Mandel et al., 2004) (Figure 2). A previous study performed on transgenic mouse model of ALS showed that an oral treatment with EGCG could improve clinical symptoms (Zhihao et al., 2006). Dietary implementation of these natural iron chelators should be taken into account as a strategy to prevent iron overload in relatives of patients with ALS, bearing the same mutation, without clinical symptoms.

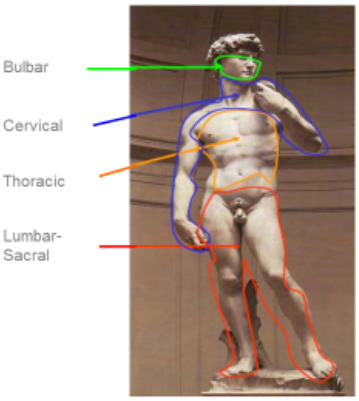

Figure 1 Regions of the body affected by motor neuron degeneration

Gene	Locus	Inheritance	Prevalence in fALS (%)	Clinical features
C9ORF72	9q21.2-p13.3	AD	35	ALS with FTD
SOD1	21q22.1	AD/AR	20	Typical ALS
FUS/TLS	16q12	AD/AR	4	Typical ALS
TARDBP	1p36	AD	5	ALS with FTD
VCP	9p13.3	AD	1-2	Adult onset, with or without FTD
UBQLN2	Xp11.21	X-linked	Rare	UMN signs proceeding LMN signs

Table 1 Main ALS causative genes - **Legend** AD: Autosomal Dominant; AR: Autosomal Recessive; fALS: Familiar Amyotrophic Lateral Sclerosis; FTD: Fronto-Temporal Dementia; UMN: Upper Motor Neuron; LMN: Lower Motor Neuron

Figure 2 Epigallocatechin gallate is the major constituent of green tea catechins

PhD Thesis Objectives and Milestones

Within the overall objective mentioned above this PhD thesis project can be subdivided into the following activities according to the Gantt diagram given in Table 2:

A1) **Sample collection** of blood, urine and liquor when available, of patients belonging to the cohort in analysis comprising mutated symptomatic patients, mutated asymptomatic patients and non mutated patients. All the mutated patients share the same mutation in TARDBP gene. Samples will be collected every 6 months.
A2) **Biochemical analysis** to evaluate micronutrients (Fe, Cu, Zn, Al, Mn) concentrations by ICP-MS in available biological fluids (A2.1) and plasmatic concentrations of Ferritin, Transferrin, Ceruloplasmin and Lactoferrin by 2D-Electrophoresis and Mass Spectrometry (A2.2). Measurements will be performed after each sample collection. Statistical analysis to assess any correlation of data obtained with the presence of the mutation in TARDBP and other vital parameters (A2.3).
A3) **Genetic analysis** of Hemocromatosis (HFE) will be performed by FRET technology in order to exclude that high levels of iron are attributable to variants in this gene (A3.1). DNA extraction, PCR amplification and sequencing of genes coding for the previously mentioned proteins will be performed with specific primers flanking the coding exons and the regulatory regions (A3.2).
A4) **Cellular models** will be created by transfecting transiently with either the wild type and the TARDBP mutated gene a human neuronal cellular model (SH-SY5Y neuroblastoma) (A4.1). Evaluation of expression and regulation of different intracellular proteins will be performed on this model (A4.2).
A6) **Writing and Editing** of the PhD thesis, scientific papers and oral and/or poster communications.

Table 2 *Gantt diagram for this PhD thesis project.*

Activity \ Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
A1) *Samples collection*																														
A2) *Biochemical analysis*																														
1) Micronutrients' concentrations																														
2) Plasmatic proteins concentrations																														
3) Statistical analysis																														
A3) *Genetic analysis*																														
1) HFE Genotyping																														
2) DNA extraction, PCR, Sequencing																														
A4) *Cellular model*																														
1) Transfection and culture																														
2) Intracellular proteins evaluation																														
A5) *Statistical analysis*																														
A6) *Thesis and Paper Preparation*																														

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XIX WORKSHOP
ON THE DEVELOPMENTS IN THE ITALIAN
PHD RESEARCH ON FOOD SCIENCE
TECHNOLOGY AND BIOTECHNOLOGY

Bari, September 24th-26th, 2014

Metals analysis in a small cohort of ALS patients originating from a restricted geographical area: preliminary data

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Introduction:

Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder with an incidence of about 1/100.000 case per year. It is characterized by a selective degeneration of both upper and lower motor neurons in the brain, brainstem, and spinal cord (Fig.1), resulting in paralysis due to muscle weakness and atrophy, leading to death in 3-5 years since the first manifestations of symptoms[1]. In a small percentage of the cases, dementia is observed. Neurodegenerative disorders such as ALS have been linked to iron and metals metabolism in different studies through the years[2-4]. Transition metal induced toxicity has been proposed to be involved in ALS[5] and higher concentrations of metals and proteins that regulate metal homeostasis have been described in ALS patients[6]. Thiophylic metals have been shown to be capable of displacing iron from reduced FeS proteins[7]. This may represent a yet underexplored target in the context of ALS pathogenesis.

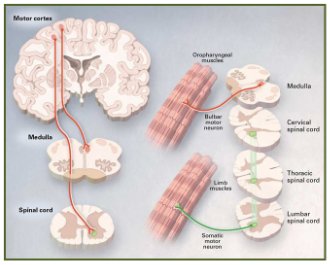

Fig.1 *Neuronal pathways affected in ALS*

Materials and Methods:

Samples of serum and urine were diluted (1:20 and 1:10, respectively) with 0.05% Triton X-100 in MilliQ water. Seronom™ Trace Elements Serum L-1 and Urine L-1 were used to build appropriate calibration curves. Samples were analyzed by ICP-MS (Bruker AURORA M90 ICP-MS). An internal standard solution (^{45}Sc, ^{89}Y, ^{159}Tb) was added to both samples and standards. Statistical analyses on the results have been carried out both with classical statistical elaborations (t-test and Principal Component Analysis) and with Auto CM algorithm, a special kind of Artificial Neural Network able to define the strength of the associations of each variable with all the others and to visually show the map of the main connections[8].

Results:

Analyses performed on serum samples highlighted elevated levels of Cr, Ni and Pb both in controls and in patients' group, if compared to literature data for the general population[9]. Higher concentrations of Ni and Pb were found in the patients' group, compared to the control's group (p-value = 0.0001 and 0.01). Surprisingly significant higher concentrations of As were found in the control's group (p-value = 0.05) (Fig. 2). Principal Component Analysis (PCA) confirmed these observations, and was able to discriminate between the two groups. The most important feature of the control group was the high concentration of As and a low concentrations of all the other metals analyzed. This observation was confirmed by Auto-CM analysis, that discriminated the two groups, clustering the control group with high levels of As (Fig. 3).

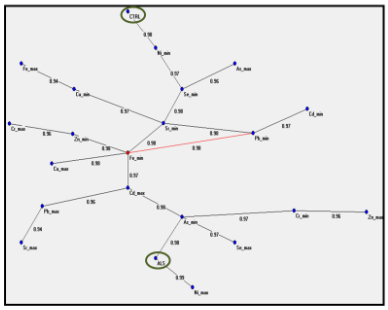

Fig.3 *Auto-CM analysis of metals' concentrations in serum*

Among the three metals that were significantly different, no one emerged as more relevant than the others in the discrimination of the control's group from the patient's group. Regarding urine analysis, all metals analyzed but Ni and Sr showed higher concentration than general population[9]. Intriguingly, Pb had low concentration both in patients' and in controls' urine, at contrast with its high levels found in serum analysis. Differences between patients and controls were significant for Fe (p = 0.01), Ni (p = 0.01), Zn (p = 0.008), As (p = 0.04), Sr (p = 0.02), Cd (p = 0.05). It must be noticed, by the way, that the control group consisted only of two subjects, different for age and geographical origin from the patients' group. PCA analysis discriminated the two groups: the control's group had low levels of metals, and the patient's group higher metal levels, with a patients' sub-group having high levels of As.

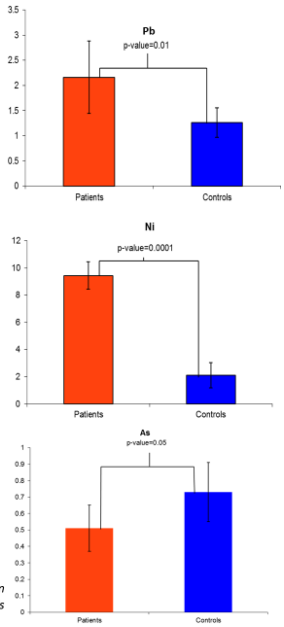

Fig.2 *Statistically significant elements in serum metal analysis*

Discussion:

Despite much research, the etiology of Amyotrophic Lateral Sclerosis still has to be clarified. Only a small percentage of the cases is attributable to genetic defects[10] and environmental factors could play a crucial role. Here we report the preliminary results of the analysis of metals' status in a small cohort of subjects originating from a restricted area, thus sharing environmental exposure. Our results confirm the hypothesis of a possible association between Pb exposure and ALS[11, 12] and provides further suggestions. The first one regards Ni, higher in ALS patients, and a second one, probably more unusual, is that high As levels have been found in the control's group. Further studies will confirm these results in other cohorts of subjects. We have also planned to perform an evaluation of serum proteins through a proteomic approach in order to expand the knowledge on the effect of dysregulated metals homeostasis on circulating proteins.

Connections to FeS proteins:

A great part of the lead damage in cellular physiology is caused by its ability to substitute for diverse polyvalent cations in their binding sites[13]. Pb and Ni have substantial affinity for protein thiols. Direct substitution of iron in various types of FeS clusters by thiophylic metals has been demonstrated [7]. Arsenic is known to interact with thiol-rich proteins, such as glutaredoxin, involved in FeS proteins biosynthesis, making it plausible that dysregulation of homeostasis of these metals could affect FeS-protein dependent events, opening a new and still unexplored area in ALS research.

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Metals and proteomics of the biological fluids in ALS patients: Preliminary data

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Introduction:

Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder with an incidence of about 2.1/100.000 case per year[1]. It is characterized by a selective degeneration of both upper and lower motor neurons in the brain, brainstem, and spinal cord, resulting in paralysis due to muscle weakness and atrophy, leading to death in 3-5 years since the first manifestations of symptoms[2]. Mutations in several genes, including SOD1, FUS and TARDBP and C9ORF72 hexanucleotide expansion have been identified as responsible for the disease in the familial forms[1]. Neurodegenerative disorders such as ALS have been linked to iron and metals metabolism in different studies through the years[3-5]. Transition metal induced toxicity has been proposed to be involved in ALS[6] and higher concentrations of metals and proteins that regulate metal homeostasis have been described in ALS patients[7]. This poster reports the preliminary results of the analyses performed on a cohort of subject with defined sporadic ALS all originating from a restricted geographical area (7 patients and 5 controls); the same environmental exposure could help to minimize the differences among the subjects under investigation.

Materials and Methods:

Blood was collected from all subjects. ALS diagnosis was according El Escorial criteria with clinically defined sporadic cases; all patients were genotyped for the main ALS genes (SOD1, FUS, TARDBP, C9ORF72) through direct sequencing and RP-PCR. Samples of serum were diluted 1:20 with 0.05% Triton X-100 in MilliQ water. Seronom™ Trace Elements Serum L-1 was used to build appropriate calibration curves. Samples were analyzed by ICP-MS (Bruker AURORA M90 ICP-MS).
For proteomic analyses, immobilized pH gradient strips for the 1st d (non linear pH range 4-10, 8 cm x 0.8 cm) were prepared and 600-700 µg of proteins, reduced with 1% 2-mercaptoethanol, were loaded near the cathode. The 2nd dimension was run on a gradient polyacrylamide gel. Image analyses of the Coomassie Blue stained gels were carried out with Image Master Software ver. 5.0. Selected spots were sent off to Mass Spectrometry (MS) analysis to identify the corresponding proteins.
Statistical analyses on the results have been carried out both with classical statistical elaborations (t-test and Principal Component Analysis) and with Auto CM algorithm, a special kind of Artificial Neural Network able to define the strength of the associations of each variable with all the others and to visually show the map of the main connections[8].

Results and Discussion:

- Genetic analyses gave negative results in all the patients, allowing us to rule out at least the most frequently mutated genes as disease causes.
- Analyses performed on serum samples highlighted elevated levels of Cr, Ni and Pb both in controls and in patients' group, if compared to literature data for the general population[9]. Higher concentrations of Ni and Pb were found in the patients' group (p-value = 0.0001 and 0.01). Surprisingly significant higher concentrations of As were found in the control's group (p-value = 0.05) (Tab 1). Auto-CM analysis, discriminated the two groups, linking the control group to high levels of As. Currently we are performing new analyses on a panel of rarer metals (Mn, Al, Co, V, U, Mo, Ag, Sn).

Element	Average Patients ± SD (µg/L)	Average Controls ± SD (µg/L)	Reference Values (µg/L)[9]
Cr	1.57 ± 0.12	1.54 ±0.06	0.07-0.28
Fe	1261.28 ± 429.00	1225.94 ± 160.00	648-1301
Ni	9.44 ± 1.02*	2.10 ± 0.92*	0.26-0.75
Cu	1130.24 ± 157.00	1141.55 ± 108.00	648-1301
Zn	811.03 ± 114.00	835.88 ± 72.40	597-1028
As	0.51 ± 0.14*	0.73 ± 0.18*	NA
Se	97.71 ± 10.20	89.54 ± 6.32	56-105
Sr	39.73 ± 12.50	34.54 ± 5.71	23-61.5
Cd	0.08 ± 0.03	0.06 ± 0.01	0.03-0.2
Pb	2.16 ± 0.72*	1.26 ± 0.29*	0.2-0.98

*Tab 1. Averages of the measures of metals concentrations in sera. *: p-value ≤ 0.05, NA: Not Available.*

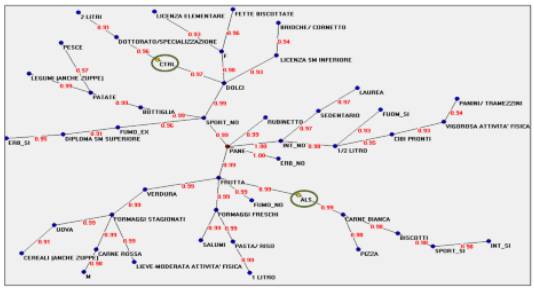

Fig 1. Semantic connectivity map showing the connections between the variables. Values on the arches refer to the strength of the association between two adjacent nodes, the range is from 0 to 1.

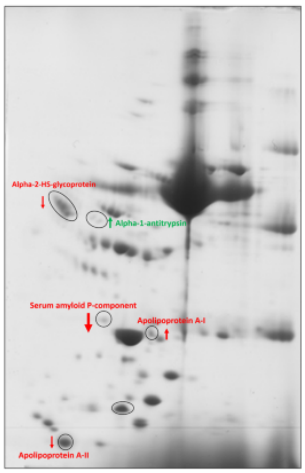

Fig 2. 2D-E gel showing the significant spots identified. Red: Negative APR proteins, Green Positive APR protein.

- Patients and controls were administered a questionnaire consisting in questions about employment, nutrition and diet, smoke and physical activity. Data were elaborated with ANNs that were able to discriminate between ALS patients and control's group, suggesting features common to the subjects belonging to each group. Interestingly, the closest connection to the disease was related to fruit consumption (Fig 1): fruit intake is quite common in the Mediterranean diet, but it could relate to the hypothesized involvement of pesticides in the etiology of the ALS disease too[10]. Further investigation in this direction seems required. We are planning to administer to the subjects a new questionnaire.
- To assess the results from 2DE experiments, the integrated volumes of the spots were compared between the controls' group and the patients' group. The statistical significance of the differences was evaluated with Student's t-test. Results are showed in Table 2. The most significant difference regards **Apolipoprotein A-II** that is decreased by 30% in patients with respect to controls. At present no literature data link this protein to ALS, but the fact that its mRNA is processed by TDP43[11], - a protein involved in ALS pathogenesis - provides a possible connection with the disease. **Alpha-2-HS-glycoprotein** and **Serum amyloid P-component** also showed a significant decrease in one group of patients. All these proteins are negative regulators of the Acute Phase Response (APR). Interestingly, the only protein significantly overexpressed in patients is **Alpha-1-antitrypsin**, a positive regulator of the APR (Fig. 2). These data give an insight into the inflammatory component of ALS disease. At present we are performing new experiments to confirm these results and to evaluate lower-abundance proteins in serum.

Protein	All	Duration ≤ 4 years (n = 4)	Duration ≥ 10 years (n = 3)	Onset ≤ 60 years (n = 4)	Onset > 60 years (n = 3)
Alpha-2-HS-glycoprotein	=	=	↓↓*	=	=
Serum amyloid P-component	=	=	↓↓↓*	=	↓↓↓*
Apolipoprotein A-I	↑**	=	↑**	=	↑**
Apolipoprotein A-II	↓*	↓*	=	↓**	↓**
Alpha-1-antitrypsin	↑**	=	↑**	=	↑*

*Tab 2. Different expression of the proteins identified with 2D-E in the comparison between patients and controls and in different subgroups. ↓protein decrease in patients, ↑ protein increase in patients, * $p < 0.05$, **$p < 0.01$*

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XX WORKSHOP
ON THE DEVELOPMENTS IN THE ITALIAN PHD RESEARCH ON FOOD SCIENCE, TECHNOLOGY AND BIOTECHNOLOGY
PERUGIA, SEPTEMBER 23TH-25TH, 2015

Metals concentrations in Amyotrophic Lateral Sclerosis patients originating from a restricted geographical area

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Introduction:

Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder with an incidence of about 1/100.000 case per year. It is characterized by a selective degeneration of both upper and lower motor neurons in the brain, brainstem, and spinal cord (Fig.1), resulting in paralysis due to muscle weakness and atrophy, leading to death in 3-5 years since the first manifestations of symptoms[1]. Neurodegenerative disorders such as ALS have been linked to iron and metals metabolism in different studies through the years[2-4]. Transition metal induced toxicity has been proposed to be involved in ALS[5] and higher concentrations of metals and proteins that regulate metal homeostasis have been described in ALS patients[6]. This poster reports the preliminary results of the analyses performed on a cohort of subject with defined ALS all originating from a restricted geographical area (7 patients and 5 controls). We applied a change in the approach to the study of ALS, by choosing to focus on a restricted cohort of subjects, in order to analyze different aspects of this multifactorial disease: the same environmental exposure could help to minimize the differences among the subjects under investigation.

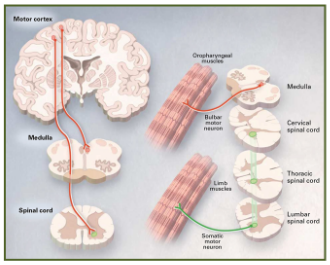


Fig.1 *Neuronal pathways affected in ALS*

Materials and Methods:

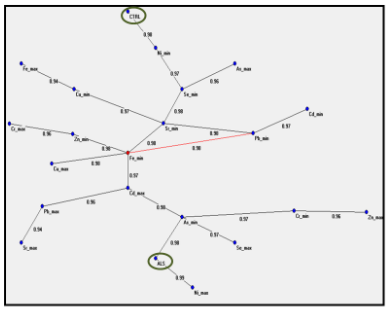
All subjects gave informed consent after genetic counselling and blood was collected. ALS diagnosis was according El Escorial criteria; genomic DNA was extracted according to standard procedures and all patients were genotyped for the main ALS genes: SOD1 whole gene, FUS exons 5-6-13-14-15, TARDBP exon 6 through direct sequencing and C9ORF72 G_4C_2 hexanucleotide repeat through RP-PCR[7]. Serum was obtained after centrifugation and stored at -80°C. Samples of serum and urine were diluted (1:20 and 1:10, respectively) with 0.05% Triton X-100 in MilliQ water and analyzed by ICP-MS (Bruker AURORA M90 ICP-MS). Statistical analyses on the results have been carried out both with classical statistical elaborations (t-test and Principal Component Analysis) and with Auto CM algorithm, a special kind of Artificial Neural Network able to define the strength of the associations of each variable with all the others and to visually show the map of the main connections, that has already been used in ALS studies[8].

Results:

Genetic analyses gave negative results in all the patients, allowing us to rule out at least the most frequently mutated genes as disease causes. Analyses performed on serum samples highlighted elevated levels of Cr, Ni and Pb both in controls and in patients' group, if compared to literature data for the general population[8]. Higher concentrations of Ni and Pb were found in the patients' group, compared to the control's group (p-value = 0.0001 and 0.01). Surprisingly significant higher concentrations of As were found in the control's group (p-value = 0.05) (Tab. 1). Principal Component Analysis (PCA) confirmed these observations, and was able to discriminate between the two groups. The most important feature of the control group was the high concentration of As and a low concentrations of all the other metals analyzed. This observation was confirmed by Auto-CM analysis, that discriminated the two groups, clustering the control group with high levels of As (Fig. 2).

Element	Average Patients ± SD (µg/L)	Average Controls ± SD (µg/L)	Reference Values (µg/L)[9]
Cr	1.57 ± 0.12	1.54 ±0.06	0.07-0.28
Fe	1261 ± 429	1225 ± 160	648-1301
Ni	9.44 ± 1.02*	2.10 ± 0.92*	0.26-0.75
Cu	1130 ± 157	1141 ± 108	648-1301
Zn	811 ± 114	835 ± 72	597-1028
As	0.51 ± 0.14*	0.73 ± 0.18*	NA
Se	97 ± 10	89 ± 6	56-105
Sr	39 ± 12	34 ± 5	23-61.5
Cd	0.08 ± 0.03	0.06 ± 0.01	0.03-0.2
Pb	2.16 ± 0.72*	1.26 ± 0.29*	0.2-0.98

Tab 1. *Averages of the measures of metals concentrations in sera.*
**: p-value ≤ 0.05, NA: Not Available.*



Among the three metals that were significantly different, no one emerged as more relevant than the others in the discrimination of the control's group from the patient's group. Regarding urine analysis, all metals analyzed but Ni and Sr showed higher concentration than general population[9]. Intriguingly, Pb had low concentration both in patients' and in controls' urine, at contrast with the high levels of this metal found in serum analysis. Differences between patients and controls were significant for Fe (p = 0.01), Ni (p = 0.01), Zn (p = 0.008), As (p = 0.04), Sr (p = 0.02), Cd (p = 0.05). However, it must be noticed that the control group consisted only of two subjects, different for age and geographical origin from the patients' group. PCA analysis discriminated the two groups: the control's group had low levels of metals, and the patient's group had higher metal levels, with only a patients' sub-group was showing high levels of As.

Fig. 2 *Auto-CM analysis of metals' concentrations in serum: Semantic connectivity map showing the connections between the variables. Values on the arches refer to the strength of the association between two adjacent nodes, the range is from 0 to 1.*

Discussion:

Despite much research, the etiology of Amyotrophic Lateral Sclerosis still has to be clarified. Only a small percentage of the cases is attributable to genetic defects[10], and environmental factors could play a crucial role. Here we report the preliminary results of the analysis of metals' status in a small cohort of subjects originating from a restricted area, thus sharing environmental exposure. Our results confirm the hypothesis of a possible association between Pb exposure and ALS[11, 12] and provides further suggestions. The first one regards Ni, higher in ALS patients, and a second one, probably more unusual, is that high As levels have been found in the control's group. A great part of the lead damage in cellular physiology is caused by its ability to substitute for diverse polyvalent cations in their binding sites[13]. Pb and Ni have substantial affinity for protein thiols. Direct substitution of iron in various types of FeS clusters by thiophylic metals has been demonstrated[14]. Arsenic is known to interact with thiol-rich proteins, such as glutaredoxin, involved in FeS proteins biosynthesis, making it plausible that dysregulation of homeostasis of these metals could affect FeS-protein dependent (or related) events, opening a new and still unexplored area in ALS research.

Further studies will be aimed at evaluating a panel of rarer metals (Mn, Al, Co, V, U, Mo, Ag, Sn). We have also planned to perform an evaluation of serum proteins through a proteomic approach in order to expand the knowledge on the effect of dysregulated metals homeostasis on circulating proteins.

We believe that the study of affected subjects in such geographic isolates would provide a representative model for the evaluation of environmental influences on Amyotrophic Lateral Sclerosis.

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Metallomics and proteomics of the biological fluids in ALS patients: preliminary data

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Introduction:

Amyotrophic Lateral Sclerosis (ALS) is a rare neurodegenerative disorder with an incidence of about 2.8/100.000 case per year in Europe. The main neuropathological features are degeneration of the corticospinal tract and extensive loss of lower motor neurons. This results in paralysis due to muscle weakness and atrophy, leading to death in 2-4 years since the first manifestations of symptoms[1-2]. Mutations in several genes, including SOD1, FUS and TARDBP and C9ORF72 hexanucleotide expansion have been identified as responsible for the disease in the familial forms[1]. Neurodegenerative disorders such as ALS have been linked to iron and metals metabolism in different studies through the years[3-5]. Transition metal induced toxicity has been proposed to be involved in ALS[6]. Proteomic studies are currently being performed to search for possible biomarkers[7]. This poster reports the preliminary results of the analyses performed on a cohort of subject with defined sporadic ALS all originating from a restricted geographical area (7 patients and 5 controls); the same environmental exposure could help to minimize the differences among the subjects under investigation.

Materials and Methods:

Blood was collected from all subjects. ALS diagnosis was according El Escorial criteria with clinically defined sporadic cases; all patients were genotyped for the main ALS genes (SOD1, FUS, TARDBP, C9ORF72) through direct sequencing and RP-PCR. Samples of serum were diluted 1:20 with 0.05% Triton X-100 in MilliQ water. Seronom™ Trace Elements Serum L-1 was used to build appropriate calibration curves. Samples were analyzed by ICP-MS (Bruker AURORA M90 ICP-MS).

For proteomic analyses, immobilized pH gradient strips for the 1st d (non linear pH range 4-10, 8 cm x 0.8 cm) were prepared and 600-700 µg of proteins, reduced with 1% 2-mercaptoethanol, were loaded near the cathode. The 2nd dimension was run on a gradient polyacrylamide gel. Image analyses of the Coomassie Blue stained gels were carried out with Image Master Software ver. 5.0. Selected spots were sent off to Mass Spectrometry (MS) analysis to identify the corresponding proteins. Statistical analyses on the results have been carried out with classical statistical elaborations

Results:

- Genetic analyses gave negative results in all the patients, allowing us to rule out at least the most frequently mutated genes as disease causes.
- Analyses performed on serum samples (Tab. 1) highlighted elevated levels of Al, Ba and V both in controls and in patients' group compared to literature data for the general population[8]. The most significant results are a higher concentration of Al in the group of ALS patients (p = 0,08) and vice versa a lower concentration of As (p = 0,007) in this group, if compared to the control's one. This last result confirms the our previous findings[9]. Hg concentration resulted low also in the patients' group. The p-value close to the statistical significance (p= 0,013), so that a possible involvement of Hg can't be excluded.
- To assess the results from 2DE experiments, the integrated volumes of the spots were compared between the controls' group and the patients' group. The statistical significance of the differences was evaluated with Student's t-test. Results are showed in Table 2. The most significant difference regards **Apolipoprotein A-II** that is decreased by 30% in patients with respect to controls. **Alpha-2-HS-glycoprotein** and **Serum amyloid P-component** also showed a significant decrease in one group of patients. All these proteins are negative regulators of the Acute Phase Response (APR). Interestingly, the only protein significantly overexpressed in patients is **Alpha-1-antitrypsin**, a positive regulator of the APR (Fig. 1).

Element	Average Patients ± SD (µg/L)	Average Controls ± SD (µg/L)	Reference Values (µg/L)*
V	0,99 ± 0,12	0,95 ± 0,12	0,03-0,11
Mn	1,30 ± 0,64	1,89 ±1,41	0,31-1,02
Co	0,50 ± 0,08	0,51 ± 0,03	0,06-0,42
Ni	2,83 ± 0,32	3,00 ±0,42	0.26-0.75
As	0,35 ± 0,03*	0,44 ± 0,07*	NA
Sn	0,14 ± 0,02	0,17 ± 0,07	0,27-1,69
Ba	14,71 ± 5,88	12,75 ± 2,14	0,32-1,37
Hg	0,83 ± 0,75	1,62 ± 0,90	0,32-2,75
Pb	1,04 ± 0,40	0,83± 0,39	0,20-0,98
U	0,03 ± 0,01	0,02 ± 0,01	NA
Al	23,9 ± 5,2*	17,2 ± 6,9*	0,4-5,3

Tab 1. *Averages of the measures of metals concentrations in sera. *: p-value ≤ 0.05, NA: Not Available.*

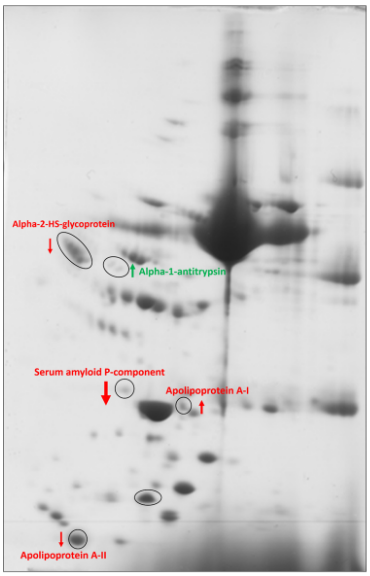

Fig 1. *2D-E gel showing the significant spots identified. Red: Negative APR proteins, Green Positive APR protein.*

Conclusions:

Altered metal's concentrations could be possibly related to environmental exposure, due to the presence in the area, where subjects involved in this study originate, of waters reported to be strongly polluted due to Acid Mine Drainage[10]. The lower levels of As found in patients is of particular interest since it is known that its metabolism in cells elicits the generation of oxidative stress[11]. Besides this, metals can compete for the binding sites of metal-containing proteins, such as those containing iron-sulfur clusters[9]. Regarding proteomics data, the most relevant result is the lower levels of APOA2 protein. At present no literature data link this protein to ALS, but the fact that its mRNA is processed by TDP43[12], - a protein involved in ALS pathogenesis - provides a possible connection with the disease. Taken together, these data give an insight into the metal status and the inflammatory component of ALS disease.

Protein	All	Duration ≤ 4 years (n = 4)	Duration ≥ 10 years (n = 3)	Onset ≤ 60 years (n = 4)	Onset > 60 years (n = 3)
Alpha-2-HS-glycoprotein	=	=	↓↓*	=	=
Serum amyloid P-component	=	=	↓↓↓*	=	↓↓↓*
Apolipoprotein A-I	↑**	=	↑**	=	↑**
Apolipoprotein A-II	↓*	↓*	=	↓**	↓**
Alpha-1-antitrypsin	↑**	=	↑**	=	↑*

Tab 2. *Different expression of the proteins identified with 2D-E in the comparison between patients and controls and in different subgroups. ↓protein decrease in patients, ↑ protein increase in patients, * p < 0.05, **p < 0.10*

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METAL AND PROTEOMIC ANALYSIS OF SPORADIC ALS PATIENTS WITH COMMON GEOGRAPHICAL ORIGIN

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Introduction:

Neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS) have been linked to iron and metals metabolism in different studies through the years[1,2]. Transition metal induced toxicity has been proposed to be involved in ALS[3]. Several researchers have analyzed different groups of patients with non similar environmental exposure by investigating metals in different tissues, but these studies have produced contrasting results[4-7]. Proteomic studies are currently being performed to search for possible biomarkers[8,9]. At present, few studies on gel-based proteomics in ALS are reported, performed on different tissues[10-13], but none on serum. This poster reports the preliminary results of a study performed on a cohort of subjects with defined sporadic ALS, all originating from a restricted geographical area (7 patients and 5 controls), so that the same environmental exposure could help to minimize the differences among the subjects under investigation.

Materials and Methods:

Blood was collected from all subjects. ALS diagnosis was according El Escorial criteria with clinically defined sporadic cases; all patients were genotyped for the main ALS genes (SOD1, FUS, TARDBP, C9ORF72). Samples of serum were analyzed by ICP-MS for metal quantification and results have been evaluated through classical statistical methods and with Auto CM algorithm[14]. For proteomic analyses, immobilized pH gradient strips for the 1std were prepared, proteins were reduced with 1% 2-mercaptoethanol. The 2nd dimension was run on a gradient polyacrylamide gel. Selected spots were identified by Mass Spectrometry (MS). Classical statistical analyses have been applied on the results.

Results:

Genetic analyses gave negative results in all the patients, allowing us to rule out at least the most frequently mutated genes as disease causes. Analyses performed on serum samples (Tab. 1) highlighted elevated levels of Al, Ni, Cr, Ba and V both in controls and in patients if compared to the reference values for the Italian population[15]. Only the As concentrations were significantly different between the two groups and, quite surprisingly, As resulted lower in patients (p = 0,01), as did Mn (p = 0,10) and Hg, two well known neurotoxic elements. Auto-CM analysis linked closely high concentrations of Al and Se to the ALS group (Fig.1). However, according to the t-test, the differences among the two groups were not statistically significant (p = 0,13 and p=0,12).

To assess the results from 2DE experiments, the integrated volumes of the spots were compared between the controls' group and the patients' group. The statistical significance of the differences was evaluated with Student's t-test. Results are shown in Table 2. In the first set of experiments (NL pH gradient 4-10) **APOA2** protein resulted decreased by 30% in patients with respect to controls. **SAMP** showed a significant decrease only in the group of patients with late onset. When we focused on the acid portion of IEF (NL pH gradient 3-7) **APOA1** and **TTHY** were decreased, the former particularly in late-onset patients. Only **ANT3** resulted increased in patients, particularly in the early-onset group. Finally, **RET4** was decreased only in the early-onset group (Fig 2). The trend in concentrations of proteins according to the years from disease onset is reported in Fig. 3. It is remarkable that some proteins undergo a quite gradual decrease (TTHY, RBP4, ZA2G), whereas others show a drastic reduction in the first 5 years of disease (APOA1, APOA2, HPTα, FETUA).

Element	Average Patients ± SD (µg/L) [n = 6]	Average controls ± SD (µg/L) [n = 5]	p-value	Reference Values (µg/L)[15]
As	0.34 ± 0.03	0.44 ± 0.07	0.01	NA
Al	23.22 ± 5.37	17.15 ± 6.88	0.13	0.4-5.3
Mn	1.33 ± 0.69	2.36 ± 1.12[a]	0.10	0.31-1.02
Se	100.2 ± 11.9	89.5 ± 8.2	0.12	56-105
Ni	2.82 ± 0.35	3.00 ± 0.42	0.45	0.26-0.75
Pb	1.09 ± 0.41	0.83 ±0.40	0.29	0.20-0.98
Hg	0.90 ± 0.80	1.62 ± 0.90	0.20	0.32-2.75
Cu	1140 ± 216	1142 ± 136	0.99	648-1301
Fe	1165 ± 521	1225 ± 202	0.81	886-2455
Zn	846 ± 151	835 ± 105	0.90	597-1028
Co	0.49 ± 0.09	0.51 ± 0.03	0.65	0.06-0.42
Cr	1.56 ± 0.18	1.54 ± 0.08	0.84	0.07-0.28
Ba	13.26 ± 4.87	12.75 ± 2.14	0.83	0.32-1.37
Sn	0.14 ± 0.03	0.17 ± 0.07	0.45	0.27-1.69
U	0.03 ± 0.01	0.02 ± 0.01	0.75	NA
V	0.96 ± 0.11	0.94 ± 0.12	0.79	0.0.-0.11
Sr	38.4 ± 19.7	34.5 ± 7.2	0.69	23-61.5

Tab 1. Averages of the measures of metals concentrations in serum.
NA: Not Available. *One subject from controls not analyzed.

Protein	All	Onset ≤ 60 years (n = 4)	Onset > 60 years (n = 3)
Apolipoprotein A-I	-17 % *	=	-22 % **
Transthyretin	-30 % *	-28 % **	-32 % **
Antithrombin-III	+71 % *	+71 % **	=
Retinol-binding protein 4	=	-25 % **	=
Serum amyloid P-component	=	=	-77 % *
Apolipoprotein A-II	-30 % *	-29 % **	-33 % **

Tab. 2 Different expression of the proteins identified with 2D-E in the comparison between patients and controls, showed as percentage variation. *p ≤ 0.05, **p ≤ 0.1

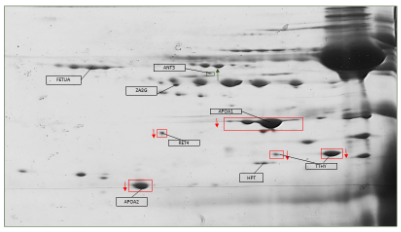

Fig.1 2D-E gel showing the significant spots identified. Red shapes: proteins decreased in patients; Green shapes: proteins increased in patients; No shape: other relevant proteins.

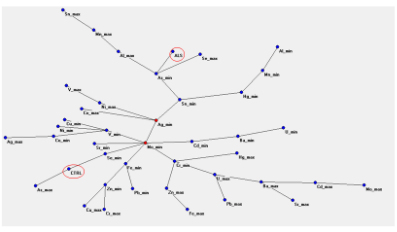

Fig.2 Semantic connectivity map showing the connections between metals concentrations, ALS group and Control group. Adjacent nodes have the strongest association.

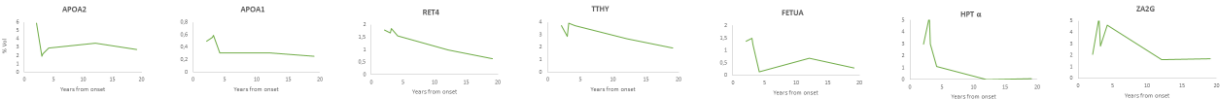

Fig.3 Graphics showing the evolution of proteins concentrations during the course of the disease. Each patient has been plotted on the x-axis according to the time from onset of the disease.

Conclusions:

Altered metals' concentrations could be possibly related to environmental exposure, due to the presence in the area, where subjects involved in this study originate, of waters reported to be strongly polluted due to Acid Mine Drainage[16]. The lower levels of As found in patients is of particular interest since it is known that its metabolism in cells elicits the generation of oxidative stress. Metals found in lower concentration in patients' sera could reflect their accumulation in some body districts/tissues, where they exert toxic effects. Besides this, metals can compete for the binding sites of metal-containing proteins, such as those containing iron-sulfur clusters[17].

Regarding proteomics data, the proteins in which we registered an alteration, are involved in the Acute Phase Response. Indeed the different expression with respect to controls could be referred to the disease status of the subject analyzed. We also noticed an alteration in some proteins related to lipid homeostasis, that is consistent with the proposed metabolic shift towards an increased peripheral use of lipids[18]. However, we would like to highlight the fact that all the proteins found differentially expressed in this study have already been described in other studies.

In this context, despite the small group analyzed here, we found our data comparable to studies involving much a much higher number of patients, strengthening our approach, based on a small number of patients but with a common environmental exposure.

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A MULTIDISCIPLINARY APPROACH TO STUDY SPORADIC AMYOTROPHIC LATERAL SCLEROSIS IN PATIENTS WITH COMMON GEOGRAPHICAL ORIGIN.

S. De Benedetti[1], G. Lucchini[2], A. Marocchi[3], S. Penco[3], C. Lunetta[4], S. Iametti[1], E. Gianazza[5], F. Bonomi[1]
[1]DeFENS, [2]DiSAA, [5]DiSFeB - University of Milan, [3]Medical Genetics Unit, Department of Laboratory Medicine,
[4]NEuroMuscular Omnicentre (NEMO), Fondazione Serena Onlus - Niguarda Ca' Granda Hospital.

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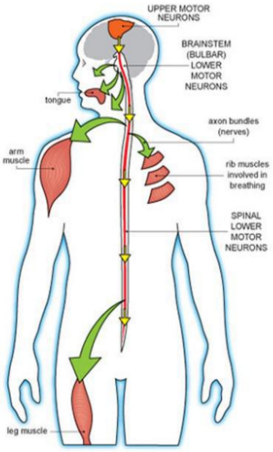
Introduction:

Neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS) have been linked to iron and metals metabolism in different studies through the years[1,2]. Transition metal induced toxicity has been proposed to be involved in ALS[3]. Several researchers have analyzed different groups of patients with non similar environmental exposure by investigating metals in different tissues, but these studies have produced contrasting results[4-7]. Proteomic studies are currently being performed to search for possible biomarkers[8,9]. At present, few studies on gel-based proteomics in ALS are reported, performed on different tissues[10-13], but none on serum. This poster reports the results of a study performed on a cohort of subjects with defined sporadic ALS, all originating from a restricted geographical area (7 patients and 5 controls), so that the same environmental exposure could help to minimize the differences among the subjects under investigation.



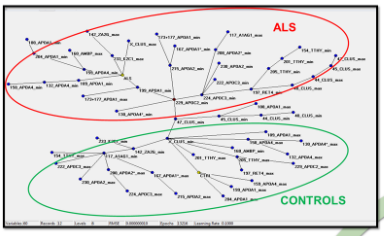
Materials and Methods:

Blood was collected from all subjects. ALS diagnosis was according El Escorial criteria with clinically defined sporadic cases; all patients were genotyped for the main ALS genes (SOD1, FUS, TARDBP, C9ORF72). Samples of serum were analyzed by ICP-MS for metal quantification and results have been evaluated through classical statistical methods and with the Auto CM algorithm[14]. For proteomic analyses, immobilized pH gradient strips for the 1st d were prepared. Both reducing conditions (1% 2-mercaptoethanol), and non reducing conditions were evaluated. The 2nd dimension was run on a gradient polyacrylamide gel. Selected spots were identified by Mass Spectrometry (MS). Comet assay was performed on 5µL of whole blood.

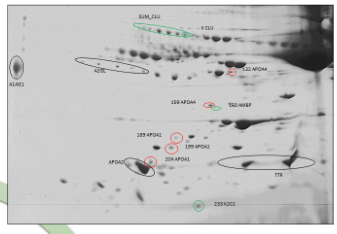
Results:

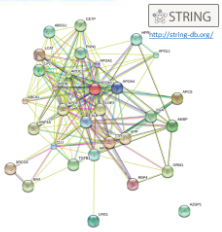
Proteomics

DECREASED ABUNDANCE IN PATIENTS	INCREASED ABUNDANCE IN PATIENTS
APOA1	ANT3
APOA2	AMBP
APOA4	K2C1
RET4	CLU
TTR	
INCREASED DURING DISEASE COURSE	
A1AG1	A2GL

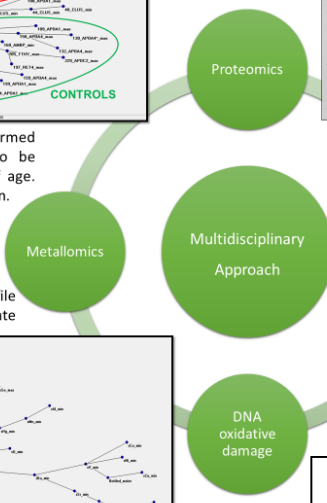


The Table reports the combined results of the 2-DEs performed in the two described conditions. RET4 was found to be decreased only in patients with onset after 60 years of age. A1AG1 and A2GL positively correlated with disease duration. TTR and APOA2 were significantly decreased in runs performed in reducing conditions and showed a negative correlation with disease course in the experiments performed in non reducing conditions.
Auto-CM analyses helped to define the peptidomic profile characteristic for ALS patients; it was also able to associate fragments of the same proteins.



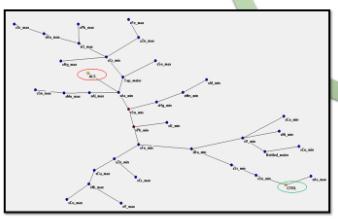


The 2DE performed in non-reducing conditions shows the localization of proteins/peptides found with differential abundance. STRING software analysis shows the close functional interconnections between them.



Metallomics

ICP-MS analyses showed high concentrations of Al, Ni, Cr, Ba and V in serum and Mn, Zn, Co and Cr in whole blood both in patients and controls, in comparison with reference values for Italian population[15] serum concentration.
Only the serum concentration of As was significantly lower in patients.
Auto-CM associated higher levels of metals analyzed with the ALS group, except for arsenic. Tap water consumption was associated with ALS group.



APOE Genotyping

Population	Allele			Genotype					
	ε2	ε3	ε4	ε2/ε2	ε2/ε3	ε3/ε3	ε2/ε4	ε3/ε4	ε4/ε4
Caucasian	0.08	0.78	0.14	0.01	0.12	0.61	0.02	0.22	0.02
Study Controls	0.10	0.80	0.10	0.00	0.20	0.60	0.00	0.20	0.00
Study Patients	0.00	0.71	0.29	0.00	0.00	0.43	0.00	0.57	0.00

APOE genotype was evaluated, since the protein is involved in lipid homeostasis and has been associated to neurological disorders. Despite the low number of subjects, frequencies in controls are comparable to those reported for the Caucasian population.
The risk allele ε4 is more frequent in the ALS cohort than in control subjects.

Comet Assay

p = 0,943

Patients % DNA in tails = 8,30 ± 2,52 ⟷ Controls % DNA in tails = 8,18 ± 3,08

No significant differences between patients and controls
No correlations with metal levels

Conclusions:

Altered metals' concentrations could be possibly related to environmental exposure, due to the presence in the area the subjects where from of waters reported to be strongly polluted due to Acid Mine Drainage[16]. The lower levels of As found in patients is of particular interest since it is known that its metabolism in cells elicits the generation of oxidative stress. Metals found in lower concentration in patients' sera could reflect their accumulation in specific (yet unknown) body districts/tissues, where they exert toxic effects. Besides, metals can compete for binding sites in some metalloproteins, such as those containing iron-sulfur clusters[17].

Regarding proteomics data, proteins found to be altered are involved in the Acute Phase Response. We also noticed an alteration in some proteins related to lipid homeostasis, that is consistent with the proposed metabolic shift towards an increased peripheral use of lipids[18]. However, we would like to highlight the fact that all the proteins found differentially expressed in this study have already been described in other studies. Higher APOE4 allelic frequency in ALS patients gives an interesting link between lipids homeostasis and neurodegeneration, at least in this cohort of subjects.

The analyses performed with Artificial Neural Networks gave very promising results in evaluating different variables at the same time, providing an insight in proteomic and metallomic profile in ALS, that must be more deeply evaluated.

In this context, despite the small group analyzed here, we found our data comparable to studies involving a much higher number of patients, strengthening our approach, based on a small number of patients but with a common environmental exposure.

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A MULTIDISCIPLINARY STUDY OF sALS IN PATIENTS ORIGINATING FROM A RESTRICTED GEOGRAPHICAL AREA.

S. De Benedetti[1], G. Lucchini[2], A. Marocchi[3], S. Penco[3], C. Lunetta[4], S. Iametti[1], E. Gianazza[5], F. Bonomi[1]

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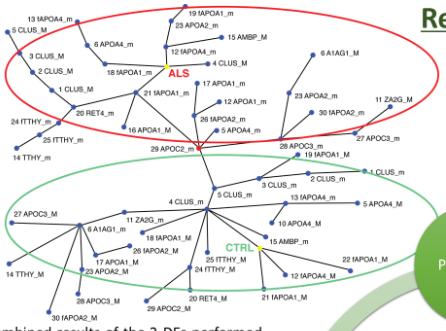

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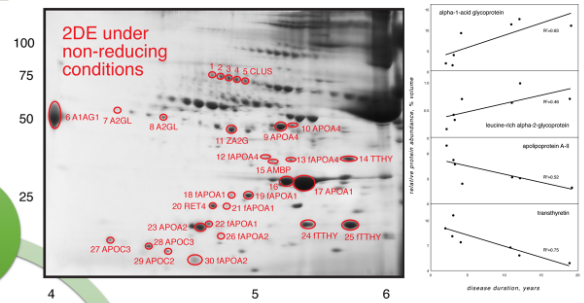

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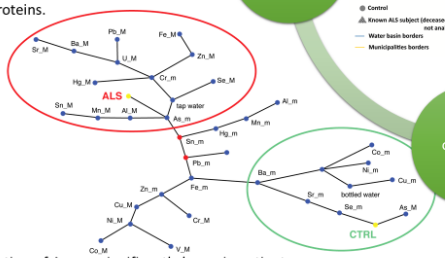

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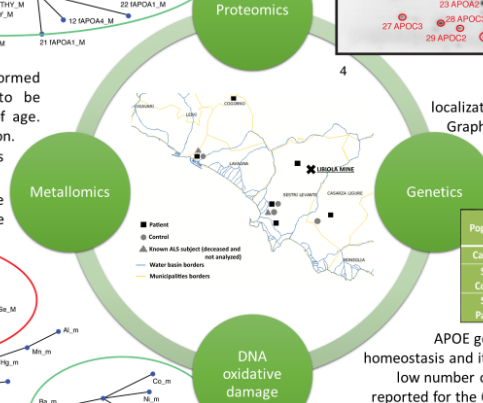

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Analyzing well defined cohorts of human patients, despite being small in number, could be a reliable model to study sporadic ALS

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