1 Introduction

Amyotrophic Lateral Sclerosis (ALS) is a rare progressive neurodegenerative disorder with an incidence of 2.1 per 100,000 person-years and an estimated prevalence of 5.4 cases per 100,000 population [1]. 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. ALS usually leads to death in 3 to 5 years upon the onset of symptoms [2]. Two forms of ALS can be recognized: the sporadic (SALS) accounts for 90−95% of the cases, while the familial form (FALS) accounts for the remaining 5−10% [3]. Both forms show similar clinical features suggesting a common pathogenic pathway.

ALS is the most common disorder among motor neuron diseases with adult onset. Despite extensive research, its etiology is still unknown. Mutations in several genes, including SOD1, FUS and TARDBP, have been identified as responsible for the disease in 25-35% of cases of the familial forms [4-6]. Recently, the pathogenic expansion of a non-coding hexanucleotide repeat sequence (GGGGCC) in the C9ORF72 gene was reported in familial and sporadic forms of ALS [7,8].

Other proposed pathogenic mechanisms involve protein aggregation, oxidative stress, impairment of mitochondrial function, transcription dysfunctions, alterations in the proteasome pathway, inflammation, excitotoxicity, and environmental influences [9]. Among the environmental factors, metals have been hypothesized to play a – still much un-clarified – role in ALS. Indeed, through the years many neurodegenerative disorders, such as Alzheimer Disease, Parkinson Disease and ALS, have been linked to iron and metal metabolism in a number of studies [10,11].

Transition metal-induced toxicity also has been proposed to be involved in the pathology [12] and different authors reported increased concentrations of metals,
as well as of proteins regulating their homeostasis, in ALS patients [13-15]. Metals are essential for different physiological processes and their concentrations must be strictly regulated since imbalance can lead to toxic effects. Environmental, occupational and nutritional levels of metals could influence their body concentrations and result in overload.

In this work, we report on a small cohort of subjects affected by the sporadic form of ALS, originating from a restricted geographical area with a high prevalence of the disease. We evaluated concentrations of different metals in serum trying to establish a correlation with ALS, assuming that the limited geographical area would allow to assess the role played by specific chemical species in the environment and in the diet of the involved subjects.

2 Methods

The study enrolled seven patients (4 men and 3 women) and five controls (2 men and 3 women, age matched – 69 years vs. 64 years – and living in the same geographical area) (Table 1 and Table 2). All patients and controls were recruited by Medical Genetics Unit of Niguarda Ca’ Granda Hospital and signed an informed consent approved by local ethics committee. Controls were represented by caregivers of the patients, known to be free from neurological disorders and living in the same geographic area. Blood was collected from all subjects. Serum was obtained by centrifugation after clotting and stored at -80°C.

ALS diagnosis was according El Escorial criteria [9] with clinically defined sporadic cases; one presenting bulbar form at the onset, while the six with spinal form. All subjects were under Riluzole treatment; two of them underwent Percutaneous Endoscopic Gastrostomy (PEG).

For metal quantitation, samples were analyzed by ICP-MS (Bruker AURORA M90 ICP-MS). Serum was 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. An aliquot of a 2 mg L⁻¹ of an internal standard solution (²⁴Sc, ⁸⁸Y, ¹⁰⁹Tb) was added to both samples and standards to give a final concentration of 20 μg L⁻¹ of each element. Typical analysis interferences were removed by using Collision-Reaction-Interface (CRI) with an H₂ flow of 70 mL min⁻¹ through the skimmer cone.

Statistical analyses were carried out with Student's t-Test and through multivariate statistical analyses tools, including Principal Component Analysis (PCA) and Auto Contractive Map algorithm (Auto-CM), a type of Artificial Neural Network (ANN) 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; this approach has already been applied to studies on the ALS disease [16].

3 Results

Serum concentrations of Cr, Fe, Ni, Cu, Zn, Se, Sr, Cd, Pb are reported in Table 3. A statistically significant difference between patients and controls was found for Ni

Table 1: 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 | Age at onset (y) | Site at onset | Duration of disease (y) | PEG | BMI at onset (m²Kg⁻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   | 26.1                  | 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            | 9.0                     | N   | 23.7                  | Manager    | N     |
| 7 SU | F      | 59               | LL            | 4.2                     | Y   | 29.3                  | Teacher    | EX    |

Table 2: Data for control subjects.

| ID  | Gender | Current age (y) | BMI (m²Kg⁻¹) | Occupation | Smoke |
|-----|--------|-----------------|--------------|------------|-------|
| 244 N | F      | 55              | 20.8         | Odontologist | N     |
| 245 N | F      | 66              | 22.3         | Teacher     | Y     |
| 246 N | F      | 65              | 25.7         | Housewife   | EX    |
| 247 N | M      | 72              | 28.1         | Professor   | N     |
| 248 N | M      | 61              | 31.1         | Manager     | EX    |
concentrations should be in the 0.26−0.75 μg L\(^{-1}\) for the ALS patients, about five times higher than in controls (2.14 μg L\(^{-1}\)). The median Pb concentration in the patients’ group was almost twice that of controls (2.16 vs 1.26 μg L\(^{-1}\)). Conversely, the median As concentration was lower in the patients’ group than in controls’ (0.51 μg L\(^{-1}\) vs 0.73 μg L\(^{-1}\)). In both groups, Ni and Pb levels were higher than the values reported for the general Italian population. Indeed, according to data reported by the Italian Superior Health Institute [17], Ni concentrations should be in the 0.26–0.75 μg L\(^{-1}\) range, and Pb concentrations in the 0.20–0.98 μg L\(^{-1}\) range.

Principal Component Analysis (PCA) was able to discriminate between the patients and the controls. The most prominent feature of the control group was a high concentration of As and a low concentrations of all the other metals analyzed. The semantic connectivity map generated with the Auto-CM system (Figure 1) confirmed this observation. This algorithm is able to visually show the connections between variables and to describe the strength of the connections with a numerical value from 0 to 1. Results clearly discriminate the two groups and define an ALS area characterized by high levels of metals, except for As. Among the three metals that were significantly different, none individually emerged as more relevant than the others in the discrimination of the controls’ group from the patients’ group. The Auto-CM analysis also indicated that high levels of Se were closely

Table 3: Averages of the measures of metals concentrations in sera. *: p-value ≤ 0.05, NA: Not Available.

| Element | Average Patients ± SD (μg/L) | Average Controls ± SD (μg/L) | Reference Values (μg/L) [17] |
|---------|-----------------------------|-----------------------------|-----------------------------|
| 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                    |

Figure 1: Semantic connectivity map obtained with Auto-CM System. The Minimum Spanning Tree (MST) shows the connections between the variables. Values on the arches of the graph refer to the strength of the association between two adjacent nodes. The range of this value is...
linked to the ALS group, whereas low levels of Se were connected with the controls. Even if Se levels did not reach statistical significance with common statistic tools \( (p = 0.12) \), artificial neural network multivariate statistical analyses point to a possible relevance of the observed differences (Figure 1).

### 4 Discussion

In the last decades, Amyotrophic Lateral Sclerosis has been the focus of intense research. Despite this, the etiology of the disease still has to be clarified. Genetic traits only partly explain this disorder [18] and, at present, fail to assess a common and clear molecular pattern that leads to the disease. Among subjects bearing a pathological mutation in one of the causative gene, a wide phenotypical heterogeneity is present [19], and this is true also in the sporadic form of the disease [20]. Clearly, some other factors must be involved in the pathogenesis of the disease; some of the triggers could be environmental influences [18]. We decided to focus our investigations on metals’ concentrations in a small group of subjects originating from a restricted geographical region due to the higher prevalence of the disease in this area, in comparison with literature data. In our intentions, limiting the investigation to a restricted area could help in pinpointing environmental or dietary contributions to specific metal species.

We did not performed any study on environmental pollution, but we are aware of the presence of a steel plant within this area. Furthermore, there is a Cu and Fe mine, closed in 1962, located 8 km behind the inhabited area, within the basin of a creek, whose waters are reported to be strongly polluted due to Acid Mine Drainage. Wastestock 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 sulfide mineralization (Fe, Cu, Zn, etc.) and host rocks (Cr, Ni, V, etc.) [21]. Among the metals analyzed, Pb resulted higher in patients’ than in controls’ group. Much evidence in the literature describes Pb as a neurotoxic agent and as being related to ALS [22,23], since high levels of this metal have been found both in blood and in cerebrospinal fluid of ALS patients [24,25]. In our analyses, Pb levels were twice higher in ALS subjects than in controls, supporting the hypothesis of a possible involvement of this metal in the etiology of the disease.

\( \text{Pb}^{2+} \) ions have a substantial affinity for protein cysteine thiols, and could be involved in direct substitution of iron in various types of FeS clusters, a property that has been demonstrated \textit{in vitro} for various thiophylic metals [26,27]. Rates and yields of cluster-metal substitution often depend on the redox state of the cluster iron [27,28] and on the accessibility of the ligands into the protein structure, as dictated by folding/unfolding rates and equilibria [27,29].

Another metal ion able to substitute for iron (in particular when ligands other than cysteine are involved) is \( \text{Ni}^{2+} \). In this study, Ni concentration was found almost five times higher in ALS patients than in controls. A Ni overload could promote oxidative stress and the production of Reactive Oxygen Species, as well as an increase in inflammatory markers [30]. However, recent evidence points to a direct role of Ni in the modulation of the biosynthetic pathways of FeS proteins in neural cells [31]. According to this report, high \( \text{Ni}^{2+} \) levels act by repressing the expression of the mitochondrial scaffold proteins IscU1 and IscU2, which are essential to FeS biogenesis [32]. According to this study, this leads to a cellular energy metabolism shift, likely as a result of an alteration in the mitochondrial respiratory chain and in the related energy fluxes [33]. The relevance of these issues is currently being investigated.

Arsenic can form both inorganic and organic compounds in the environment and in the human body, as either arsenite (\( \text{As}^{3+} \)) or arsenate (\( \text{As}^{5+} \)). Since absorbed arsenate is mostly reduced to arsenite in blood, the effect of the two forms appears to be very similar [30]. 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 [34]. In the light of this, the result obtained is unexpected, as the levels in our study controls were higher than in patients. One possible interpretation is that low levels of As are a feature of this peculiar group of ALS patients, without hypothesizing that an As overload may exert a protective role in control subjects. On the other hand, He and Ma reported that, in mouse hepa1c1c7 cells, \( \text{As}^{5+} \) induces the transcription of metallothionein I (MT1) through direct interaction with metal-activated transcription factor 1 (MTF1) [35], and this could possibly result in a protective effect, in case of non-toxic levels of As overload.

Auto-CM analysis also pointed to a possible relationship between ALS and Se levels in serum, although statistical significance was close to the limit of acceptability and expanding the number of subjects is still needed for verification. Abnormal levels of various Se species have been found in cerebrospinal fluid of ALS patients [36] and circulating levels of Se, usually bound to...
serum albumin, could reflect the Se levels present in the Central Nervous System. Finally, we were not able to find a correlation between Fe levels and ALS in the group of patients considered in this study, although several reports previously assessed this relationship [14,37-39].

Despite these phenomenological reports, the role of these metals in the pathogenesis of ALS remains unclear. We plan to evaluate it in a wider cohort of subjects while investigating a broader panel of metals. Impaired metal homeostasis, hypothetically attributable to environmental exposure, in the end could lead to overload of different elements. Besides promoting oxidative stress, metals can compete for the binding sites of metals-containing proteins, such as those containing iron-sulfur clusters. This damaging effect could be exerted by all those metals having affinity for thiols, like mercury(II), silver(I), cobalt(II), lead(II), cadmium(II), nickel(II), zinc(II) and cobalt(II) whose toxicity in bacteria has been shown to be proportional to the affinity for sulfur [40].

The role of iron-sulfur clusters biosynthetic machinery in ALS is still poorly explored. Upregulation of Frataxin (Fxn) and of the human ortholog 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 [38], whereas Ni overload results in the repression of IscU biosynthesis, as discussed above [31]. However, further investigation is 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 [35,38,41]) and on proteins involved in controlling the intracellular redox potential and/or the concentration of active chemical species (such as peroxides and superoxides [19]) also remain to be assessed.

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