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

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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.

Concentrazione dei metalli e proteomica dei fluidi biologici in pazienti affetti da SLA: Dati preliminari.

Le analisi biochimiche descritte nel progetto di dottorato sono state condotte su un piccolo gruppo di soggetti affetti da SLA sporadica tutti provenienti da un'area geografica circoscritta. Nel dettaglio sono state misurate le concentrazioni di alcuni metalli nelle urine e nel siero; le analisi proteomiche sono state eseguite sulle urine con SDS-PAGE e sul siero con elettroforesi bidimensionali. I dati raccolti con un questionario sullo stile di vita e nutrizione sono stati analizzati con un software basato sulle Reti Neurali.

Key words: Sporadic ALS, metals, micronutrients, proteomic analyses, Artificial Neural Networks

1. Introduction

This poster reports the preliminary results of the biochemical analyses performed on a cohort of subject with defined sporadic Amyotrophic Lateral Sclerosis (ALS), all originating from a restricted geographical area; the same environmental exposure could help to minimize the differences among the subjects under investigation. In detail the experiments performed can be divided into three groups:

- With ICP-MS the concentrations of Fe, Zn, Se, Sr, Cd, Pb, Ni, Cu, Cr and As have been evaluated in serum of seven patients and five controls, those of Fe, Zn, Se, Sr, Cd, Pb, Ni, Cu and As have been evaluated in six patients and two controls.
- Statistical analyses on the results have been carried out both with classical statistical elaborations (t-test and Principal Component Analysis) and with Auto Contractive Map algorithm (Auto-CM). It is 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 (Buscema and Grossi, 2008). This analysis combined the results of metal analyses and the information collected with a survey on lifestyle and nutrition.
- Proteomic analyses were performed on serum of patients and controls through two-dimensional electrophoresis (2D-E) using for the 1st d home-made IPG strips, covering non linearly the pH range 4-10; the analysis on urine was carried out with SDS-PAGE after precipitation and concentration of proteins.

2. 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^{TM} 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 aliquot of a 2 mg L^{-1} of an internal standard solution (^{45}Sc, ^{89}Y, ^{159}Tb) was added to both samples and standards to give a final concentration of 20 µg L^{-1}. Typical analysis interferences were removed by using CRI (Collision-Reaction-Interface) with an H_2 flow of 70 mL min^{-1} through the skimmer cone.

For SDS-PAGE analysis, after removal of the cell debris by centrifugation, proteins in urine were precipitated with 10% TCA solution (1 hour at 4°C), followed by centrifugation (13000 rpm, 15 min at 4°C). The precipitate was washed twice with cold (-20°C) acetone. The protein pellets were suspended in adequate volumes of Tris/HCl with 2% SDS and 2% 2-mercaptoethanol, and boiled for 10 minutes, then the same amount of proteins per sample was loaded on the gel.

Immobilized pH gradient strips for the 1st d (non linear pH range 4-10, 8 cm x 0.8 cm) were prepared according to the protocol published by Gianazza (The Protein Protocols Handbook, 2nd Edition, chapter 23). 600-700 µg of proteins, reduced with 1% 2-mercaptoethanol, were loaded near the cathode. The 2nd d was run on a 7.5-17.5% T gradient polyacrylamide gel, at 75 mA per gel. Image analyses of the Coomassie Blue stained gels were carried out with Image Master Software ver. 5.0.

3. Results and Discussion

3.1 Metals analyses

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 (ISTISAN). Higher concentrations of Ni and Pb were found in the patients' group, compared to the controls' 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). 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, linking the control group to high levels of As. 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. Intriguingly, Pb had low concentration both in patients' and in controls' urine, at contrast with the high levels of 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 ($p = 0.05$). 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.

Transition metal induced toxicity has been proposed to be involved in the pathology (Carrì et al., 2003) and higher concentrations of metals have been described in ALS patients (Roos et al., 2012; Goodall et al., 2008). However, further analyses are required, focusing on even rarer metals and on investigating other homogeneous cohorts of patients, thus allowing to collect a higher number of subjects and to investigate further possible geographical issues.

3.2 ANNs elaboration of the lifestyle and nutrition questionnaire

Contextually with the sample collection, patients and controls were administered a questionnaire aimed at collect data about their lifestyle and nutrition habits. The questionnaire consists in several questions about employment, nutrition and diet, smoke and physical activity. Information about the disease were collected too.

ANNs 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. This could be related to the fact that 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 (Kamel et al. 2012, Malek et al. 2012) too. Further investigation in this direction seems required.

The analysis of metals concentrations and diet was not able to link these variables to ALS. Both groups were linked to the metals' levels, confirming the analyses previously described, but no significant relationship to eating habits was evident, if for fruit consumption, as stated above.

3.3 Proteomic analyses

SDS-PAGE performed on urine samples showed very similar patterns for all subjects. Control samples had obvious bands only for albumin and, presumably, Tamm-Horsfall protein, as expected from healthy subjects. In patients' samples a higher number of proteins were visible. Among them, a band was evident at about 80 kDa, that could probably be transferrin, a protein related to iron transport and homeostasis. Three patients showed bands at very low molecular weights, including probably (hemo)globin chains. The differences between the control group and the patients' one could probably be related to the different ages of the subjects (69 vs 28) and stem from likely concomitant infections to the urinary tract, frequent in bedridden patients.

To assess the results from 2DE experiments, the integrated volumes of the spots were compared between the controls' group and the patients' group, and the statistical significance of the differences was evaluated with the Student's t-test. Visual identification of the proteins was performed with reference to literature data (Anderson and Anderson, 1977). As soon as possible, the spots of interest will go through MS analysis to positively identify the proteins they contain.

From this preliminary comparison two spots clearly emerged with a lower concentration in serum of the patients' group ($p = 0.05$ and $p = 0.03$, respectively). One spot appears to correspond to apolipoprotein AII (APOA2). At present no literature data link this protein to ALS, but the fact that its mRNA is processed by TDP43 (Mercado et al. 2005), a protein involved in ALS pathogenesis (Tetsuaki et al. 2006) provides a possible connection with the disease.

Since the group of test patients showed a high interpersonal variability in disease status, other analyses were performed stratifying the patients' group according to duration and age at onset of the disease. For the first analysis patients were divided between subjects with less than 4 years (n=4) of disease and more than 10 years (n=3). When compared to the control group, two new proteins were found to be less expressed in the group of

patients with more than 10 years of disease with statistical significance (p=0.03 and p=0.002). These proteins have yet to be identified. At difference from the previous analysis of APOA2 abundance in healthy and patients' groups, the APOA2 content appears lower in the subgroup of patients with less than 4 years of disease. No "new" proteins have been identified as significantly different when patients were grouped according to age of onset (age ≤ 60 y, n = 4, and age > 60 y, n = 3).

In all the analyses, a small group of acidic proteins with an approximate molecular weight of 55-60 kDa, resulted markedly much more expressed in patients than in controls. These proteins will have to be identified, and will be the focus of further activities. Again, further studies will require expanding the groups of subjects being considered, in order to strengthen possible evidence gathered in these preliminary studies.

4. References

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