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

Stefano De Benedetti (stefano.debenedetti@unimi.it)
DeFENS - Department of Food, Environmental and Nutritional Sciences, University of Milan, Italy
Tutor: Prof.ssa Stefania Iametti / Prof. Francesco Bonomi

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.*

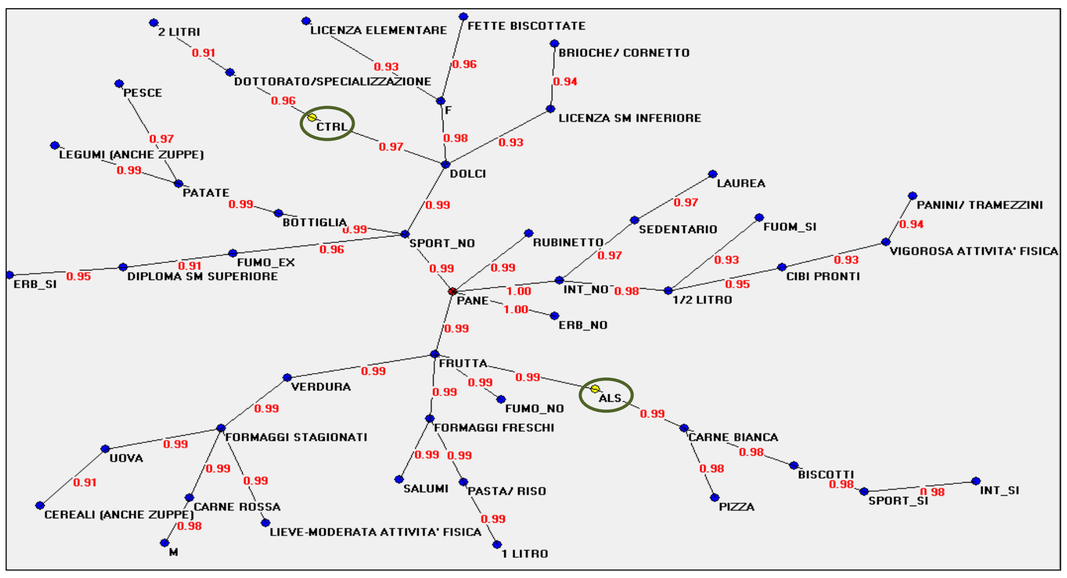

Fig 1. *Semantic connectivity map showing the connections between the variables. Values on the arches refer to the strength of the association between two adjacent nodes, the range is from 0 to 1.*

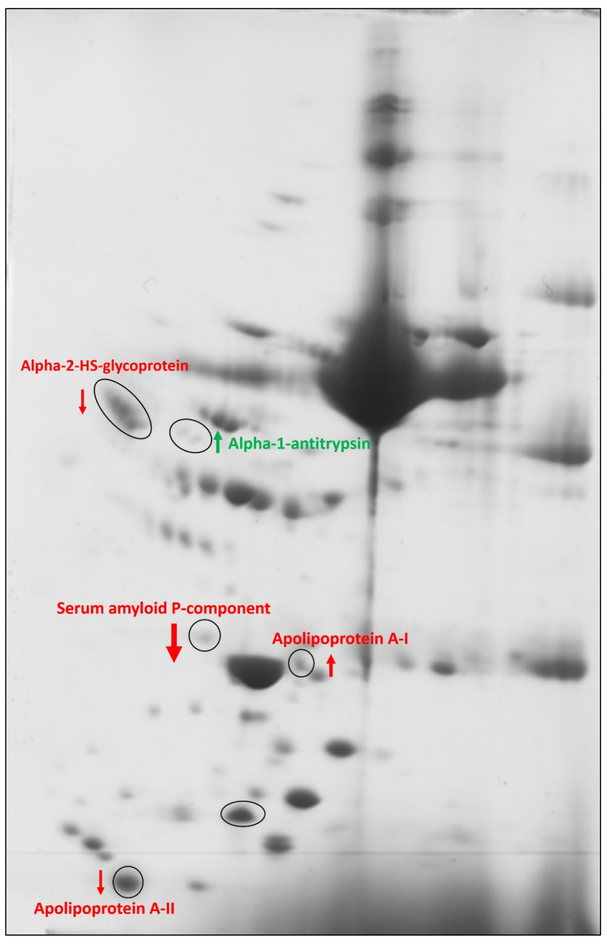

Fig 2. *2D-E gel showing the significant spots identified. Red: Negative APR proteins, Green Positive APR protein.*

- 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 < 0.10*

Bibliography:

1. Chiò et al. (2013) Global epidemiology of amyotrophic lateral sclerosis: a systematic review of the published literature. *Neuroepidemiology.*
2. Mitchell et al. (2007) Amyotrophic lateral sclerosis. *Lancet*
3. Crichton et al. (2006) Metal-based neurodegeneration. *England: John Wiley&Sons.*
4. Hadzhieva et al. (2013) Dysregulation of iron protein expression in the G93A model of amyotrophic lateral sclerosis. *Neuroscience.*
5. Hadzhieva et al. (2014) Review: iron metabolism and the role of iron in neurodegenerative disorders. *Neuropathol Appl Neurobiol.*
6. Carrí et al. (2003) Neurodegeneration in amyotrophic lateral sclerosis: the role of oxidative stress and altered homeostasis of metals. *Brain Res .*
7. Roos et al. (2012) Manganese in cerebrospinal fluid and blood plasma of patients with amyotrophic lateral sclerosis. *Exp Biol Med.*
8. Buscema et al. (2012) A Novel Mathematical Approach to Define the Genes/SNPs Conferring Risk or Protection in Sporadic Amyotrophic Lateral Sclerosis Based on Auto Contractive Map Neural Networks and Graph Theory. *Neurol Res Int.*
9. ISTISAN et al. (2010) Report 10/22: Biomonitoraggio della popolazione italiana per l'esposizione ai metalli: valori di riferimento 1990–2009. Accessed May 2015.
10. Kamel et al. (2012) Pesticide exposure and amyotrophic lateral sclerosis. *Neurotoxicology.*
11. Mercado et al. (2005) Depletion of TDP 43 overrides the need for exonic and intronic splicing enhancers in the human apoA-II gene. *Nucleic Acids Res*.

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