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ABSTRACT

Spinocerebellar ataxia type 2 (SCA2) is a neurological disorder characterized by cerebellar dysfunction. The possible association between metals and neurodegenerative diseases is under constant investigation, with particular focus on their involvement in oxidative stress and their potential role as biomarkers of these pathologies.

Whole blood samples of SCA2 patients and of healthy individuals were subjected to multi-elemental analysis by inductively coupled plasma-mass spectrometry (ICP-MS). Reduced levels of manganese and copper were found in SCA2 patients, while zinc and vanadium concentrations were significantly higher in patients compared to controls. Copper, manganese and zinc are cofactors of many enzymes (such as superoxide dismutase, SOD) involved in the cellular antioxidant response, whereas vanadium is a transition metal able to produce reactive radicals.

A marked decrease of the antioxidant response has been previously reported in SCA2 patients. We suggest that an unbalance of transitional elements in the blood may reflect altered antioxidant homeostasis in SCA2 patients and could constitute a future peripheral biomarker for this disease. In addition, we suggest a possible role of vanadium in the altered lipid metabolism of SCA2 patients.

Introduction

Autosomal dominant spinocerebellar ataxias (SCAs) are genetically heterogeneous neurological diseases characterized by Purkinje cell degeneration causing cerebellar dysfunction. Spinocerebellar ataxia type 2 (SCA2) is the second most prevalent spinocerebellar ataxia subtype worldwide after spinocerebellar ataxia type 3 [1]. The highest world prevalence rate of SCA2 was registered in Holguin province in Cuba [2].

In Italy, the proportion of SCA patients with SCA2 is 47% (the Ataxia Center, Chicago University). This rare neurodegenerative disease is characterized by uncoordinated movements, decreased muscle tone, tremors, poor tendon reflexes, nystagmus, polyneuropathy, dysphagia, chorea, Parkinsonism and dementia [3]. The severity and the age of onset of SCA2 vary, in the majority of cases it becomes symptomatic from the third to the fourth decades of life; however, SCA2 is more rapidly progressive when onset happens in the second decade of life [4].

SCA2 is caused by the expansion of a CAG triplet repeat located in the 5'coding region of the ataxin-2 gene; the mutant protein then contains a segment of polyglutamines [5]. The pathogenic effects of SCAs involve accumulation of a mutated/misfolded cytoplasmatic protein - Ataxin 2, which is located in several tissues and neurons. Ataxin 2 interacts with poly(A)binding protein 1 and assembles into polyribosomes, which probably have a function in RNA metabolism and in regulating cellular mRNA turnover [6]. Intracellular protein aggregates and widespread neuronal loss in the cerebellum have been observed in the brains of SCA2 patients [7].

In recent years, oxidative stress has been shown to be related to several neurodegenerative disorders such as Friedreich ataxia (FA), Huntington disease (HD) [8], and Ataxia-Telangiectasia (A-T) disease [9]. Oxidative stress is the end point of chronic neurodegenerative disease, but is also related to Autism Spectrum Disorder and other neuropsychiatric disorders [10]. The cellular antioxidant system, designed to control the flux of reactive oxygen species (ROS), consists of several antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx)

and catalase (CAT). An excess of ROS generation results in a wide range of neural disorders and also aging; toxicity of free radicals causes damage to proteins and DNA and leads to cellular apoptosis [11].

SCA2 has been previously related to oxidative stress, particularly to altered activity of key antioxidant enzymes [4, 12-13]. In particular, Cornelius *et al.* investigated the level of mitochondrial oxidative stress by assessing superoxide levels in fibroblasts from SCA2 patients and controls, and found that SCA2 cells displayed a significantly increased amount of oxidative stress compared to control cells.

Altered homeostasis of trace elements has been linked to the etiology of many neurodegenerative syndromes [14]. In particular, Bocca and coauthors [15] found a lower manganese concentration in the blood of Amyotrophic Lateral Sclerosis (ALS) patients compared to healthy individuals; while Roos and coauthors [16] reported an increased level of copper in the blood of ALS patients. Several investigations have reported higher levels of copper in the blood of Alzheimer's disease (AD) patients and high manganese levels in the blood of Parkinson's disease (PD) subjects relative to controls [17].

Some essential elements are, in fact, essential cofactors for antioxidant enzymes such as cytoplasmic Zn/Cu-SOD (SOD1) and mitochondrial Mn-SOD (SOD2), which act as bulk scavengers of superoxide radicals.

In a previous investigation, we discovered altered metal concentrations in the blood of Ataxia Telangiectasia (A-T) patients, another neurodegenerative disease in which ataxia is one of the main symptoms. In particular, we found that copper levels were significantly higher in A-T patients and zinc levels were significantly lower [9]. Moreover, a reduction of Cu/Zn-SOD and Mn-SOD activities were also observed in A-T lymphoblastoid cell lines (LCLs), suggesting that altered homeostasis of zinc and copper may play a role in the pathology of A-T. In this study, we measured the levels of 15 metals (arsenic, beryllium, cadmium, cobalt chromium, copper, iron, manganese,

nickel, lead, antimony, selenium, thallium, vanadium and zinc) in the blood of SCA2 patients, with the aim of assessing metal concentrations in the patients' blood compared to controls, and investigating whether transitional metals involved in the oxidative stress response could constitute a possible peripheral biomarker of this disease.

Patients and methods

Subjects

We enrolled 20 SCA2 adult patients (10 males and 10 females) diagnosed with SCA2, which was confirmed by genetic testing, and 18 healthy adult individuals as controls (10 males and 8 females). Subjects were age-matched, with a mean age of 49 years in patients and 47 years in healthy individuals.

The study was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) 1964), and was approved by the committee of the Medical Sciences Dept. (DSM-ChBU). Patients (or their legal representative) provided their informed consent.

Venous blood was collected, as previously described [9]; briefly, 1 mL of blood was added to HNO_3, microwave digested (ETHOS 1 system, Milestone S.r.l, Italy), and processed together with a certified reference material (Seronorm trace elements whole blood level 2). Metal concentration analyses were performed with A Thermo X series II ICP-MS instrument, as previously described [9], utilizing a Collision Cell Technique (CCT), to remove interferences.

Statistical analysis

The D'Agostino-Pearson normality test was utilized to determine the distribution of values, which were normally distributed. The Student's t-test was utilized for comparing between the control group and the patient group. Results were considered statistically significant using p values of < 0.05. Graph Pad Statistics Software Version 6.0 (GraphPad Software, Inc., USA) was utilized for statistical calculations.

106

107 **Results**

108 Whole blood concentrations of the analyzed metals in SCA2 patients and healthy individuals are

109 shown in Table 1, and were expressed as μg/L± standard deviation (S.D).

Table 1. Metal concentrations (μg L^{-1} ± S.D) in the blood of SCA2 patients and controls.

Metals	*Patients (N=20)*	*Controls (N=18)*	*Statistics (Unpaired t-test)*
As	3.4 (±2.9)	2.0 (±2.2)	n.s.
Be*			
Cd	0.80 (±0.53)	0.71 (±0.40)	n.s.
Co	0.57 (±0.35)	0.56 (±0.19)	n.s.
Cr	22 (±1.5)	19 (±1.2)	n.s.
Cu	**866 (± 45)**	**1116 (±68)**	**p=0.0001**
Fe	523648 (±5041)	495857 (±4523)	n.s.
Mn	**16 (± 3.1)**	**21 (± 4.2)**	**p=0.001**
Ni*			
Pb	62 (±8.5)	70 (±12)	n.s.
Sb	4.2 (±1.7)	3.4 (± 1.2)	n.s.
Se	119 (± 19)	125 (± 35)	n.s.
Tl*			
V	**6.5 (±1.0)**	**3.7 (± 0.8)**	**p=0.03**
Zn	**5860 (± 900)**	**5189 (± 999)**	**p=0.04**

110 Note: statistically significant results are indicated in bold.
111 *Concentrations of these elements were below the limit of quantitation of the method.
112
113

114 Of all the essential trace elements, copper and manganese levels were significantly lower in the

115 blood of SCA2 patients (p = 0.0001 and 0.001, respectively) while zinc levels were significantly

116 higher (p = 0.04) (Table 1, Figure 1).

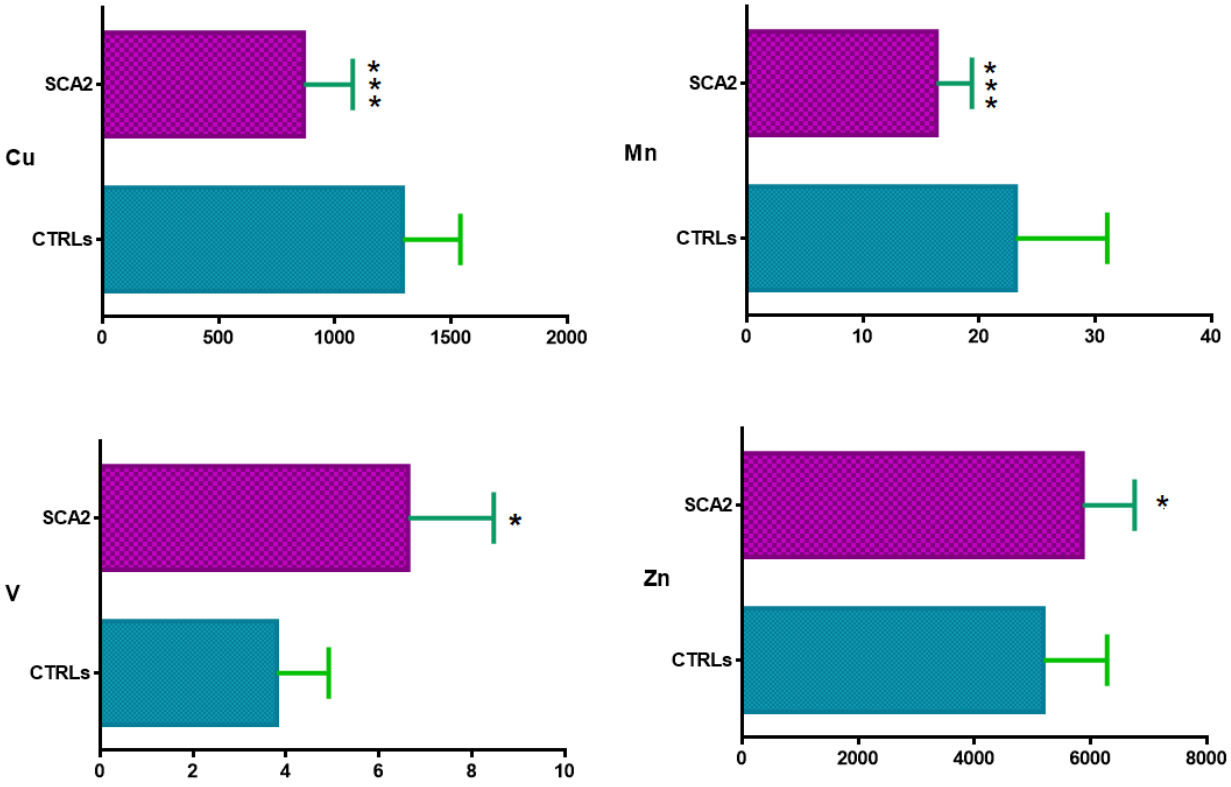

Figure 1 Box-plot diagrams of copper, manganese, vanadium and zinc (mean ± SD) in the blood of SCA2 patients and controls (μg L^{-1}).

Levels of the other essential elements, i.e. cobalt, chromium, iron and selenium did not significantly differ between patients and controls.

Arsenic, antimony, cadmium, lead, thallium and vanadium are metals with no recognized biological functions, and are thus considered non-essential for life. Among them, only vanadium levels (Figure 1) were found significantly higher in SCA2 patients (p=0.03).

DISCUSSION

Many previous investigations have linked the origin of neurodegenerative disorders (such as AD, PD, HD, ALS, Friedreich's Ataxia and AT) to increased oxidative stress in neuronal cells, thus suggesting the involvement of microelements such as Zn, Cu and Fe [9, 14-17].

Essential trace elements play a crucial role in the correct functioning of the human nervous system. Neuronal cells do not function properly when there is a copper imbalance, and Cu dyshomeostasis

has been linked to AD and ALS [20]. Moreover, in Mendelian disorders such as Wilson and Menkes diseases, the progressive neurological dysfunction is due to a disturbance of Cu metabolism, with an accumulation of copper in the brain and other organs [20].

Zinc is the second most abundant microelement in nervous tissue, after iron. Decreased Zn concentrations have been implicated in the pathophysiology of PD, AD, Friedreich's Ataxia [21] and, recently, AT disease [9].

Superoxide dismutases are antioxidant enzymes that are able to catalyze the conversion of superoxide anions to hydrogen peroxide, providing the cells with a defense mechanism against ROS [22]. Cu and Zn are co-factors of the main SOD isoform, SOD1, which exerts most of the total cellular SOD activity in the cytosol. An equilibrated molar ratio between Cu and Zn is necessary for the correct functioning of this antioxidant enzyme. When we studied A-T patients [9], we argued that the lower zinc levels, together with the higher copper levels found in patients, could be the cause of the functional reduction of SOD1 observed in A-T cells.

Conversely, in the blood of SCA2 patients, we observed a higher zinc concentration and a lower copper level. The presence of oxidative stress and the alteration of SOD levels in spinocerebellar ataxia type 2 have been extensively documented by Guevara-Garcia and coauthors [12], who observed an increase of SOD1 levels.

The manganese superoxide dismutase (Mn-SOD or SOD2) exerts its antioxidant function in mitochondria. SOD2 has been proposed to be involved in the progression of several neurodegenerative disorders, as well as having a probable role in stroke and age-related cognitive decline [23]. Cornelius and coauthors [13] suggested that oxidative stress and mitochondrial dysfunction may be contributory factors to the pathophysiology of SCA2; moreover, they determined the level of mitochondrial oxidative stress by assessing SOD2 levels in fibroblasts, showing a significant increase in SOD2 production in patients. Mitochondria control the primary pool of cellular Mn, and it is well known that, in humans, this essential element is a required

158 cofactor in enzymes involved in neuronal and glial cell functions, and in neurotransmitter synthesis
159 and metabolism [24].
160 In this study, we found that blood Mn concentrations were lower in SCA2 patients than in controls.
161 Furthermore, we suggest that dyshomeostasis of the three essential elements - copper, zinc and
162 manganese - could be linked to the altered stress oxidative status already demonstrated in SCA2
163 subjects.
164 Vanadium (V) is the 21st most abundant element in the Earth's crust; vanadate is able to replace
165 phosphate in apatite, due to the similarity between vanadate and phosphate [25]. In fact, vanadate
166 can substitute phosphate in many enzymes such as phosphatases, phosphomutases, diesterases,
167 ATPases, ribonucleases and kinases, resulting in inhibition of these enzymes [25, 26]. Vanadium
168 compounds are transported in the blood by serum transferrin (Tf), and red blood cells take part in
169 the transport and distribution of this metal; Tf also mediates the transport of several other metals
170 such as Cu, Mn and Zn [27]. The first medical applications of vanadium were carried out in a
171 French hospital in 1897, after discovering that V compounds overcame dysfunctions in glucose and
172 lipid metabolism, by enhancing insulin action; it was then utilized to cure diabetes [25]. However,
173 redox active metals, like V, undergo redox cycling reactions in cells and can produce ROS in
174 biological systems; by producing ROS, transition elements may cause DNA damage and apoptosis
175 [25]. Regarding vanadium, studies have suggested that V may cause central nervous system
176 depression, tremors and neurasthenia [29-31], even if the pathways that lead to neurological
177 diseases are not well elucidated.
178 In SCA2 patients, we observed higher levels of V in the blood compared to healthy individuals. V
179 produces ROS such as hydroxyl free radicals [32] and superoxide [33]. The presence of higher
180 concentrations of V in cells could lead to an increase in ROS, and upregulation of SOD enzymes
181 may counteract the increase of oxidative stress. Vanadium species and, in particular, the oxido-

vanadium cation VO^{2+} have been previously reported to be involved in the annihilation and formation of ROS [26].

The ability of V to inhibit glycogenolysis and lipolysis, stimulating lipogenesis [25] is a particularly interesting aspect. Vanadium compounds in fact, were found to ameliorate dysfunctions in glucose and lipid metabolism in diabetes, and mimic or enhance the insulin action [25].

The neurodegenerative disease SCA2 is caused by mutations in the gene ATXN2 that codes for the cytoplasmic protein ataxin-2, which has been recently proposed to play a key role in insulin sensitivity and cellular stress responses [34]. In animal models, such as ATXN2 knock-out mice, signs of dyslipidemia were observed, along with alterations in lipid metabolism-related molecules in the cerebellum, suggesting that the absence of ataxin-2 causes deregulation of lipids [34].

Lipid pathology is a feature of SCA2 and, as already observed in insulin resistance syndromes [35], the absence of ataxin-2 leads to abdominal obesity and hepatosteatosis, along with reduced insulin receptor expression in the liver and cerebellum, and increased insulin levels in the pancreas and blood serum [35]. Thus, we can speculate that the increase of vanadium found in the blood of SCA2 patients could reflect an attempt, at a cellular level, to counteract the metabolic imbalance of lipids. Further studies are required to fully understand the role of V on lipid metabolism in SCA2 patients.

CONCLUSIONS

Neurodegenerative processes have been previously related to metal imbalances, with altered levels of different elements in serum or blood samples. Here we found altered levels of copper, manganese, zinc and vanadium in the blood of SCA2 patients, and we hypothesized that the dyshomeostasis of these transitional metals could be related to the oxidative stress typically found in SCA2 patients.

Additional investigations are necessary to confirm our results, but we can suggest that the characterization of the metal profiles in the blood of SCA2 patients is particularly important to find

potential markers of this disease. Moreover, we suggest that the increase of vanadium could be related to the SCA2-associated lipid metabolism pathology, due the ability of this element to enhance insulin action.

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Conflicts of interest: the authors declare that there are no conflicts of interest.

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