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# UNIVERSITÀ DEGLI STUDI DI TORINO

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1 **Blood metal levels and related antioxidant enzyme activities in**  
2 **patients with Ataxia-Telangiectasia**

3  
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17  
18 Key words: Ataxia-Telangiectasia; metals; biomarker; essential trace elements; Cu; Zn; SOD; catalase

19  
20 Running title: metals in Ataxia-Telangiectasia

23 **ABSTRACT**

24 Transition metals are co-factors for a wide range of vital enzymes, and are directly or indirectly  
25 involved in the response against reactive oxygen species (ROS), which can damage cellular  
26 components. Their altered homeostasis has been studied in neurodegenerative disorders such as  
27 Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS), but  
28 no data are available on rarer conditions.

29 We aimed at studying the role of essential trace elements in Ataxia-Telangiectasia (A-T), a rare  
30 form of paediatric autosomal recessive cerebellar ataxia with altered antioxidant response. We  
31 found an increased level of copper (Cu,  $p=0.0002$ ), and a reduced level of zinc (Zn,  $p=0.0002$ ) in  
32 the blood of patients (n. 16) compared to controls, using inductively coupled plasma mass  
33 spectrometry (ICP-MS). Other trace elements involved in the oxidative stress response, such as  
34 manganese (Mn) and selenium (Se) were unaltered. Cu/Zn-dependent superoxide dismutase  
35 (SOD1) was shown to have a 30% reduction in gene expression and 40% reduction in enzyme  
36 activity upon analysis of lymphoblastoid cell lines of patients (Student's t-test,  $p=0.0075$ ). We also  
37 found a 30% reduction of Mn-SOD (SOD2; Student's t-test,  $p=0.02$ ), probably due to a feedback  
38 regulatory loop between the two enzymes. The expression of antioxidant enzymes, such as  
39 erythrocyte glutathione peroxidase (*GPXI*), and *SOD2* was unaltered, whereas catalase (*CAT*) was  
40 increased in A-T cells, both at the mRNA level and in terms of enzyme activity (~25%). Enhanced  
41 *CAT* expression can be attributed to the high ROS status, which induces *CAT* transcription.

42 These results suggest that alterations in essential trace elements and their related enzymes may play  
43 a role in the pathogenesis of A-T, although we cannot conclude if altered homeostasis is a direct  
44 effect of A-T mutated genes (*ATM*). Altered homeostasis of trace elements may be more prevalent  
45 in neurodegenerative diseases than previously thought, and it may represent both a biomarker and a  
46 generic therapeutic target for different disorders with the common theme of altered antioxidant  
47 enzyme responses associated with an unbalance of metals.

48

## 49 INTRODUCTION

50 Ataxia-Telangiectasia (A-T) is an autosomal recessive multisystem disorder with a frequency of  
51 1/40,000–1/100,000 (Swift et al., 1986). Main clinical features include progressive cerebellar  
52 degeneration leading to severe neuromotor dysfunction, oculocutaneous telangiectasia, profound  
53 immunodeficiency of both humoral and cellular compartments, gonadal dysgenesis, growth  
54 retardation in some patients, predisposition to malignancies (primarily lymphoreticular), high levels  
55 of serum alpha-fetoprotein, and acute radiosensitivity. A-T cells show chromosomal instability,  
56 premature senescence, accelerated telomere shortening, sensitivity to the cytotoxic and clastogenic  
57 effects of ionizing radiation and radiomimetic chemicals, and defective activation of cell-cycle  
58 checkpoints by these agents (Boder and Sedgwick, 1970; Gatti, 2001; Shiloh, 2006). The A-T  
59 mutated gene (*ATM*) encodes for a serine/threonine protein kinase recruited and activated by DNA  
60 double-strand breaks. ATM phosphorylates several key proteins that initiate the activation of DNA  
61 damage checkpoints, leading to cell cycle arrest, DNA repair or apoptosis. Although the  
62 neurodegenerative phenotype has been attributed to a defective response to DNA breaks in pre- and  
63 post-mitotic neurons (Lee et al., 2001), oxidative stress and reduced anti-oxidant defence may also  
64 play a role (Biton et al., 2006).

65 Correlation of the A-T phenotype with oxidative stress has been determined by molecular, *in vitro*  
66 and animal studies. A-T patients show persistent oxidative stress at the cellular level, and *ATM*  
67 knock-out mice exhibit increased signatures of reactive oxygen species (ROS) (Chen et al., 2003;  
68 Kamsler et al., 2001; Liu et al., 2005; Watters, 2003). At the molecular level, persistent oxidative  
69 stress in A-T cells was recently associated with the activation of ATM in response to oxidants (Guo  
70 et al., 2010), a mechanism clearly distinct from activation by DNA breaks. Therefore, ATM plays a  
71 role in redox-sensing and signalling, and the loss of redox balance in A-T may be central to the  
72 neuro-pathological phenotype. Several reports have demonstrated the protective effects of low

73 molecular weight antioxidants on the A-T phenotype (Browne et al., 2004; Reliene et al., 2008;  
74 Reliene and Schiestl, 2007).

75 Multiple antioxidant defence systems are present in the human body to escape the damage caused  
76 by reactive oxygen species (ROS). Superoxide dismutase (SOD) acts by degrading the superoxide  
77 anion ( $O_2^{\cdot-}$ ), while catalase (CAT) and glutathione peroxidase (GPx) detoxify hydrogen peroxide  
78 ( $H_2O_2$ ). Trace elements are required in small concentrations as essential cofactors for the  
79 antioxidant enzymes. GPx, cytoplasmic Zn/Cu-SOD (SOD1), mitochondrial Mn-SOD (SOD2) and  
80 CAT enzymes contain Se, Zn/Cu, Mn and Fe as cofactors, respectively.

81 Trace metals therefore play important roles in a wide variety of biological processes, and their  
82 altered homeostasis has been implicated in the aetiology of several neurodegenerative disorders  
83 (Bush, 2003; Jellinger, 2013). Metals interfere with cell signalling pathways and affect growth  
84 receptors, tyrosine and serine/threonine kinases, as well as nuclear transcription factors, by reactive  
85 oxygen species (ROS)-dependent and ROS-independent mechanisms. In neurodegenerative  
86 disorders, it is now recognized that the main underlying cause is increased oxidative stress,  
87 substantiated by the findings that the protein side-chains are modified either directly by ROS or  
88 reactive nitrogen species (RNS), or indirectly, by the products of lipid peroxidation. Hydroxyl  
89 radical ( $OH^{\cdot}$ ) is the primary ROS implicated in neurodegenerative stress, and although peroxynitrite  
90 appears to be capable of hydroxyl-like activities, hydroxyl radicals mostly reflect the Fenton  
91 reaction between reduced transition metals, usually iron or copper, and hydrogen peroxide ( $H_2O_2$ )  
92 (Taddeo et al., 2003).

93 In Alzheimer's disease, the increased level of oxidative stress in the brain is reflected by elevated  
94 levels of iron (Fe) and copper (Cu) in the brain, both of which are capable of stimulating free  
95 radical formation via the Fenton reaction (Jomova et al., 2010).

96 Breakdown of metal-ion homeostasis can lead to metals binding to protein sites which are not  
97 intended for metal-binding, replacement of other metals from their natural binding sites (Nelson,

98 1999), or to uncontrolled metal-mediated formation of deleterious free radicals (Gutteridge, 1995;  
99 Valko et al., 2007). In particular, essential trace elements such as copper, zinc and manganese, play  
100 a major role in metabolic pathways, and they have been studied in many diseases, including  
101 autoimmune, neurological and psychiatric disorders.  
102 Currently, only scarce information is available, on *in vivo* redox abnormalities in A-T patients  
103 (Aksoy et al., 2004; Reichenbach et al., 2002; Reichenbach et al., 1999).  
104 Here, we have studied the blood concentration of trace elements in A-T patients. We further  
105 explored the expression of *CAT*, *GPXI*, *SOD1* and *SOD2*, and the activity of *CAT*, *SOD1* and  
106 *SOD2* enzymes in A-T lymphoblastoid cell lines, to verify if changes in the metal concentration  
107 correlate with antioxidant enzyme activity, which is probably secondary to the alterations in their  
108 cofactor concentrations.

109

## 110 **MATERIALS AND METHODS**

### 111 *Patients*

112 We enrolled 16 A-T patients (9 males and 7 females; median age 10.6 years, range 3-23 years)  
113 diagnosed with A-T according to the diagnostic criteria of the European Society for  
114 Immunodeficiencies (ESID), and subsequently confirmed by genetic testing. None of the patients  
115 had acute infections at the time of sample collection. The control group consisted of 18 healthy  
116 individuals (10 males and 8 females, median age 13.2, age range 3-23 years old). The study was  
117 carried out in accordance with the ethical standards specified in the 1964 Declaration of Helsinki,  
118 and was approved by the internal review board of the Department of Medical Sciences (DSM-  
119 ChBU). Informed consent was obtained from patients or their legal representative.

120

### 121 *Blood sampling and analysis of metals*

122 Venous blood was collected in heparinized vacutainer BD tubes (Becton Dickinson Labware,  
123 Franklin Lakes, USA), and stored at -20°C until required for analysis. A sub-aliquot of 1 mL of  
124 blood was transferred into a 15 mL plastic tube (Falcon, Becton, Franklin Lakes, USA), then added  
125 to 2mL of super-pure concentrated HNO<sub>3</sub> (Romil Ltd., Cambridge, UK) and microwave (MW)  
126 digested in an ETHOS-Mega II oven (FKV, Bergamo, Italy), following the temperature ramp  
127 program: 45 min to reach 100°C and 4 h at 100°C. The system was equipped with an optical sensor  
128 to regulate the temperature. In each digestion, a blank reagent and a blood certified reference  
129 material was also processed (Serorm trace elements whole blood level 2, Sero AS, Billingstadi,  
130 Norway).

131

### 132 *Instruments*

133 A Thermo X series II ICP-MS instrument (Thermo Scientific, Germany), with interface Ni sampler  
134 and skimmer cone, was equipped with a CETAC ASX 500 Model 520 (CETAC Technologies,  
135 USA) auto sampler and a peristaltic pump nebulizer.

136 Operating conditions were as follows: forward power 1.40 kW, coolant gas flow rate 13.0 L/min,  
137 auxiliary gas flow rate 0.70 L/min, nebulizer gas flow rate 0.90 L/min, dwell time 10 ms, with five  
138 replicates. Torch position, ion lenses and gas output were optimized daily with a tuning solution.

139 The Collision Cell Technique (CCT), performed with a Helium/Hydrogen mixture (95/5) at a flow  
140 rate of 3.5 ml/min, was used to remove interferences.

141 An ETHOS 1 microwave digestion system (Milestone S.r.l, Italy) was used for acid digestion of  
142 cereals and reference materials. The recoveries are shown in Table S1. The Limit Of Quantification  
143 (LOQ) is the lowest concentration of the analyte that can be correctly quantified in the sample. The  
144 percentage of recovery (range 80-120) is determined by dividing the value observed in the  
145 analytical procedure by the reference value of the Reference Material (Table S1).

146



147

148

**Table S1.** Quantification limit values ( $\mu\text{g/L}$ ), reference material values and percentages of recovery.

Element	LOQ	Seronorm trace elements whole blood level 2	Percentage of recovery
As	10	14.3 $\pm$ 2.9	107
Be	5	5.68 $\pm$ 0.23	108
Cd	5	5.8 $\pm$ 0.2	105
Co	5	5.8 $\pm$ 1.2	90
Cr	10	11.8 $\pm$ 2.4	108
Cu	10	1,330 $\pm$ 270	109
Fe	100	331,000 $\pm$ 17,000	106
Mn	10	29.9 $\pm$ 6.0	103
Ni	20	17.9 $\pm$ 3.6	n.a.
Pb	10	310 $\pm$ 62	102
Sb	10	30.5 $\pm$ 6.1	92
Se	10	112 $\pm$ 23	98
Sn	10	5.7 $\pm$ 1.2	n.a.
Tl	10	10.3 $\pm$ 0.3	90
Zn	100	6,500 $\pm$ 300	90

Note: n.a: not applicable because &lt;LOQ

149

150 *Cell culture*

151 Five A-T lymphoblastoid cell lines (LCLs) were obtained from blood samples of patients by  
 152 Epstein-Barr virus (EBV) infection (Table S2). Six gender-matched control LCLs were obtained  
 153 from the Human Genetics Foundation of Torino (HuGeF). LCLs were grown at 37°C and 5% CO<sub>2</sub>  
 154 in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 2 mM L-  
 155 glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin.

**Table S2.** ATM mutations at protein level in tested cell lines.

Gender	Protein change
M	p.[(Arg2506fs)];[(Arg2506fs)]
F	p.[(Trp2109*)];[(Trp412*)]

F p.[(Ser1037fs;Lys2643\_Lys2671del)];[(p.Trp1814)]  
 F p.[(Met2938fs)];[\*3057Glyext\*28)]  
 M p.[(Met1\_Pro938dup)];[(Arg62\_Arg111del)]

156

157 *Gene expression*

158 Total RNA was extracted using Direct-zol according to the manufacturer's protocol (Zymo  
 159 Research Corporation, Irvine, USA); one milligram was retro-transcribed using M-MLV Reverse  
 160 Transcriptase (Life Technologies Europe, Monza, Italy). Quantitative real-time RT-PCR to evaluate  
 161 *CAT*, *GPX1*, *SOD1* and *SOD2* expression was carried out on an ABI-Prism7500 Fast instrument  
 162 (Life Technologies, Europe) using the TaqMan® Universal PCR Master Mix, Universal Probe  
 163 Library (UPL) technology (Roche Diagnostics, Mannheim, Germany) (Table S3).

**Table S3.** Primers and probes used for gene expression analysis by real-time qPCR

Assay	Primer F	Primer R	UPL probe
<i>CAT</i>	5'-gctcatttgaccgagagaga	5'-tgacctcaaagtagccaaagg	#68
<i>GPX1</i>	5'-caaccagtttgggcatcag	5'-tctcgaagagcatgaagttgg	#77
<i>SOD1</i>	5'-tcacaaatttcgagcagaagg	5'-gcaggccttcagtcagtcc	#60
<i>SOD2</i>	5'-ctggacaaacctcagcccta	5'-tgatggcttccagcaactc	#22

164

165 Experimental Ct values were normalized to the human *GUSB* gene (beta glucuronidase) or *TBP*  
 166 (TATA-binding protein) Endogenous Controls (VIC®/TAMRA Probe, Life Technologies Europe).  
 167 Gene expression was calculated in each sample relative to the mean of controls, using the delta-  
 168 delta Ct method as described (Livak and Schmittgen, 2001). Each sample was examined in  
 169 triplicate.

170

171 *Analysis of SOD isoforms activities*

172 A total of  $1 \times 10^7$  cells, obtained from five A-T cell lines and four control cells, were collected and  
 173 homogenized in lysis buffer (20 mM Hepes pH 7.2, 1 mM EGTA, 210 mM mannitol and 70 mM

174 sucrose). Cells were then centrifuged at 1,500 g for 5 min at 4°C. To separate the two enzymes  
175 (cytosolic and mitochondrial), supernatants were centrifuged at 10,000 g for 15 min at 4°C; the  
176 supernatant contained the cytosolic SOD, whereas the pellet was washed and resuspended in ice  
177 cold lysis buffer to measure mitochondrial SOD. To evaluate SOD activities in the two lysates, we  
178 used the Superoxide Dismutase Assay kit (Cayman, MI, USA, #706002). Analysis was performed  
179 by reading the absorbance at 440-460 nm on the microplate Reader, Model 680 (Bio-  
180 Rad Laboratories S.r.l., Segrate, Italy). To obtain SOD activity quantification, we compared  
181 absorbance values to a Standard curve with the range 0-0.25 U/mL of the SOD standard (assayed in  
182 each experiment in triplicate). Each sample was assayed in duplicate and in at least three  
183 independent experiments.

184

#### 185 *Analysis of CAT activity*

186 The day of the experiment, a total of  $5 \times 10^6$  cells, obtained from three A-T cell lines and three  
187 control cells, were collected by centrifugation, washed twice in PBS and homogenized in ice cold  
188 lysis buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.0). Cells were centrifuged at 10,000  
189 g for 15 minutes at 4°C. Supernatant was stored on ice for the assay. To evaluate CAT activities, we  
190 used the Catalase Assay kit (Cayman, MI, USA, #707002) following manufacture's protocol.  
191 Analysis was performed by monitoring the absorbance at 540 nm on a xMark microplate Reader  
192 (Bio-Rad Laboratories S.r.l., Segrate, Italy). CAT activity was defined as the amount of CAT  
193 enzyme able to produce 1.0 nmol of formaldehyde per minute at 25°C, interpolating the values in a  
194 standard curve of formaldehyde ranging 0-75  $\mu$ M. Each sample was assayed in duplicate and in at  
195 least three independent experiments.

196

#### 197 *Statistical analysis*

198 For analysing metal concentrations, we performed the D'Agostino-Pearson normality test to  
 199 determine the distribution of the values. Mean values of variables with normal distribution were  
 200 reported, and comparison between control group and the patient group was conducted using the  
 201 Student's t-test. If the distribution of data was not normal, variables were presented as median  
 202 values (Cr, Fe, Mn, Cu, Se and Zn) and differences between the two groups were studied using the  
 203 Mann-Whitney *U* test. The median concentration of each quantifiable element is shown with its  
 204 standard deviation. Results were considered statistically significant at *p* values of < 0.01.  
 205 Significance of gene expression and enzyme activity data was calculated using the Student's t-test  
 206 (unpaired). Statistical calculations were performed using Graph Pad Statistics Software Version 6.0  
 207 (GraphPad Software, Inc., USA).

208

## 209 RESULTS

210 We measured whole blood concentration of 15 metals in A-T patients and healthy controls by using  
 211 ICP-MS (Table 1).

**Table 1.** Median concentration of metals ( $\mu\text{g/L} \pm \text{S.D}$ ) in the blood of A-T patients and controls.

<i>Metals</i>	<i>Patients</i> ( <i>N</i> =16)	<i>Controls</i> ( <i>N</i> =18)	<i>P</i>
Cr	17.3 ( $\pm 2.0$ )	20.7 ( $\pm 3.5$ )	0.3201
<b>Cu</b>	<b>1,460 (<math>\pm 353</math>)</b>	<b>935 (<math>\pm 260</math>)</b>	<b>0.0002</b>
Fe	431,350 ( $\pm 1,020$ )	425,271 ( $\pm 2,015$ )	0.5011
Mn	34.2 ( $\pm 2.1$ )	39.0 ( $\pm 1.4$ )	0.4787
Se	110 ( $\pm 50.8$ )	130 ( $\pm 39.9$ )	0.2140
<b>Zn</b>	<b>4,370 (<math>\pm 435</math>)</b>	<b>5,760 (<math>\pm 577</math>)</b>	<b>0.0002</b>

212 Note: In bold statistically significant metal ions.

213

214 As, Be, Cd, Co, Ni, Pb, Sb, Sn and Tl levels were below the limit of quantitation of the method.

215 Copper levels were significantly higher in A-T patients ( $p = 0.0002$ ) and zinc levels were

216 significantly lower ( $p = 0.0002$ ). As copper and zinc are co-factors for ROS detoxifying enzymes,

217 we measured the activity of cytosolic (Cu/Zn-isoform, SOD1), mitochondrial superoxide dismutase  
218 (Mn-isoform, SOD2) and catalase (CAT) by ELISA assay in A-T LCLs. We showed a 40%  
219 reduction of Cu/Zn-SOD isoform activity (A-T median dose:  $0.66 \pm 0.06$  U/ml;  $n = 5$  vs. CTRLs  
220 median dose:  $1.0 \pm 0.09$  U/ml;  $n = 5$ ; Student's t-test,  $p = 0.0075$ ) and a 30% reduction of Mn-  
221 SOD (A-T median dose:  $0.67 \pm 0.08$  U/ml;  $n = 5$  vs. CTRLs median dose:  $1.1 \pm 0.13$  U/ml;  $n = 5$ ;  
222 Student's t-test,  $p = 0.02$ ). CAT activity was increased by ~25% in patients' cells compared to  
223 controls (A-T mean dose:  $1.24 \pm 0.08$ ; vs. CTRLs mean dose:  $1.0 \pm 0.8$ ; Student's t-test,  $p = 0.039$ )  
224 (Figure 1A).

225 Considering the feedback regulatory loop of detoxifying enzymes, we measured the expression of  
226 the same genes and *GPXI* involved in the ROS response. Using real-time quantitative PCR, we  
227 showed a decreased SOD1 mRNA level in A-T cells vs. CTRLs (A-T median dose:  $0.7 \pm 0.03$ ;  $n =$   
228  $4$ . CTRLs median dose:  $1.0 \pm 0.05$ ;  $n = 4$ ) (Student's t-test,  $p = 0.0001$ ). *GPXI* and *SOD2* showed  
229 levels similar to healthy controls (*GPXI*: A-T median dose:  $0.95 \pm 0.04$ ;  $n = 5$  vs. CTRLs median  
230 dose:  $1.1 \pm 0.08$ ;  $n = 5$ ; Student's t-test,  $p = 0.02$ , data not shown. *SOD2*: A-T median dose:  $1.1 \pm$   
231  $0.06$ ;  $n = 5$  vs. CTRLs median dose:  $1.0 \pm 0.03$ ;  $n = 5$ ; Student's t-test,  $p = 0.10$ ). Catalase mRNA  
232 level was increased in A-T cells vs. CTRLs (A-T median dose:  $1.5 \pm 0.09$ ;  $n = 5$  CTRLs median  
233 dose:  $1.0 \pm 0.07$ ;  $n=5$ ) (Student's t-test,  $p = 0.0012$ ) (Figure 1B).

234

## 235 **DISCUSSION**

236 The current knowledge in the field of neurodegenerative diseases indicates that metal-induced and  
237 metal-enhanced formation of free radicals and other reactive species can be regarded as a common  
238 factor in determining toxicity induced by metals (Jomova et al., 2010). Many reports link the origin  
239 of Alzheimer's disease (AD), and to a lesser extent Parkinson's disease (PD), to increased oxidative  
240 stress of the brain. A role for metals in these diseases and other disorders, such as Huntington's

241 disease, amyotrophic lateral sclerosis, and prion diseases such as Creutzfeldt-Jakob disease has been  
242 proposed (Bush and Curtain, 2008; Jomova et al., 2010).

243 We focused our attention on a rare form of paediatric ataxia, Ataxia-Telangiectasia, because of our  
244 interest in the genetics and molecular pathogenesis of this disease (Cavalieri et al., 2008; Cavalieri  
245 et al., 2006; Cavalieri et al., 2012). Among the pleiotropic features of A-T, neurodegeneration and  
246 premature aging are strongly associated with accumulation of oxidative damage which may  
247 contribute to degenerative processes observed in this disease (Reichenbach et al., 2002). *In vitro*, A-  
248 T cells are under a constant state of oxidative stress with high ROS levels, and have an abnormal  
249 response to agents inducing oxidative stress (Watters, 2003). Several groups have documented the  
250 presence of high levels of oxidative damage in A-T patients, confirming previous observations  
251 made in *Atm*<sup>-/-</sup> mice which displayed increased levels of oxidative stress and damage (Hoche et al.,  
252 2012; Kamsler et al., 2001; Schubert et al., 2004; Stern et al., 2002). In fact, brains or astrocytes  
253 from *Atm*-deficient mice present high ROS levels and an increased activation of the ERK1/2 redox-  
254 sensitive kinases (Liu et al., 2005). (Reliene and Schiestl, 2007) showed that the antioxidant N-  
255 acetylcysteine suppresses ERK signalling and protects Purkinje cells from oxidative stress-induced  
256 degeneration in *Atm*-deficient mice. Furthermore, (Stern et al., 2002) found a significantly impaired  
257 level of nicotinamide adenine dinucleotide phosphate, a cofactor of antioxidant enzymes, in  
258 cerebellar neurons of *Atm*<sup>-/-</sup> mice. Developing neurons are rapidly proliferating and potentially able  
259 to accumulate high levels of oxidants; therefore the above data provide extensive evidence that at  
260 least a part of the neurological phenotype in A-T may result from ROS-deficient homeostasis  
261 (Hoche et al., 2012).

262 We found increased copper and reduced zinc levels in the blood of A-T patients. Alterations in  
263 copper levels may reflect many physiological and pathological conditions, including dietary factors,  
264 hepatic disease, and acute and chronic infections, or it may be suggested they are associated directly  
265 with ATM impairment.

266 Copper is toxic when present in excessive amounts as it can directly induce ROS production,  
267 through Fenton and Haber-Weiss reactions (Halliwell, 2006). Therefore, free Cu levels must be  
268 precisely regulated in the cell in order to minimize damage. The excess of Cu reported in A-T  
269 patients may promote free radical-mediated pathways that, in turn, give rise to an antioxidant  
270 response. In our patients, an increase in mRNA and activity of catalase may represent the first  
271 process to escape an excess of ROS. However, this response seems to be insufficient.

272 Indeed, maintenance of appropriate copper levels in neurons is critical for their correct development  
273 and/or function; specifically, release of copper into the synaptic cleft regulates the excitability of  
274 neurons and also helps protect the neurons from excitotoxicity (Marmolino and Manto, 2010). Cu  
275 dyshomeostasis has been related to neurodegenerative disorders such as Alzheimer's, and  
276 amyotrophic lateral sclerosis (ALS), and it is directly involved in Mendelian disorders such as  
277 Wilson and Menkes diseases (Ahuja et al., 2014).

278 Cu is also an essential component of Complex IV of the mitochondrial respiratory chain and part of  
279 the ROS scavenging cell repertory, being a co-factor of the superoxide dismutase isoform present in  
280 the cytosol (Cu/Zn-SOD or SOD1). SOD1 is the predominant superoxide dismutase in most cells  
281 and tissues, accounting for 70–80% of the total cellular SOD activity. Its primary function is to act  
282 as an antioxidant enzyme, lowering the steady-state concentration of superoxide. Over 100 different  
283 mutations have been identified in the *SOD1* gene in patients diagnosed with the familial form of  
284 AML (Valentine et al., 2005).

285 An equilibrated molar ratio between Cu and Zn is essential for correct function of SOD1. In the  
286 presence of a Cu/Zn unbalance, equimolar Cu/Zn-SOD rapidly forms heterodimers with Zn-  
287 deficient SOD leading to SOD1 deficiency. The stabilization of Zn-deficient SOD with Cu/Zn-SOD  
288 has been suggested to contribute to the dominant inheritance of ALS mutations (Roberts et al.,  
289 2007). We suggest that the impaired molar ratio of Cu/Zn seen in A-T patients may be the basis of  
290 the SOD1 functional reduction in A-T cells.

291 Eukaryotic systems have evolved defence mechanisms against free radicals and the manganese  
292 superoxide dismutase (Mn-SOD or SOD2) is a key mitochondrial antioxidant enzyme, coded by the  
293 *SOD2* gene, which catalyses the conversion of superoxide anions to hydrogen peroxide (Flynn and  
294 Melov, 2013). Loss of SOD2 activity can result in numerous pathological phenotypes in  
295 metabolically-active tissues, particularly within the central nervous system. SOD2 is potentially  
296 involved in the progression of neurodegenerative diseases, such as stroke and Alzheimer's and  
297 Parkinson's diseases, as well as its potential role in "normal" age-related cognitive decline (Clausen  
298 et al., 2010). In this study, we found that blood manganese concentrations were comparable in  
299 patients and controls, although SOD2 enzyme activity assays showed a 30% decrease in  
300 comparison to the control group.

301 Conversely, reduction of zinc may lead to several deleterious effects. The decrease of Zn  
302 concentrations in the blood could be a result of Zn accumulation in tissues, along with dietary  
303 factors. Zinc is an essential metal implicated in the functioning of more than 200 enzymes; it plays  
304 an important role in axonal and synaptic transmission and is necessary for nucleic acid metabolism  
305 and brain tubulin growth and phosphorylation. In physiological concentrations, zinc exhibits  
306 neuroprotective activity, although an unbalance of zinc homeostasis has been reported in a number  
307 of brain processes, which can then lead to the onset of chronic pathologies such as depression,  
308 schizophrenia, AD, PD, aging, or ALS (Szewczyk, 2013). High concentrations of zinc are  
309 neurotoxic (Choi et al., 1988; Cote et al., 2005; Perry et al., 1997; Plum et al., 2010), and its  
310 deficiency has been reported in the plasma of AD patients, and it is hypothesized that a deficiency  
311 of Zn could be one of the contributing factors in the development of AD (Constantinidis, 1991;  
312 Religa et al., 2006).

313 It is interesting to note that many A-T patients display a primary immunodeficiency, and zinc  
314 deficiency may play a role in modulating this phenotype (Lynn and Wong, 1997; Prasad et al.,  
315 2007). Zinc is essential for the maintenance of immune function and for the development and



316 function of neutrophils, macrophages, and natural killer cells. Thus, zinc deficiency leads to the  
317 reduction of thymulin, interleukin-2, and interferon-gamma, and the increase in production of pro-  
318 inflammatory cytokines: zinc deficiency is also associated with a higher incidence of infections.  
319 A hypothesis for the protective role of antioxidants considers the induction of synthesis of  
320 metallothioneins (MTs). These proteins contain a large number of thiol groups, which are effective  
321 in the reduction of ROS formation (Valko et al., 2005). MTs are zinc-binding proteins involved in  
322 the regulation of the transport, storage and transfer of zinc to various enzymes and transcription  
323 factors (DiGirolamo et al., 2010; Liuzzi and Cousins, 2004).  
324 Finally, similarly to our findings, zinc was found to be significantly reduced in the plasma of  
325 children with Type 1 Diabetes Mellitus (T1DM) (Salmonowicz et al., 2014), the activity of SOD1  
326 was significantly reduced and CAT activity was significantly increased. (Shukla et al., 2006)  
327 proposed that in diseases associated with chronic oxidative stress, ROS impair the Cu/Zn-SOD  
328 function by reducing intracellular Cu ions found in protein compounds such as metallothionein and  
329 SOD.

330

## 331 **CONCLUSIONS**

332 Mutations that inactivate wide-ranging regulators such as *ATM*, the gene mutated in A-T, are  
333 expected to affect many cellular systems and cause serious disruption of cellular homeostasis. The  
334 clinical and cellular phenotypes of such disorders also indicate ongoing deleterious processes,  
335 marked by slowly progressing degeneration of specific tissues and occasionally by signs of  
336 premature aging. A possible contributor to these processes, which are also seen in various  
337 neurodegenerative diseases and aging tissues, is oxidative stress, reflected by elevated levels of  
338 ROS. A-T cells show poor cellular anti-oxidant defences and increased oxidant sensitivity  
339 compared to normal cells, and *ATM* partly functions as an oxidative stress sensor. Accumulating  
340 evidence suggests that oxidative stress is involved in the pathogenesis of A-T.

341 In line with these observations, we detected alterations in the essential trace elements copper and  
342 zinc, which are involved in the oxidative-stress response, in the blood of A-T patients, which was  
343 associated with transcriptional and functional alterations of ROS-detoxifying enzymes (CAT, SOD1  
344 and SOD2) in patients' cell lines.

345 Our results suggest that zinc and copper homeostasis may play a role in the pathology of A-T, as  
346 summarized in figure 2. The initial determinant of the dyshomeostasis of these metals is unknown,  
347 and may be *ATM* deficiency itself. In turn, they contribute to an altered antioxidant defence system,  
348 impairing SOD1 and SOD2 activity. Enhanced catalase expression is a marker of increased ROS  
349 activity. Indeed Zn depletion, due to its antioxidant properties, may act to further increase oxidative  
350 damage.

351 Cu/Zn alterations may therefore be suggested as biomarkers of A-T, and open to future works on  
352 other ataxias of genetic origin. Furthermore, given the unbalance and the antioxidant properties of  
353 Zn, dietary supplementation with this metal, which has also been proposed in other studies (Bao et  
354 al., 2008; Prasad et al., 2007), would be an interesting therapeutic possibility that should be  
355 explored in future experiments.

356

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362

363

364 **FIGURE LEGENDS**

365 **Figure 1.** Enzymatic activity and expression analysis of ROS-detoxifying enzymes in A-T  
366 lymphoblasts. A. In the upper panels, enzymatic activity of the related proteins was measured by  
367 ELISA assay and expressed as relative activity vs. controls (Superoxide Dismutase Assay kit,  
368 Cayman, MI, USA, and Catalase Assay kit, Cayman, MI, USA, #707002). CAT showed a 20%  
369 increase in activity in A-T patients. Both SOD1 and SOD2 enzymes showed a reduced activity of  
370 30-40% in A-T cell lines (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ). B. In the lower panels, expression of *CAT*,  
371 *SOD1* and *SOD2* genes was tested by real-time PCR. Reference genes for normalization were  
372 *GUSB* for *CAT*, and *SOD2* and *TBP* for *SOD1*. Fold-changes were significant for *CAT* and *SOD1*  
373 genes and concordant with activity (\* =  $p < 0.05$ ). CTRLs: controls; A-T: Ataxia-Telangiectasia  
374 patients.

375

376 **Figure 2.** Schematic summary of the role of Cu and Zn metals in A-T cells. A Cu/Zn unbalance,  
377 which may be directly associated with *ATM* mutations, or secondary to unknown causes, can affect  
378 A-T cell survival by increasing ROS directly via Cu oxidative damage, and by SOD1/SOD2  
379 impairment. ROS increase induces a higher *CAT* transcription in the attempt to reduce ROS stress.

380

381

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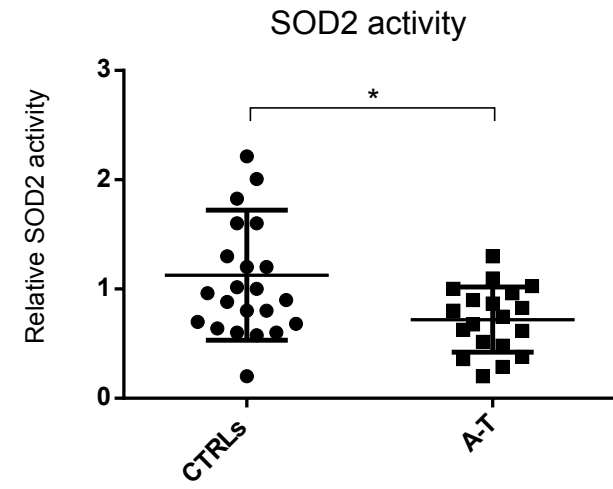
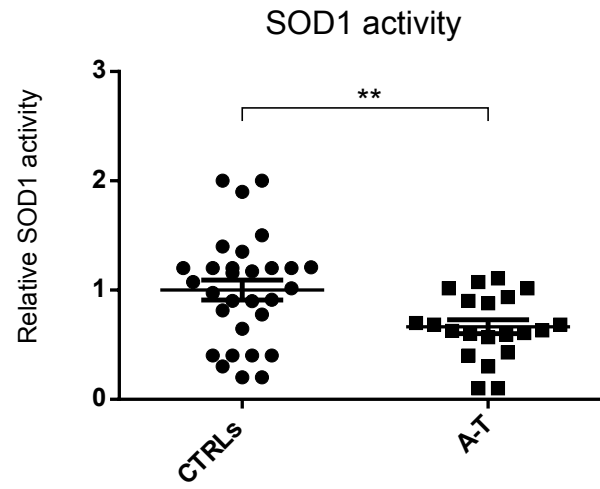
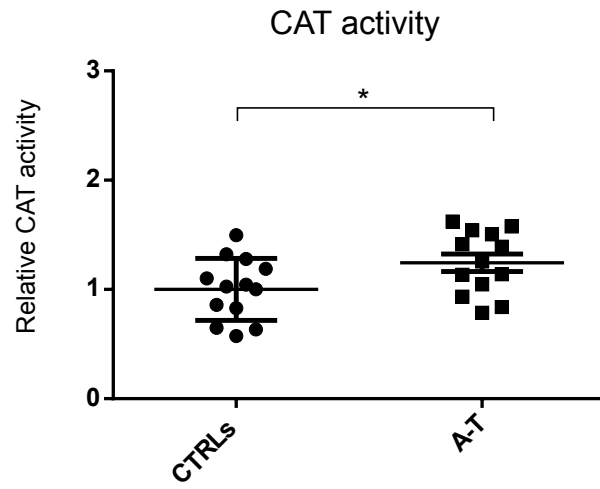
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- 484

Figure 1.

A

Enzymes activity (ELISA assay)



B

Gene expression (real-time PCR assay)

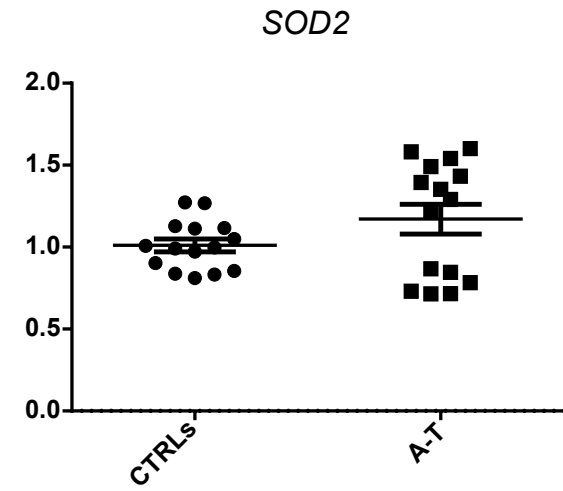
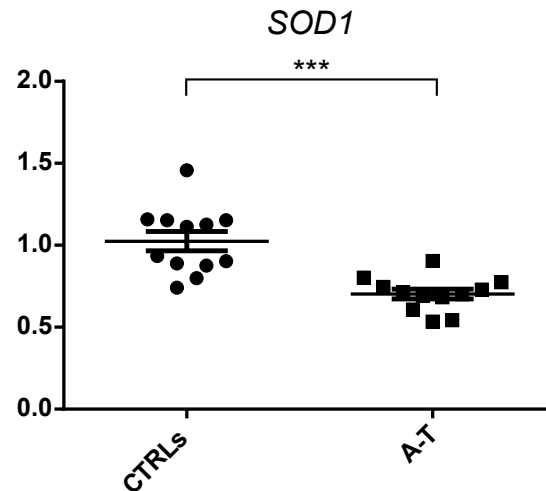
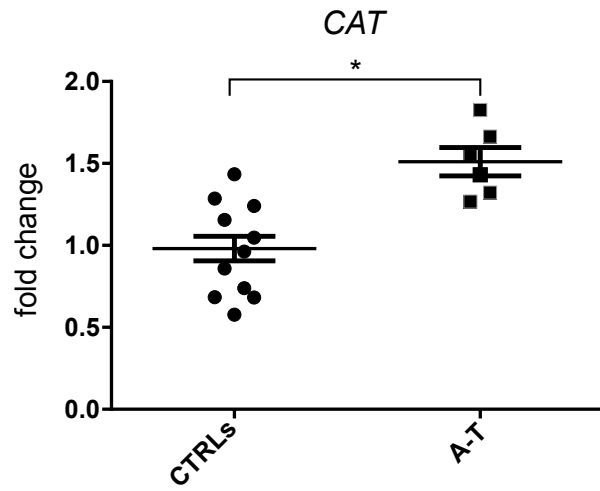




Figure 2

