Blood metal levels and related antioxidant enzyme activities in patients with ataxia telangiectasia

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ABSTRACT

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

We aimed at studying the role of essential trace elements in Ataxia-Telengiectasia (A-T), a rare form of paediatric autosomal recessive cerebellar ataxia with altered antioxidant response. We found an increased level of copper (Cu, p=0.0002), and a reduced level of zinc (Zn, p=0.0002) in the blood of patients (n. 16) compared to controls, using inductively coupled plasma mass spectrometry (ICP-MS). Other trace elements involved in the oxidative stress response, such as manganese (Mn) and selenium (Se) were unaltered. Cu/Zn-dependent superoxide dismutase (SOD1) was shown to have a 30% reduction in gene expression and 40% reduction in enzyme activity upon analysis of lymphoblastoid cell lines of patients (Student's t-test, p=0.0075). We also found a 30% reduction of Mn-SOD (SOD2; Student's t-test, p=0.02), probably due to a feedback regulatory loop between the two enzymes. The expression of antioxidant enzymes, such as erythrocyte glutathione peroxidase (*GPX1*), and *SOD2* was unaltered, whereas catalase (*CAT*) was increased in A-T cells, both at the mRNA level and in terms of enzyme activity (~25%). Enhanced *CAT* expression can be attributed to the high ROS status, which induces *CAT* transcription.

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

INTRODUCTION

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

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

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

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

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

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

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

1999), or to uncontrolled metal-mediated formation of deleterious free radicals (Gutteridge, 1995; Valko et al., 2007). In particular, essential trace elements such as copper, zinc and manganese, play a major role in metabolic pathways, and they have been studied in many diseases, including autoimmune, neurological and psychiatric disorders.

Currently, only scarce information is available, on *in vivo* redox abnormalities in A-T patients (Aksoy et al., 2004; Reichenbach et al., 2002; Reichenbach et al., 1999).

Here, we have studied the blood concentration of trace elements in A-T patients. We further explored the expression of *CAT, GPX1, SOD1* and *SOD2*, and the activity of CAT, SOD1 and SOD2 enzymes in A-T lymphoblastoid cell lines, to verify if changes in the metal concentration correlate with antioxidant enzyme activity, which is probably secondary to the alterations in their cofactor concentrations.

MATERIALS AND METHODS

Patients

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

Blood sampling and analysis of metals

Venous blood was collected in heparinized vacutainer BD tubes (Becton Dickinson Labware, Franklin Lakes, USA), and stored at -20°C until required for analysis. A sub-aliquot of 1 mL of blood was transferred into a 15 mL plastic tube (Falcon, Becton, Franklin Lakes, USA), then added to 2mL of super-pure concentrated HNO_3 (Romil Ltd., Cambridge, UK) and microwave (MW) digested in an ETHOS-Mega II oven (FKV, Bergamo, Italy), following the temperature ramp program: 45 min to reach 100°C and 4 h at 100°C. The system was equipped with an optical sensor to regulate the temperature. In each digestion, a blank reagent and a blood certified reference material was also processed (Seronorm trace elements whole blood level 2, Sero AS, Billingstadt, Norway).

Instruments

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

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

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

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

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Table S1. Quantification limit values (µg/L), reference material values and percentages of recovery.

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

Note: n.a: not applicable because <LOQ

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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_2
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)]

Gene expression

Total RNA was extracted using Direct-zol according to the manufacturer's protocol (Zymo Research Corporation, Irvine, USA); one milligram was retro-transcribed using M-MLV Reverse Transcriptase (Life Technologies Europe, Monza, Italy). Quantitative real-time RT-PCR to evaluate *CAT*, *GPX1*, *SOD1* and *SOD2* expression was carried out on an ABI-Prism7500 Fast instrument (Life Technologies, Europe) using the TaqMan® Universal PCR Master Mix, Universal Probe 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
CAT	5'-gctcattttgaccgagagaga	5'-tgacctcaaagtagccaaagg	#68
GPX1	5'-caaccagtttgggcatcag	5'-tctcgaagagcatgaagttgg	#77
SOD1	5'-tcatcaatttcgagcagaagg	5'-gcaggccttcagtcagtcc	#60
SOD2	5'-ctggacaaacctcagcccta	5'-tgatggcttccagcaactc	#22

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

Analysis of SOD isoforms activities

A total of $1x10^7$ cells, obtained from five A-T cell lines and four control cells, were collected and homogenized in lysis buffer (20 mM Hepes pH 7.2, 1 mM EGTA, 210 mM mannitol and 70 mM

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

Analysis of CAT activity

The day of the experiment, a total of $5x10^6$ cells, obtained from three A-T cell lines and three control cells, were collected by centrifugation, washed twice in PBS and homogenized in ice cold lysis buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.0). Cells were centrifuged at 10,000 g for 15 minutes at 4°C. Supernatant was stored on ice for the assay. To evaluate CAT activities, we used the Catalase Assay kit (Cayman, MI, USA, #707002) following manufacture's protocol. Analysis was performed by monitoring the absorbance at 540 nm on a xMark microplate Reader (Bio-Rad Laboratories S.r.l., Segrate, Italy). CAT activity was defined as the amount of CAT enzyme able to produce 1.0 nmol of formaldehyde per minute at 25°C, interpolating the values in a standard curve of formaldehyde ranging 0-75 µM. Each sample was assayed in duplicate and in at least three independent experiments.

Statistical analysis

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

RESULTS

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

Table 1. Median concentration of metals (μg/L ± S.D) in the blood of A-T patients and controls.

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

Note: In bold statistically significant metal ions.

As, Be, Cd, Co, Ni, Pb, Sb, Sn and Tl levels were below the limit of quantitation of the method. Copper levels were significantly higher in A-T patients ($p = 0.0002$) and zinc levels were significantly lower ($p = 0.0002$). As copper and zinc are co-factors for ROS detoxifying enzymes,

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

Considering the feedback regulatory loop of detoxifying enzymes, we measured the expression of the same genes and *GPX1* involved in the ROS response. Using real-time quantitative PCR, we showed a decreased SOD1 mRNA level in A-T cells vs. CTRLs (A-T median dose: 0.7 ± 0.03; n = 4. CTRLs median dose: 1.0 ± 0.05; n = 4) (Student's t-test, p = 0.0001). *GPX1* and *SOD2* showed levels similar to healthy controls (*GPX1*: A-T median dose: 0.95 ± 0.04; n = 5 vs. CTRLs median dose: 1.1 ± 0.08; n = 5; Student's t-test, p = 0.02, data not shown. *SOD2*: A-T median dose: 1.1 ± 0.06; n = 5 vs. CTRLs median dose: 1.0 ± 0.03; n = 5; Student's t-test, p = 0.10). Catalase mRNA level was increased in A-T cells vs. CTRLs (A-T median dose: 1.5 ± 0.09; n = 5 CTRLs median dose: 1.0 ± 0.07; n=5) (Student's t-test, p = 0.0012) (Figure 1B).

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

function of neutrophils, macrophages, and natural killer cells. Thus, zinc deficiency leads to the reduction of thymulin, interleukin-2, and interferon-gamma, and the increase in production of pro-inflammatory cytokines: zinc deficiency is also associated with a higher incidence of infections.

A hypothesis for the protective role of antioxidants considers the induction of synthesis of metallothioneins (MTs). These proteins contain a large number of thiol groups, which are effective in the reduction of ROS formation (Valko et al., 2005). MTs are zinc-binding proteins involved in the regulation of the transport, storage and transfer of zinc to various enzymes and transcription factors (DiGirolamo et al., 2010; Liuzzi and Cousins, 2004).

Finally, similarly to our findings, zinc was found to be significantly reduced in the plasma of children with Type 1 Diabetes Mellitus (T1DM) (Salmonowicz et al., 2014), the activity of SOD1 was significantly reduced and CAT activity was significantly increased. (Shukla et al., 2006) proposed that in diseases associated with chronic oxidative stress, ROS impair the Cu/Zn-SOD function by reducing intracellular Cu ions found in protein compounds such as metallothionein and SOD.

CONCLUSIONS

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

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

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

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

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FIGURE LEGENDS

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

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

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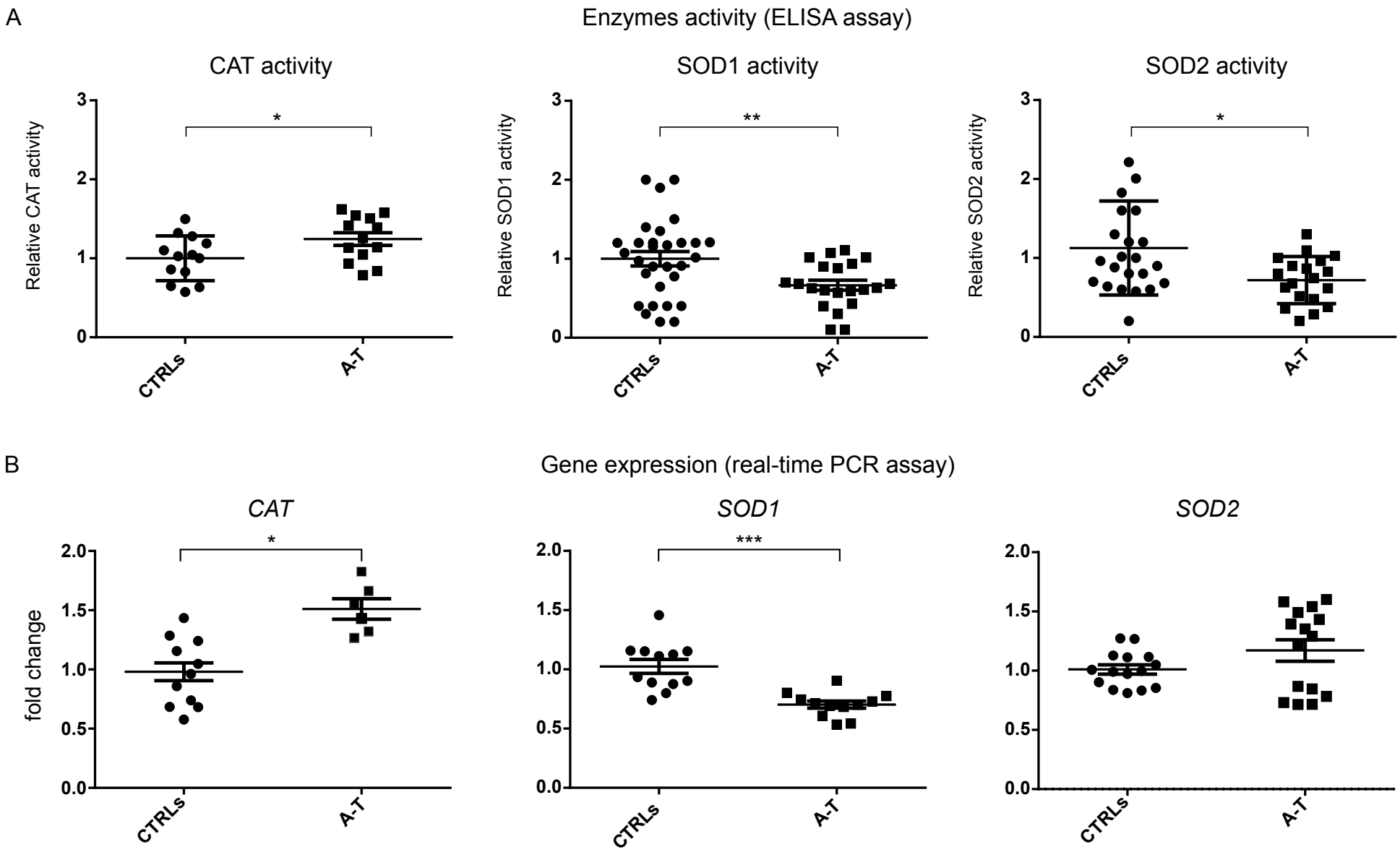
484

Figure 1.


A
Enzymes activity (ELISA assay)
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SOD2 activity
Relative CAT activity
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Gene expression (real-time PCR assay)
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SOD2
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Figure 2