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Original article

Non-protein-bound iron and 4-hydroxynonenal protein adducts in classic autism

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Abstract

A link between oxidative stress and autism spectrum disorders (ASDs) remains controversial with opposing views on its role in the pathogenesis of the disease. We investigated for the first time the levels of non-protein-bound iron (NPBI), a pro-oxidant factor, and 4-hydroxynonenal protein adducts (4-HNE PAs), as a marker of lipid peroxidation-induced protein damage, in classic autism. Patients with classic autism ($n = 20$, mean age 12.0 ± 6.2 years) and healthy controls ($n = 18$, mean age 11.7 ± 6.5 years) were examined. Intraerythrocyte and plasma NPBI were measured by high performance liquid chromatography (HPLC), and 4-HNE PAs in erythrocyte membranes and plasma were detected by Western blotting. The antioxidant defences were evaluated as erythrocyte glutathione (GSH) levels using a spectrophotometric assay. Intraerythrocyte and plasma NPBI levels were significantly increased (1.98- and 3.56-folds) in autistic patients, as compared to controls ($p = 0.0019$ and $p < 0.0001$, respectively); likewise, 4-HNE PAs were significantly higher in erythrocyte membranes and in plasma (1.58- and 1.6-folds, respectively) from autistic patients than controls ($p = 0.0043$ and $p = 0.0001$, respectively). Erythrocyte GSH was slightly decreased (−10.34%) in patients compared to controls ($p = 0.0215$). Our findings indicate an impairment of the redox status in classic autism patients, with a consequent imbalance between oxidative stress and antioxidant defences. Increased levels of NPBI could contribute to lipid peroxidation and, consequently, to increased plasma and erythrocyte membranes 4-HNE PAs thus amplifying the oxidative damage, potentially contributing to the autistic phenotype.

Keywords: Neurodevelopment disorders; Oxidative stress; Free iron; Lipid peroxidation; 4-hydroxynonenal protein adducts

1. Introduction

Autistic spectrum disorders (ASDs) are a complex group of neurodevelopment disorders, still poorly understood and treatment-refractory. Specifically, ASDs refer to five different disorders characterized by delay in the development of multiple basic functions: classic autistic disorder (also referred as "classic autism"), a milder form known as Asperger syndrome,

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Table 1
Oxidative stress markers and antioxidant defenses in ASDs: summary of the current knowledge.

Antioxidant defences	Oxidative stress markers
Total antioxidant capacity ↓ Plasma TAOS [5*]	**Pro-oxidant factors** Altered serum Cu/Zn ratios [19**] ↑ Plasma NO [7**,20] ↑ Erythrocyte NO [15] ↔ Plasma homocysteine [5*,6] ↔ Plasma Fe, Cu and Zn [5*]
Glutathione ↓ Plasma GSH [6,7**,8-9,10*] ↓ Plasma GSH/GSSG ratio [6,8,10] ↓ GSH/GSSG ratio in lymphoblastoid cells [11] ↓ Erythrocyte GSH [12*] ↔ Erythrocyte GSH [5*]	
Glutathione Peroxidase ↓ Plasma and erythrocyte GSH-Px [13] ↓ Plasma GSH-Px [14] ↑ Plasma GSH-Px [9,15] ↔ Erythrocyte GSH-Px [5*]	**Lipid peroxidation markers** ↑ Plasma MDA [20-22] ↑ Plasma F_2-isoprostanes [14] ↔ Plasma F_4-neuroprostanes [23] ↔ Plasma TBARS [5*,15] ↑ Erythrocyte TBARS [16]
Superoxide Dismutase ↔ Plasma SOD [5*,15] ↑ Erythrocyte SOD [9,16] ↓ Erythrocyte SOD [13]	**Protein oxidation markers** ↑ Plasma 3-nitrotyrosine [6,10*] ↑ Plasma Protein carbonyls [20]
Catalase ↔ Plasma catalase [9] ↓ Erythrocyte catalase [16] ↔ Erythrocyte catalase [5*]	
Antioxidant proteins (metal-binding proteins) ↓ Serum ceruloplasmin [7**] ↔ Plasma ceruloplasmin [5*] ↓ Serum transferrin [7**] ↔ Plasma transferrin [5*]	**DNA oxidative damage** ↑ Leukocyte 8-OHdG [6]
Vitamins ↓ Plasma Vitamin E [9] ↔ Plasma Vitamin E [5*] ↔ Plasma Vitamin A [5*] ↔ Plasma Vitamin B12 [5*,6] ↔ Vitamin C [9]	
GSH pathway genes variations Glutathione pathway genes variation and risk of ASDs [17] Polymorphism of GSH-Px 1 [18*]	**Mitochondrial dysfunction** ↑ Plasma lactate/pyruvate ratio [20]

Symbols legend: ↑ increased; ↔ unchanged; ↓ decreased. *Including also data from patients with Asperger syndrome and/or Pervasive Development Disorder not otherwise specified (PDD/NOS); **Review article.
Legend: TAOS, total antioxidant status; GSH, reduced glutathione; GSSG, oxidized glutathione; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; NO, nitric oxide; MDA, malondialdheide; TBARS, thiobarbituric acid reactive substances; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

the rare genetic condition known as Rett syndrome (RTT), childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS) [1].

The onset of clinical manifestation in ASDs is usually within the 2nd year of life and mainly consists in the triad (1) impairment in language, (2) social behaviour and (3) stereotyped or repetitive behaviour. ASDs are considered to be the result of a complex interaction between a genetic background and environmental factors [2], and appear to be steadily increasing in frequency, according to some authors. In fact, autism diagnosis has been reported to be rising by some counts from 1 in 5000 in the mid 1970s, to 1 in 110 in 2009 [3]. If from one side, the rise in autistic patients can be explained for *ca.* 50% of the observed growth because of increased awareness, diagnosis and social factors, the reasons for about 46% of the increase remains unexplained.

Oxidative stress (OS) is a well-known pathogenic mechanism involved in several human pathologies. By definition, OS occurs when the antioxidant response is insufficient to balance the production of reactive oxygen species (ROS), potentially causing cell death by apoptosis or necrosis via an array of signaling pathways. Brain is particularly vulnerable to ROS damage compared to other organs due to its high metabolic rate combined with a relatively low concentration of antioxidant

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proteins. OS and mitochondrial dysfunction have been implicated in all major neurodegenerative disorders (i.e., amyotrophic lateral sclerosis, Parkinson's and Alzheimer's disease) [4].

A potential relationship between OS and ASDs has been repeatedly explored [5–23] (Table 1) and generally found to be related to either increased OS or altered antioxidant defences, although this link remains highly controversial, with opposing views regarding its role in the development of the autistic phenotype. In particular, at this time, it is unclear whether OS is a cause or consequence of autism [24], and the issue is further complicated by the heterogeneity of patients series (often mixing classic autism with Asperger syndrome and/or PDD-NOS; see Table 1 legend) and the employed OS biomarkers.

Recently, we have shown increased OS marker levels, i.e., non-protein-bound iron (NPBI: free redox-active iron) [25] and 4-hydroxynonenal protein adducts (4-HNE PAs) [26] in RTT, a particular form of ASDs that belongs to those small group of the autistic conditions known to be the result of a single gene mutation.

To date, no information exists regarding NPBI, a pro-oxidant factor, and 4-HNE PAs, a biochemical marker of lipid peroxidation-induced protein damage, in classic autism.

NPBI acts as a pro-oxidant, causing release of hydroxyl radicals as the results of the Fenton reaction [4]. In turn, the hydroxyl radicals can initiate lipid peroxidation of polyunsaturated fatty acids (PUFA), producing several compounds of degradation, one of the most studied is 4-HNE, which can covalently bind proteins, phospholipids, and DNA. This highly reactive aldehyde reacts readily with nucleophilic groups of protein amino acidic side chains, and its covalent attachment to proteins, lead to alteration in their structure and biological activity [27]. Thus, 4-HNE, depending on its concentration and location, may be considered as a "second toxic messenger", which disseminates and augments initial free radical events.

In the present study, we evaluated the levels of NPBI and 4-HNE PAs in plasma and erythrocytes (RBCs) of patients with classic autistic disorder.

2. Subjects and methods

2.1. Subjects

A total of 20 patients with classic autistic disorder (mean age: 12.0 ± 6.2 years, range 4–30), as well as 18 healthy controls of comparable age (mean age: 11.7 ± 6.5 years, range 4–30) participated to the study.

All the patients were consecutively admitted to the Child Neuropsychiatry Unit of the University Hospital of Siena (Head: J.H.). Patients with diagnosed RTT, X-fragile or tuberous sclerosis were excluded from the present study. In addition, subjects with perinatal adverse events and/or brain abnormalities on magnetic resonance imaging (MRI) were excluded for the present study. All the subjects were on a typical Mediterranean diet.

None of the autistic patients had active epilepsy at the time of blood sampling. A total of 4 patients (16%) experienced seizures early in the course of their disease, which were no longer present. In particular, none of the patients exhibited complications of epileptic seizures. Antiepileptic drug (AED) treatment (i.e., either carbamazepine or valproic acid), as monotherapy, was assumed by 8 patients (32%) as mood stabilizers at an average dose of 10 mg/b.w./day.

The autistic patients were diagnosed by Diagnostic and Statistical Manual of mental disorders IV (DSM-IV) and evaluated using Autism Diagnostic Observation Schedule (ADOS), and Autism Behaviour Checklist (ABC). Childhood Autism Rating Scale scores (CARS) [28] were calculated and Intelligence quotient (I.Q.) estimated in both the autistic and control group by an experienced child neuropsychiatrist (J.H.). Blood samplings in the control group were carried out, during routine health checks or blood donations, always followed by written informed consent. The study was conducted after the approval by the Institutional Review Board and all written informed consents were obtained from either the parents or the legal tutors of the enrolled patients.

2.2. Blood sampling

All samplings were carried out around 8 AM after overnight fasting. Blood was collected in heparinized tubes and all manipulations were carried out within 2 h after sample collection. The blood samples were centrifuged at 2400*g* for 15 min at 4 °C; the platelet poor plasma was saved and the buffy coat was removed by aspiration. RBCs were washed twice with physiological solution (150 mM NaCl). An aliquot of packed erythrocytes was resuspended in Ringer solution [125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 5 mM glucose, 1 mM $CaCl_2$], pH 7.4 as a 50% (vol/vol) suspension for the determination of intraerythrocyte NPBI. The remaining volume of packed erythrocytes was used for erythrocyte membranes preparations (i.e., haemoglobin-free ghosts) for 4-HNE PAs determinations. Plasma was used for the NPBI assay and Western blotting.

2.3. Intraerythrocyte and plasma NPBI

Intraerythrocyte NPBI was determined as a desferrioxamine (DFO)–iron complex (ferrioxamine), as previously reported [25]. Briefly, 25 μM DFO was added to

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the samples (1 ml erythrocyte suspension), the erythrocytes were then lysed by adding water (1 vol) and freezing (−70 °C) and thawing. The hemolysate was ultrafiltered at 3373*g* for 30 min in centrifugal filters with a 30-kDa molecular weight cut-off (VIVASPIN 4, Sartorius Stedim Biotech GmbH, Goettingen, Germany) and the ultrafiltrate was stored at −20 °C until analysis. The DFO excess was removed by silica (Silicagel; 25–40 μm) column chromatography. The DFO–iron complex was determined by HPLC and the detection wavelength was 229 nm. Plasma NPBI was determined as reported for intraerythrocyte NPBI. DFO (25 μM) was added to plasma diluted (1:1; vol:vol) with Ringer solution, then the samples were ultrafiltered and stored at −20 °C until analysis as above. The calibration curve correlation for intraerythrocyte NPBI and plasma NPBI was adequate ($r^2 = 0.994009$), the minimum detection limit was 0.1 nmol/ml, and mean intra- and inter-observer coefficients of variation of ⩽2.5% and ⩽5%, respectively.

2.4. Erythrocyte membrane preparation

An aliquot (600 μl) of packed RBC was lysed in Dodge buffer, and erythrocyte membranes were prepared, according to Dodge [29], by repeated washing until the "ghosts" were pearly white. Samples were kept frozen at −70 °C until used for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.5. Western blot for erythrocyte and plasma 4-HNE protein adducts

Western blot protocols were performed as previously described [26]. Erythrocyte membrane and plasma proteins (30 μg protein, determined using Bio-Rad protein assay; BioRad, Hercules, CA, USA) were resolved on 4–20% SDS–PAGE gels (Lonza Group Ltd., Switzerland) and transferred onto a hybond ECL nitrocellulose membrane (GE Healthcare Europe GmbH, Milan, Italy). After blocking in 3% non-fat milk (BioRad, Hercules, CA, USA), the membranes were incubated overnight at 4 °C with goat polyclonal anti 4-HNE adduct antibody (cod. AB5605; Millipore Corporation, Billerica, MA, USA). Following washes in TBS Tween and incubation with specific secondary antibody (mouse anti-goat horseradish peroxidase-conjugated, Santa Cruz Biotechnology Inc., CA, USA) for 1 h at RT, the membranes were incubated with ECL reagents (BioRad, Hercules, CA, USA) for 1 min. The bands were visualized by autoradiography. Quantification of the significant bands was performed by digitally scanning the Amersham Hyperfilm™ ECL (GE Healthcare Europe GmbH, Milan, Italy) and measuring immunoblotting image densities with ImageJ software.

2.6. Erythrocyte glutathione

Glutathione (GSH) content determinations in RBCs were carried out according to the spectrophotometric method of Beutler et al. [30]. After RBCs lysis and subsequent metaphosphoric acid protein precipitation, the method involved GSH oxidation by sulfhydryl reagent 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to form the derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm.

2.7. Statistical analysis

Variables of interest were tested for normal distribution (D'Agostino–Pearson test) and data are presented either as means ± SD or medians (inter-quartile range), as appropriate. As the variables tested were continuous normally distributed, average values from autistic patients and the control population were compared by using independent-sample *t* test. Associations between continuous parametric variables were tested using linear regression analysis. The MedCalc version 12.1.4 statistical software package (MedCalc Software, Mariakerke, Belgium) was used, and two-tailed *p* values of less than 0.05 were considered significant.

3. Results

3.1. CARS and IQ ranges

CARS scores were 50.3 ± 7.6 in the autistic patients vs. 19.5 ± 2.4 in the control group ($p < 0.0001$). Estimated IQs were 30.0 ± 5.1 in the autistic patients and 97.5 ± 5.9 in the control group ($p < 0.0001$). In the autistic group, CARS score was positively related with chronological age [$r = 0.7695$ (0.5902 to 0.8765), $p < 0.0001$] and inversely correlated with IQ [$r = -0.6773$ (−0.8074 to −0.4843), $p < 0.0001$]. Likewise, in the autistic patients IQ was inversely related to age [$r = -0.6195$ (−0.7858 to −0.3697), $p < 0.0001$]. In contrast, no significant correlations between CARS or IQ and age were present in the control group.

3.2. Intraerythrocyte and plasma NPBI

The levels of intraerythrocyte NPBI was significantly higher in autistic patients as compared with the control RBCs ($p = 0.0019$), with a *ca.* 1.98-fold increase (Fig. 1A). Likewise, an 3.56-fold increase in plasma NPBI was detected in autistic patients as compared to healthy controls ($p < 0.0001$) (Fig. 1B).

3.3. Erythrocyte membrane and plasma 4-HNE protein adducts

4-HNE PAs were examined by Western blot analysis and the specificity of 4-HNE antibody was assessed by

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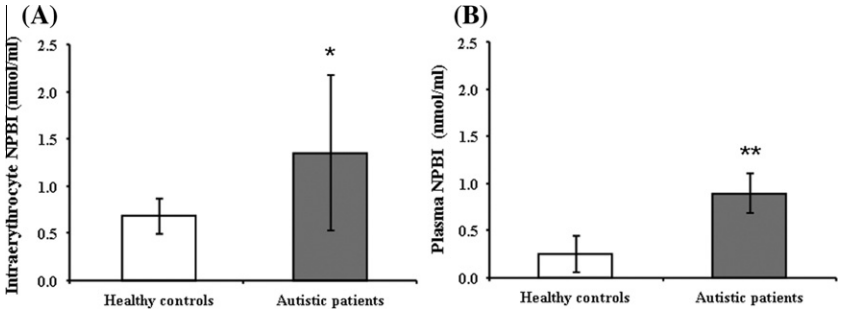

Fig. 1. Non-protein-bound-iron (NPBI) in erythrocytes and plasma from patients with autism. (A) Intraerythrocyte NPBI in autistic patients ($n = 20$) and healthy controls ($n = 18$). Intraerythrocyte NPBI was expressed as nmol/ml packed erythrocytes. (B) Plasma NPBI in patients with autism ($n = 20$) and healthy controls ($n = 18$). Statistical significant differences were reported. The values are reported as means ± SD. $^{*}p = 0.0019$ significantly different from controls. $^{**}p < 0.0001$ significantly different from healthy controls.

dot-blot analysis (data not shown), as previously described [26]. 4-HNE PAs signal was clearly increased in the erythrocyte membranes of autistic patients respect to the healthy controls ($p = 0.0043$) (Fig. 2A). Quantification of the bands (Fig. 2C) shows that erythrocyte membrane 4-HNE PAs were significantly increased of 1.58-folds. As illustrated in Fig. 2B and D, the trend showed in the erythrocyte membrane 4-HNE PAs was also observed in the plasma of autistic patients (1.6-fold increase; $p = 0.0001$ as compared to healthy controls). In addition, in Fig. 2A and B, it possible to appreciate the physiological variability between the some representative samples shown, demonstrating a certain grade of 4-HNE PAs signal also in the healthy controls.

3.4. Intraerythrocyte GSH

Autistic patients showed slightly reduced erythrocyte GSH levels compared to those of the healthy controls (−10.34%; $p = 0.0215$) (Fig. 3).

3.5. Correlations between OS markers, erythrocyte GSH, CARS and IQ values

We observed a positive correlation between intraerythrocyte NPBI and plasma NPBI levels [$r = 0.7289$ (0.4998 to 0.8626), $p < 0.0001$]. We also observed a significant positive correlation between intraerythrocyte NPBI and erythrocyte membrane 4-HNE PAs

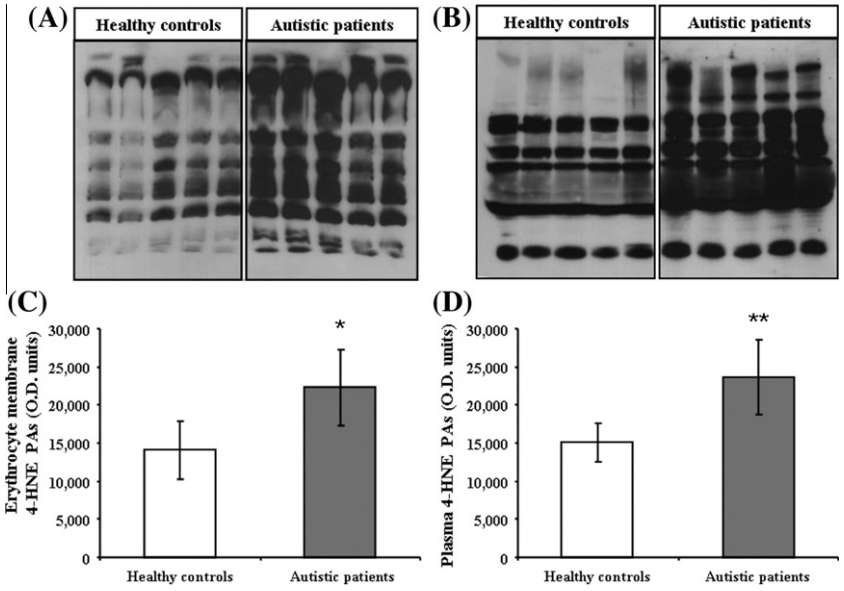

Fig. 2. Immunochemical detection of 4-hydroxynonenal protein adducts (4-HNE PAs) PAs in erythrocyte membrane and plasma samples from autistic patients. Panels A and B depict Western blot for 4-HNE PAs in erythrocyte membranes and plasma from healthy controls ($n = 18$) and autistic patients ($n = 20$). Shown is a representative of four replicate experiments. In the bottom panels (C and D) is shown the quantification of 4-HNE PAs bands, determined by densitometric analysis of the scanned images. Average values were expressed as arbitrary units. The values are reported as medians (inter-quartile range). $^{*}p = 0.0043$ significantly different from healthy controls. $^{**}p = 0.0001$ significantly different from healthy controls.

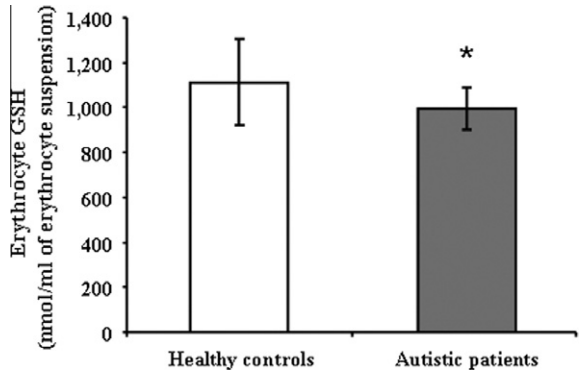

Fig. 3. Erythrocyte glutathione (GSH) levels in patients with autism. The values are reported as means ± SD; $^{*}p = 0.0215$ significantly different from healthy controls.

[$r = 0.7862$ (0.1829 to 0.9594), $p = 0.0207$] and a trend for the correlation between plasma NPBI and 4-HNE PAs [$r = 0.5685$ (−0.0079 to 0.8614) and $p = 0.0537$], whereas no significant correlations were observed between intraerythrocyte GSH and the examined OS biomarkers ($p \geqslant 0.5004$, data not shown). No significant correlations were observed between the examined OS biomarkers, erythrocyte GSH, CARS and IQ (classic autism: r values range: −0.3185 to 0.2325; p values range: 0.2889 to 0.9508; controls: r values range: −0.2869 to 0.2406; p values range: 0.4761 to 0.9378).

In addition, no statistical significant differences ($p \geqslant 0.78$) in the levels of the examined OS markers were observed between the AED- treated and AED-untreated sub-populations.

4. Discussion

Our data showed a clear and significant increase of the evaluated OS markers along with a decrease of erythrocyte GSH in autistic patients, thus confirming that OS can play a role in the pathogenesis of autism. However, it is difficult to determine whether OS is a primary contributor to the development of the disease or whether it is a secondary consequence of the original insults that have been proposed to contribute to phenotypical features of disorder. As increased NPBI and 4-HNE PAs have also been reported in RTT [25,26], a genetically determined condition in which autistic traits are invariably present in the regression stage of the natural progression of the disease, it is conceivable that, at least partially, some common mechanisms are involved in the development of the autistic phenotype. The observed decrease in the erythrocyte GSH content, indicating lower antioxidant defences, is in line with prior reports [6–12]. It is interesting to note that the inclusion or exclusion of autistic sub-types different from classic autistic disorder does not significantly change the current knowledge on the oxidative derangement previously reported in autism [5–23] (Table 1). Taken together, the data from the literature and our findings strongly support the notion that OS may be involved in the pathogenesis of autism.

It is well established that redox-active iron is one of the most active sources of OS. In particular, iron is intimately linked to OS [31]. Several studies have established an involvement of disruption of iron regulation as an important etiological factor in neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, Huntington's disease and Friedreich's ataxia [32]. We have previously demonstrated that OS induces in RBCs iron release in a free redox active form accompanied by methemoglobin (Met-Hb) formation [33]. Our results show an increase in the intraerythrocyte NPBI levels in autistic patients and, as part of free iron could diffuse out of RBCs [34], this fact may account also for the observed increase of plasma NPBI. Indeed, the significant positive correlation observed between intraerythrocyte and plasma NPBI levels in our patients would suggest this possibility.

NPBI causes a release of hydroxyl radical, an extremely powerful oxidizing species, which, in turn, is the main trigger for lipid peroxidation [4].

4-HNE PAs are to be considered not only a reliable marker of OS in that, once the aldehyde forms the adducts to the proteins, they show a biological impact by likely changing protein function. The presence of 4-HNE PAs represents a consequence of lipid peroxidation and therefore plasma proteins can be considered a target of increased OS status in classic autism. These irreversible protein modifications have been extensively investigated in research on neurological diseases, such as Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis, and Parkinson's disease [35] and also on neurodevelopmental disorders, such as RTT [26].

Whether the enhanced OS observed in autistic patients is exogenous or endogenous is, of course, still unknown.

Several authors have suggested a link with environmental stressors [2]. This idea is supported by the epidemiologic evidence that exposure, near roadways, to traffic-related air pollutants, not only during pregnancy but also in early postnatal life, augments the genetic risks implicated in autism aetiology [36]. The work by Volk et al. [37] showed higher incidence of autism in the children living within 309 m of the freeway around the time of birth. This could be a consequence of the pro-oxidant effect that air pollution has on central nervous system during developmental process, a period of particular vulnerability and susceptibility to any environmental insult for brain. For example, it is well demonstrated that diesel exhaust particles, benzo[α]pyrene and ozone could affect neuro-maturation in animal models through their induced oxidative damage [38,39].

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Alternatively, as previously shown for RTT, it is possible that OS detected in autistic patients derives from an endogenous source. In fact, our group showed that the probable origin of oxidant insult in RTT could be an impaired pulmonary gas exchange with a consequence chronic hypoxia condition [25]. Although there are no solid data showing a clear relationship between systemic hypoxia and autism, it is possible that the similar phenomena observed in RTT occurs also in autistic patients, at least during or after gestation, or locally in cerebral tissue. For example, the work of Kolevzon et al. [40], on the basis of epidemiological evidence, has advanced the possibility that neonatal or foetal hypoxia are implicated in autism; while Burstyn et al. [41] has suggested a weak effect of foetal hypoxia on risk of autism. Furthermore, even if only at the brain level, it has been shown that autism is characterized by a hypoperfusion condition with the resulting brain hypoxia [42].

Our results show a statistically significant increased levels of erythrocyte membrane and plasma 4-HNE PAs in autistic patients as compared to their non-autistic siblings. In addition, we found a linear positive correlation between 4-HNE PAs and NPBI, although this correlation was significant for RBCs, but not plasma. The reason of this discrepancy would be due to the diluting factor of the high blood volume or to the limited number of patients.

Several factors, ultimately leading to enhanced OS in autism, may all be connected to increased levels of 4-HNE PAs, such as alterations in antioxidant enzymes, abnormal iron and copper metabolism, imbalance in homocysteine and methionine metabolism, increased xanthine oxidase activity, and mitochondrial dysfunction [7].

Recent evidence indicates that the activity of the main enzyme involved in the 4-HNE detoxification, which is glutathione-S-transferases (GST), is significantly decreased in autistic RBCs [43], causing an impaired ability to metabolize 4-HNE PAs. This condition might lead to an increase in the susceptibility of plasma and RBCs membrane proteins to 4-HNE "attack", explaining the increased 4-HNE PAs levels observed in autistic patients.

Significant alterations in the fatty acid profiles in individuals with ASDs has been reported, in both plasma and membrane RBCs [44]. It is possible that pro-oxidant environment present in autistic patients could contribute to fatty acid deficiencies; particularly, lower arachidonic acid (AA) concentration recorded in children with ASDs [45] is consistent with the increased levels of 4-HNE PAs, as 4-HNE is a oxidation product generated, among others, from AA by a free radical initiated process. Moreover, NPBI-induced lipid peroxidation and the consequent 4-HNE PAs formation could lead to the loss of membrane function and integrity in RBCs as observed in a recent study, where the fluidity of the erythrocyte membrane of children with autism was lower than the controls [7].

The interest of the role of peroxidation in neurodegenerative diseases and also in neurodevelopment disorders has been greatly increased due to the particular composition of brain. In fact, the brain is highly vulnerable to OS due to its limited antioxidant capacity, higher energy requirement, and higher amounts of lipids and iron [4,31]. Therefore, neurons are more susceptible to OS since they are the first cells to be affected by the increase in ROS and shortage of antioxidants. The issue of using biological samples other than the brain tissue or the cerebrospinal fluid (CSF) for monitoring the redox status of patients with a neurological disease, in which the OS is involved, is a long-standing one and a question not easy to solve. However, our OS prior studies in a rare disease included in the ASDs group, i.e. RTT, indicate that blood may be considered a suitable biological sample in order to monitor an autistic disorder [23,25,26].

Taken together, these findings suggest that a systemic oxidant status, triggered and possibly amplified by NPBI release, may play a key role in the pathogenic mechanisms of classic autistic disorder. It is possible that a vicious cycle is activated where the presence of NPBI induces peroxidation and subsequently, RBCs exposed to lipoperoxidation products, such as 4-HNE, are damaged and further iron is released from haemoglobin.

A critical question regards the issue of whether increased OS is a primary contributor or just a secondary consequence of autism. In the lack of an universally accepted experimental model of autism it is currently impossible to solve this puzzle at this time. Future investigations in this animal models are certainly needed in order to better dissect at the molecular level the relationship between OS damage and neurodevelopment. Nevertheless, our study, as well as many other reports of the literature, indicates a pro-oxidant/antioxidant balance shift toward the pro-oxidant arm in classic autism, thus paving the way towards new therapeutical strategies aimed at modulating OS in these patients.

In conclusion, our data suggest that, whatever the source, an oxidative-driven brain damage could play a key role in the pathogenesis of autism.

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None.

Conflicts of interest

All authors declare that they have no conflicts of interest related to the present study.

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