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The biological basis of Autism Spectrum Disorders:

Evaluation of oxidative stress

and erytrocyte membrane alterations

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Abstract

Oxidative stress may play a role in the pathogenesis of Autism Spectrum Disorders (ASD). We evaluated a high number of peripheral oxidative stress parameter, erythrocyte and lymphocyte membrane functional features, morphology and membrane lipid composition of erythrocyte. The study was conducted in two successive steps (First Study and Second Study), involving a total of 29 autistic children (Au) aged 5 to 12 years, and 28 gender and age-matched typically developing children (TD), some of which have took part in both studies (4 Au and 4 TD) [First study results has been published in Plos One (Ghezzo A et al. 2013)].

Erythrocyte TBARS (MDA), **Peroxiredoxin II, Protein Carbonyl Groups** and urinary **HEL and Isoprostane** levels **were elevated in AU**, thus confirming the occurrence of an imbalance of the redox status of Au; other oxidative stress markers or associated parameters (urinary 8-oxo-dG, plasma Total antioxidant capacity and plasma carbonyl groups, erythrocyte SOD and catalase activities) were unchanged, whilst peroxiredoxin I showed a trend of elevated levels in red blood cells of Au children.

A very significant **reduction** of both **erythrocyte and lymphocyte Na+, K+-ATPase activity (NKA)** ($P<0,0001$), a reduction of **erythrocyte membrane fluidity**, a reduction of **Phospatydylserine** exposition on erythrocyte membranes, an alteration in erythrocyte fatty acid membrane profile (**increase in MUFA and in ω6/ω3 ratio due to decrease in EPA and DHA**) and a reduction **of cholesterol** content of erythrocyte membrane were found in Au compared to TD, without change in erythrocyte membrane sialic acid content and in lymphocyte membrane fluidity. **Some Au clinical features appear to be correlated** with these findings; in particular, **hyperactivity** score appears to be related with some parameters of the lipidomic profile and membrane fluidity, and **ADOS and CARS** score are inversely related to peroxiredoxin II levels.

Oxidative stress and erythrocyte structural and functional alterations may play a role in the pathogenesis of ASD and could be potentially utilized as peripheral biomarkers of ASD.

Key words

Autism Spectrum Disorders (ASD); Na+, K+-ATPase activity; Fatty Acid Profile; Membrane Fluidity; Oxidative stress markers; Erythrocyte membrane

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Introduction

Autism Spectrum Disorders (ASD) are neurodevelopmental disorders characterized by a marked decrease in social interaction and communication. Anatomical, ultrastructural and/or functional alterations have been identified in brain areas such as Broca's area, the brainstem, cerebellum, the limbic system, the parietal lobe and the corpus callosum. Complex immune system abnormalities were also found.

Diagnosis of ASD is only based on clinical features, and at present there are no validated biomarkers for diagnostic and/or screening purposes (Momeni N et al. 2012). The causes of autism are still unknown. The nature of the disorder, in fact, and the complex relationships involving mind-brain, does not allow to refere to the sequential model etiopathogenesis, commonly used in medical disciplines: etiology -> pathology -> pathogenesis -> symptomatology (Rapin, 2004). It should also be considered that autism, which is defined exclusively in terms of behavior, appears as the final event of various pathological conditions of different etiology (Baird et al., 2003) and therefore presents significantly heterogeneous clinical pictures (Moreno et al., 1982).

Genetic susceptibility, immunologic alterations, toxicological and environmental factors have been proposed to play an etio-pathogenic role in ASD (Theoharides TC et al. 2009). Recent studies showed an impairment of mirror neurons system (Cattaneo L et al. 2007), that maps visual descriptions of actions done by others onto the observer's motor representations of the same actions. No curative pharmacological treatment are available. It has been suggested that oxidative stress may play a role in the etio-pathogenesis of ASD [James SJ et al. 2004; Chauan A and chauan V, 2006; James SJ et al. 2006).

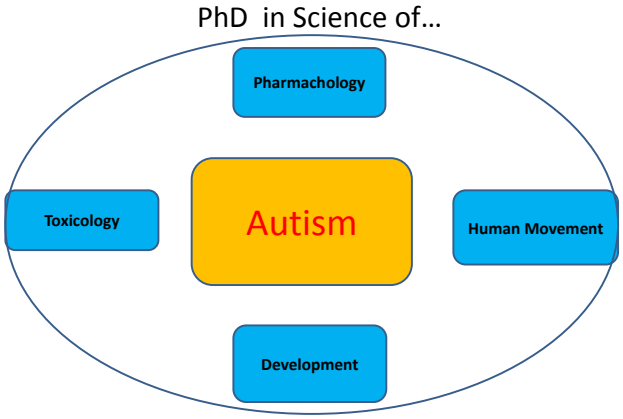

Fig. 1: Autism encopasses the four components of the PhD School in Pharmacological, Toxicological, Developmental ad Human Movement Science

1. Oxidative stress

Oxidative stress is defined as the disruption of the normal intracellular balance between reactive oxygen species (ROS), produced either during aerobic metabolism or as a consequence of pathologic processes and antioxidant defence mechanisms (Sies H, 1985; Rokutan K er al., 1989). Oxidative stress, in turn, induces the secretion of numerous vasoactive and pro-inflammatory molecules [Frossi B et al. 2003] leading to neuroinflammation [Theoharides TC et al. 2009].

Thanks to an antioxidant system, in physiological conditions the organism is able to defend itself from the presence of oxidizing agents; the antioxidant system includes enzymes and molecules which counterbalance the production of reactive oxygen or nitrogen species (ROS and NOS respectively). If the generation of oxidants exceeds the antioxidant capacities of the cell, it is established a condition called oxidative stress (H Sies, 1997).

ROS are molecules characterized by a remarkable chemical reactivity: they are in fact constituted by an atom or a molecule that has at least one unpaired electron in the orbital more external. They are then able to bind other radicals or subtract an electron to other neighboring molecules to balance their electromagnetic charge. This mechanism gives rise to new unstable molecules, triggering a chain reaction that, if not stopped in time, ends up damaging the cell structures. In fact, ROS are able to oxidize the hydrocarbon chains of unsaturated fatty acids, the amino acid residues of the protein and the nitrogenous bases of nucleic acids. These changes alter the functionality of these biomolecules thus compromising the biological functions. ROS are neutralized by anti-oxidants used by the body to protect itself from the damaging effects of free radicals. The anti-oxidant enzymes are a wide range of enzymes that transform ROS into harmless substances. In this category are included Superoxide Dismutase (SOD) and Catalase. The SOD is the first defense against ROS and in particular transforms the superoxide radical in hydrogen peroxide. The latter is eliminated by the action of catalase which converts it into water and oxygen. There are other enzymes such as glutathione reductase and glutathione transferase involved in GSH synthesis. Besides the antioxidant enzymes, there are anti-oxidants devoid of catalytic activity that are defined secondary antioxidants. One of the most important antioxidants is glutathione (GSH); GSH is the most powerful and important of antioxidants produced by the body. It is a tripeptide consisting of cysteine, glycine and glutamate. Glutathione is necessary to maintain a reducing environment capable of preserving nuclear proteins and protect DNA from oxidizing agents. At the

level of red blood cells GSH plays an important role, protecting these cells from oxidative phenomena that would cause hemolysis. Thanks to its structure GSH is able to give electrons to the oxidized molecules by oxidizing in turn. Glutathione, once oxidized, can become extremely toxic to the cell, which is, however, able to control the balance between reduced glutathione and oxidized glutathione. In fact, the oxidized GSH (GSSG) is reduced to GSH by the action of the enzyme glutathione reductase (GSR) containing the NADPH. The enzyme transfers the electrons transferred from NADPH to oxidized glutathione (GSSG) turning it into reduced glutathione (GSH). In addition to GSH, important secondary antioxidants are vitamin A, fat soluble vitamin present in foods of animal origin, vitamin C, water-soluble vitamin, and vitamin E, which is considered the most potent lipophilic antioxidant; part of the secondary antioxidants are also carotenoids, plant pigments lipid present in foods of plant origin, polyphenols, especially abundant in fruits and vegetables and the catechins contained in cocoa, wine and tea; also copper and zinc are antioxidants of mineral origin and are contained in legumes and in the meat (Sies H, 1993). Oxidative stress can be detected by studying a panel of different markers [Frustaci A et al. 2012], some of which, such as DNA, proteins and polyunsaturated fatty acid (PUFA) residues, are pathognomonic of oxidative damage of biomolecules.

2. Autism Spectrum Disorders (ASD)

Autism is a behavioral syndrome caused by a developmental disorder biologically determined, with onset in the first three years of life. The areas mainly concerned are those relating to social interaction, the ability to communicate ideas and feelings and the ability to establish relationships with others (Baird et al., 2003; Berney, 2000; Szatmari, 2003 a and b).

Autistic Disorder is characterized by qualitative impairment of reciprocal social interaction, verbal and nonverbal communication, behavior and imaginative activity. A characteristic feature of the syndrome is the presence of stereotyped behaviors, in particular gestural stereotypes.

The Cognitive impairment is frequent (70-90% of patients have an IQ below 70, a 40% is within the range of moderate to severe mcognitive impairment). Epilepsy, especially of partial type, shows a higher incidence than the general population.

Autism was first described in 1943 by psychiatrist Leo Kanner, who noted that some children had a pathological behavior different than children with mental retardation or schizophrenia. In particular, the children described by Kanner showed an inability to relate to other people. He

called this syndrome "Early Infantile Autism" (Kanner 1943). The disease is characterized by a variety of symptoms and is quite difficult to provide a comprhensive clinical definition, so that it comes to autism spectrum disorders (or Autistic Spectrum Disorders), just to indicate a number of diseases or syndromes with defined behavioural features. Autism symptoms and severity vary from one individual to another. Autistic people have, first of all, deficit and atypia of social interaction: they tend to isolate themselves from the outside world and refuse contact with others. Often have abnormal responses to sounds, touch and other sensory stimuli. The lack of communication is nonverbal (gaze, facial expressions, gestures) and verbal (language), which in some cases can lead to a total failure of expression or use of an incomprehensible language. They showed also repetitive and stereotyped behavior such as the use of certain sequences of movements, specific rituals or habits. Some subjects showed a marked resistance to change that can occur if the subject is removed from its environment or if the environment is changed. Finally, they may show outbursts, sudden laughter or crying without any reason (Filipek PA et al., 1999). The causes of this disease are still unknown; one hyopethesis is that environmental and genetic factors may contribute to its development. Several studies suggest an important role of oxidative stress in pathogenesis of autism (A Chauhan et al., 2006; Rossignol DA et al., 2012). In particular, in recent years, various studies have shown alterations in some markers of oxidative stress and antioxidant molecules (Goldani AA et al., 2014). The disease is diagnosed around or before the 3 years of a child's life. The diagnosis of autism is based primarily on clinical parameters, by using specific International Classification Scales; the diagnosis is based on the presence of a number of behavioral features identified after the observation of the subject. To date there are still no molecular markers able to diagnose this disease. Studies are directed to search for specific markers able to make an early diagnosis in order to promptly intervene with specific habilitation-rehabilitation approaches.

2.1 .Clinical aspects of ASD

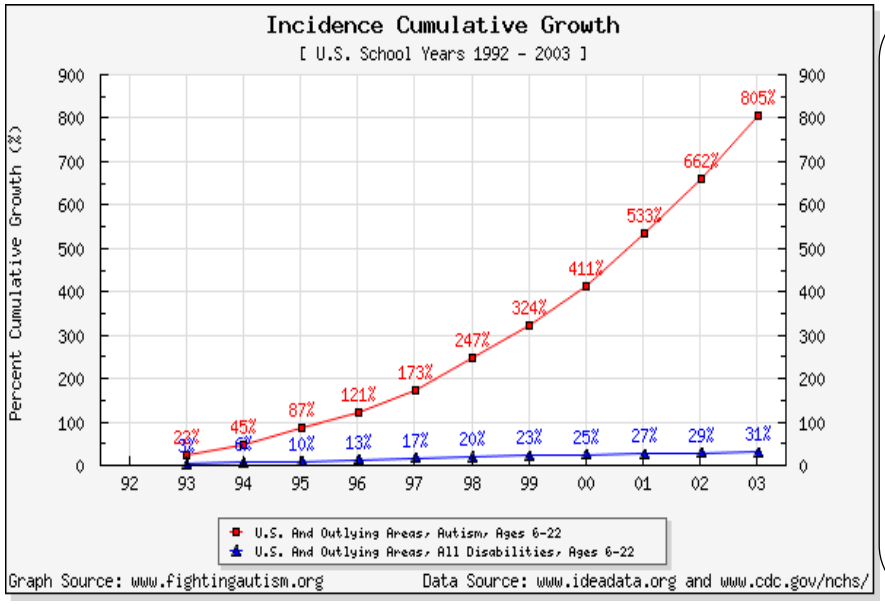

Fig 2: Diagnosis of autism Spectrum Disorder **<u>Is increasing</u>** worldwide.

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Enormous social and health impact.

Diagnosis of autism Spectrum Disorder is increasing worldwide (Fig 2). The most recent epidemiological studies by the Centers for Disease Control and Prevention (CDC) of Atlanta, reported that in USA one child in 68 is affected by ASD. As for Italy was highlighted that the prevalence of at least 5 cases out of 10 000 for children attending the elementary school rises above 7 out of 10 000 (CDC, 2006). More recent data have shown an increase in cases of autism in Italy. This increase is thought to be due by the improvement of diagnosis and by a greater awareness of autism by operators. Autism affects mainly males at a rate four times higher than the female.

In the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and in 'International Classification of Mental and Behavioral Disorders (ICD-10), autism is part of Pervasive Developmental Disorders (Pervasive Developmental Disorders, PDD), that is characterized by severe and pervasive impairment in social, behavioral and communication. The diagnosis of ASD is

usually made between 2 and 3 years of age. The diagnosis is based on parameters using behavioral rating scales as the ADOS (Autism Diagnostic Observation Schedule), the ADI-R (Autism Diagnostic Interview-Revised (Lord et al., 1994, 2000) and CARS (Childhood Autism Rating Scale) (Schopler et al., 1988). It is very important to make an early diagnosis in the early years of life, to allow a specific intervention in a period of life when the brain has a certain plasticity. The role of parents in the identification of the first symptoms (such as anomalies in the behaviour) appears to be crucial (De James A. et al., 1998).

Autistic symptoms vary from one individual to another and they display various levels of severity. Common Clinical characteristics are:

• **Difficulties in social interaction**: the subject shows an apparent lack of interest to other people, or to what is happening around him; prefer not to be touched or embraced; shows a high tendency to isolation and closure and difficult to participate in group games. It is not unusual that children with autism are initially diagnosed as deaf because they do not show any reaction when called by name (Filipek PA et al., 1999).

• **Difficulty in verbal communication**: the individual repeats the same words or phrases repeatedly; answers questions by repeating the question (echolalia); uses language incorrectly (phonological and morpho-symptatic errors); has difficulty in communicating needs and desires; does not understand simple instructions, requests and questions; interprets what he is told in a very literal way (not able to understand the irony and sarcasm) (Amato CAH et al., 2010).

• **Difficulty level of nonverbal communication**: avoid eye contact, use facial expressions that are not consistent with what they are saying and react unusually to some visual and auditory stimuli (Amato CAH et al., 2010; Jones W et al., 2008).

• **Presence of rigid and stereotyped behaviors**: autistic children present rigid behavior (for example: insist on doing the same road to go to school); have difficulty in adapting to any change in the day (e.g. to eat at a different time than usual) or of the environment (e.g.moving furniture); they show an unusual attachment to objects or particular games; they align obsessively objects; repeat the same actions or movements over and over again. The autistic person may explode in fits of tears and laughter, they can become self-defeating, hyperactive and aggressive toward others or toward objects.

People with autism often have abnormal responses to sounds, touch and other sensory stimuli. Many show reduced sensitivity to pain, but they can also be extraordinarily sensitive to other sensations. This altered sensitivity may contribute to behavioral symptoms such as resistance to be embraced.

2.2 Neurological abnormalities

Neuropathological feature of ASD are characterized by changes concerning the development of atypical certain brain structures and the levels of connection between the different areas as well as dysfunction of neurotransmitters in the central nervous system and immunological abnormalities.

Neuroimaging studies showed several features including an increased density of nerve cells at the level of certain brain structures such as the cerebellum, hippocampus and amygdala; the latter, is a brain structure, part of the limbic system, that plays a role in the emotional integration, and instinctive behavior of the individual (Vivanti G. 2007; Amaral DG et al., 2008). Furthermore MRI studies have shown that the volume of the amygdala and hippocampus is increased in children with autism compared to controls. The cause of these changes, however, is still unknown (Schumann CM et al., 2004).

Studies have been conducted that showed abnormalities in the connections between neurons in specific brain areas such as the auditory cortex (Roberts TP et al., 2010; Roberts TP et al., 2011), and changes in the volume and symmetry of the two cerebral hemispheres (Wallace GL et al., 2010; Kosaka H et al., 2010).

At the cellular level have been highlighted anomalies in the functioning of nerve synapses, mainly those γ-aminobutyric acid-mediated (GABA). Moreover, there were defects in the synthesis and release of the neurotransmitter serotonin (5HT), involved in the modulation of mood and behavior of the individual: a deficiency of serotonin in the frontal lobes can be correlated with impulsivity and obsessive-compulsive symptoms typical of autism (Lambiase M, 2004). Functions such as perception and attention, which are regulated by the neurotransmitter dopamine system, are compromised in individuals with autism.

Recently, a defect of neural pruning has been shown in pyramidal cells of the layer V in the temporal cortex; this defect is associated with a deficiency of macroautophagy, in turn due to excessive activation of the kinase mTOR (Tang G et al., 2014).

Alterations of the immune system and neuroinflammatory processes were showed int the cerebral cortex and cerebellum (Vargas DL et al., 2005).

In children with ASD was highlighted the presence of certain gastrointestinal disorders such as abdominal pain, constipation, chronic diarrhea, vomiting and gastroesophageal reflux (JF White, 2003). The association between DSA and gastrointestinal symptoms remains unclear. The high presence of gastrointestinal disorders in individuals with autism is associated with a selective behavior towards the food (Ibrahim SH et al., 2009; Smith RA et al ., 2009).

2.3 Etiopathogenesis of Autism Spectrum Disorders

Autism is a complex disorder characterized by a heterogeneous phenotype; it is believed that autism may be a multifactorial disorder in which genetic and environmental factors are intertwined each other.

2.3.1 Environmental Factors

The exposure to a large number of complications in the pre- and postnatal period may increase the risk of autism; among the factors that were associated with the risk of developing the disease, there are, for example, complications due to the umbilical cord, size and weight for gestational age, medications taken during pregnancy and neonatal anemia (Gardener H et al., 2010).

It is known that exposure to environmental toxicants, such as mercury, lead, arsenic and toluene, can lead to neurological disorders, such as psychiatric disorders, schizophrenia and depression (Rossignol DA and Frye RE, 2011). In recent years, several studies have shown a possible link between ASD and exposure to environmental toxins such as heavy metals, pesticides and chemicals. These substances can damage nerve cells, causing the increase of oxidative stress, reduced glutathione and the impairment of cellular signaling. A study carried out in 2014 confirmed the association between ASD and exposure to toxic substances: the results of this research suggest that, at least in a subset of children, there are complex interactions between some environmental toxicants and genetic factors and that they may act synergistically (or in

parallel) during the critical periods of neurological development, to increase the likelihood of developing autism (Rossignol DA et al., 2014).

In recent years, vaccines containing mercury (Thimerosal as a preservative) have been the subject of study and controversy. In 1998, the British doctor Andrew Wakefield suggested a possible association between vaccines and autism spectrum disorders. Wakefield was referring in particular to the MMR triple vaccine against measles, mumps and rubella (Godlee F. et al., 2011). The hypothesis advanced by Wakefield was soon disproved. To date, no significant results were obtained on a possible link between MMR vaccine and autism (Hertz-Picciotto I et al., 2009; Holmes AS et al., 2003).

2.3.2 Genetic factors

The hypothesis that genetic factors may be implicated in the pathology of autism was confirmed primarily through studies of monozygotic and dizygotic twins (Bailey A et al., 1995). The data obtained showed in monozygotic twins a high concordance rate for autism. From these studies it was assumed that genes could play a primary role in the manifestation of the disorder. The identification of the genetic determinants underlying autism is a complex issue: the pathogenesis is probably heterogeneous, not attributable to the action of a single gene, but several genes may contribute together to the development of autism.

To date, the identification of genes involved in the disease is still very complicated; most of the changes highlighted was indeed found in sporadic cases. Some research groups have used an approach based on the analysis of the entire genome to identify susceptibility loci (Lamb J et al. 2002). The strategy is to analyze the human genome through genetic studies of linkage, to study the inheritance of DNA markers in families with at least two individuals with autism. This experimental approach allows to identify which regions of the genome are "more similar" between the pairs of affected siblings in each family: in fact, if a region of a chromosome contains a gene involved in autism, this region will be inherited, with some frequency, by both affected siblings. The comparison of the results obtained by different research groups showed certain chromosomal regions in which is probably located a susceptibility locus for autism. The most significant results concerning chromosomes 2q and 7q (IMGSAC, 2001). Particularly interesting is a chromosomal region identified on chromosome 7q22-q23, where are located several genes

involved in disorders of speech and language, wich are very important clinical signs in autistic patients.

Promsing studies are directed towards the search for "candidate genes", ie genes involved in brain development, neurotransmitters or neuromodulators located in chromosomal regions with evidence of linkage. The genes involved in the metabolism of serotonin or other neurotransmitters have been the subject of study by several research groups (Maestrini E et al., 1999). The identification of genes involved in autistic disorder will be of great importance for the understanding of the neuropathological mechanisms underlying the disorder, the development of more accurate methods of diagnosis and the identification of specific drug targets.

2.3.3. Metabolic alterations

Molecular and behavioral evidence indicates an association between sex hormones and steroids in autism. In some subgroups of individuals with autism was found an altered metabolism of steroid hormones, particularly testosterone (Ruta L et al., 2011) and cholesterol (Aneja A et al., 2008). This finding may explain the high rate of autism in males than females. Furthermore, conditions related to altered levels of testosterone, such as polycystic ovary syndrome, are reported in women with autism. Cholesterol is essential for the production of neuroactive steroids and for a normal embryonic and fetal development. It also modulates the oxytocin receptor and the serotonin receptor. A deficit of cholesterol can disrupt these biological mechanisms and, therefore, contribute to the ASD.

Recently, it has been suggested that oxytocin may play a role in the manifestation of the social deficits of autism. Oxytocin is a hormone considered a major regulator of social behavior in humans. Clinical studies have shown that treatment with oxytocin is able to change some aspects of behaviour as the ability to participate in a game where interaction with others is required (Andari E et al., 2010). Many studies have tried to associate the presence of autistic disorders with genetic disorders involving the gene of oxytocin and/or its receptor. Mutations in the gene encoding for the oxytocin receptor, as well as epigenetic modifications leadind to an alteration in the phenotypic level, has been shown (Gregory SG et al, 2010).

2.4 Therapy

Currently there is no cure for autism. The therapies used are chosen according to the specific symptoms of each subject and include mainly a psycho-educational program (which aims to improve cognition and behavior of the child) and, to a lesser extent, a drug treatment. Such interventions do not cure autism, but it can often lead to an improvement in the quality of life of the child. The educational and behavioral treatments are designed to help the child to develop social skills and language. This type of therapy should begin as soon as possible: an early intervention has a good chance to positively influence the brain development. Drug therapy is aimed primarily at reducing some of the symptoms associated with autism such as poor attention and obsessive-compulsive behaviors. In particular, drugs that regulate the serotonin, as inhibitors of serotonin reuptake, reduce repetitive and aggressive behaviors (McDougle CJ, et al., 1996; Gordon, 1993). Other medications inhibit impulse transmission in dopaminergic neurons, reducing some behavioral manifestations (Anderson et al., 1989). Finally, the use of a nutraceutical approach may be useful in autistic subjects, as it could lead to an improvement in antioxidant capacity with consequent reduction of oxidative stress (Abdulrahman SA, 2013).

2.5 Autism and oxidative stress

Oxidative stress has been suggested to underlie several mental disorders, including schizophrenia and bipolar disorder [Andreazza AC et al. 2008; Ng F et al., 2008; Tsaluchidu S et al. 2008], and neurodegenerative pathologies such as Alzheimer disease [Porter MM et al., 1995; Sultana R et al. 2010]. In autism there is growing evidence of increased oxidative stress. Glutathione redox status has been found to be decreased in autistic patients, also in the post-mortem analysis of Autistic brain tissues (Rose S et al. 2012). Low levels of exogenous antioxidants vitamin C, vitamin E, vitamin A in plasma, zinc and selenium in erythrocytes, has been shown (SJ James et al. 2004). The imbalance of membrane fatty acid composition and PUFA loss can affect ion channels and receptors [Murphy MG 1990]. In particular, Ca2+ channel deficiency was found in Au [Krey JF and Dolmetsch RE 2007], but never correlated to membrane parameters. A reduced level of energy metabolism was also found in autistic subjects; reduced synthesis of ATP (Chugani et al., 1999) and high levels of lactate and pyruvate (Coleman and Blass, 1985), elements that may suggest an

altered mitochondrial metabolism in autism (Graf et al., 2000; Filipek et al., 2003). Oxidative stress is one of the major causes of reduced mitochondrial metabolism (Packer, 1984). In addition to the high levels of oxidative stress, neuroinflammation (activation of microglia and elevated levels of proinflammatory cytokines) may play a role in the development of histopathological changes in Autism (Vargas et al. 2005).

Tables 1A and 1B show a summary of main published results on oxidative stress and on fatty acid composition of the erythrocyte membrane in Autism, respectively.

Tab 1A: Summary of main published results on oxidative stress involvement in autism.

1) Anti oxidant enzymes

Author	year	Plasma, urine or cells	Results
Yorbik, Sayal, Akay, Akbiyik, Sohmen	2002	Plasma per GPX e SOD1, RBC per GPX	Plasma GPX↓, plasma SOD1↓, RBC GPX↓
Sogut, Zoroglu, Ozyurt, Yilmaz, etc	2003	Plasma, RBC per NO	Plasma GSH↑, SOD1=, TBARS=, GPX↑, RBC NO↑
Zoroglu, Armutcu, Ozen Gurel, etc	2003	RBC	TBARS↑, SOD1↑, XO↑, CAT↓, TBARS=
Chauhan Chauhan, Brown, Cohen	2004	Plasma	MDA↑, lipids peroxidates↑, transferrine↓, cerulopasmine↓
Ranjbar A, Rashedi V, Rezaei M.	2014	Urine	↑total antioxidant concentration (TAC), ↓catalase activity (CAT) and↓ total thiol molecules (TTM)
Ngounou Wetie AG, Wormwood K, et al.	2014	Serum	↑apolipoproteins ApoA1 and ApoA4 and paraoxanase/arylesterase 1
Kovač J, Macedoni Lukšič M et al.	2014	DNA	Rare single nucleotide polymorphisms in the regulatory regions of the superoxide dismutase genes
Zhang QB, Gao SJ, Zhao HX	2015	Serum	↑thioredoxin

2) Lipids and oxidative stress markers

Author	year	Plasma, urine or cells	Results
Vancassel, Durand, etc	2001	Plasma	PUFA↓
Chauhan Chauhan, Brown, Cohen	2004	Plasma	Lipids peroxidates↑
Ming, Stein, Brimacombe, etc	2005	Urine	isoprostane↑, 8-OHdG≈↑
Bu, Ashwood, Harvey, etc	2006	RBC	Eicosanoic Acid ↑, erucic acido ↑
Tostes MH, Teixeira HC, Gattaz WF, Brandão MA, Raposo NR.	2012	Plasma	↑VIP, IFN-γ and NO , ↓NT-3
Ming X, Stein TP, Barnes V, Rhodes N, Guo L.	2012	Urine	↓glycine, serine, threonine, alanine, histidine, taurine carnosine
El-Ansary A, Al-Ayadhi L.	2012	Plasma	↑PGE2, leukotrienes and isoprostanes
Pecorelli A, Leoncini S, De Felice C, et al.	2013	Plasma and erythrocyte	↑non-protein-bound iron (NPBI), and 4-hydroxynonenal protein adducts (4-HNE PAs)
Napoli E, Wong S, Giulivi C.	2013	MitochondrialDNA(peripheral blood monocytic cells)	↑mtDNA deletions
Gorrindo P, Lane CJ,	2013	Plasma	↑ F2t-Isoprostanes
Frye RE, Delatorre R	2013	Plasma	↑3-chlorotyrosine (3CT, measure of chronic immune activation), ↓GSH
Gu F, Chauhan V et al.	2013	Brain (frontal cortex)	defects in complexes I and III (sites of mitochondrial free radical generation) and complex V (adenosine triphosphate synthase),↓ Piruvate Dehydrogenase
Kuwabara H, Yamasue H et al.	2013	Plasma	↑arginine and taurine ↓5-oxoproline and lactic acid
Ciccoli et al.	2013	RBC	RBCs altered morphology ↑F2-isoprostanes and 4-hydroxynonenal protein adducts. oxidative damage of β-actin protein
Napoli E, Wong S, Hertz-Picciotto I, Giulivi C	2014	Granulocytes	defects in oxidative phosphorylation, immune response, and antioxidant defense

3) transmethylation/transsulfuration metabolism

Author	year	Plasma, urine or cells	Results
Jill James, Cutler, etc	2004	Plasma	Metionina↓, SAM↓, omocisteina↓, cistationina↓, GSH↓, SAH↑, adenosina↑, GSSG↑
Jill James, Melnick, etc	2006	Plasma	Differenze nella frequenza allelica di geni correlati
Muratore CR, Hodgson NW et al.	2013	Brain	↓methionine synthase mRNA
Hodgson NW, Waly MI,	2014	Serum	↓ glutathione ↑homocysteine and S-adenosylhomocysteine

4) nutraceutical and antioxidant approaches

Authors	year	Supplement	Results
Richrdson, Montgomery	2005	ω3, ω6	Improvement
Amminger, Berger, etc	2006	ω3	Improvement
Frye RE, Rossignol DA. (review)	2014	l-carnitine and a multivitamin containing B vitamins, Carnosine, antioxidants, vitamin E, and co-enzyme Q10 ubiquinol, folinic acid, methylcobalamin with and without folinic vitamin C and N-acetyl-l-cysteine tetrahydrobiopterin.	Improvement
Singh K, Connors SL, et al.	2014	Sulforaphane	Improvement

Tab 1B: Summary of main published results on fatty acid composition of the erythrocyte membrane (from Ghezzo et al., 2013, modified)

	fatty acid composition of erythrocyte membrane	Patients
Highly unsaturated fatty acids (HUFA)	decreased	1 ASD patient (Bell JG et al., 2000)
Stearic acid (18:0) Arachidic acid (24:0) Total SFA Oleic acid (18:1 n-9) Nervonic acid (24:1) Total MUFA Linoleic acid (18:2 n-6) Docosapentaenoic acid (22:5 n-6) ARA (20:4 n-6) DPA (22:5 n-3) Total ω3 Arachidonic acid (ARA):EPA ratio (20:4 n-6/20:5n-3)	increased increased increased decreased increased decreased increased increased decreased decreased decreased increased	18 Au children with developmental regression (Bell JG et al, 2004)
Stearic acid (18:0) Arachidic acid 24:0 Nervonic acid (24:1) Docosapentaenoic acid (22:5 n-6) DPA (22:5 n-3) Total ω3 Arachidonic acid (ARA):EPA ratio (20:4 n-6/20:5 n-3)	increased increased increased increased decreased decreased increased	11 children with Classical autism or Asperger (Bell JG et al, 2004)
eicosenoic acid (20:1n9) erucic acid (22:1n9) total MUFA	increased increased increased	20 Au children with developmental regression (mean age 3.5 years) (Bu B et al., 2006)
α-Linolenic acid (18:3 n-3) Arachidonic acid (ARA):EPA ratio (20:4 n-6/20:5 n-3)	decreased increased	49 Au children (mean age 7.5 years) (Bell JG et al. 2010)

3. Research project

3.1 Objective

The aim of our study was to evaluate

a) a broad spectrum of oxidative stress markers in Au children, in order to assess the possible imbalance of their redox status, taking into account the correlation between peripheral and central nervous system markers of oxidative stress [Skoumalova A et al. 2012];

b) structural, functional and morphological features of erythrocytes, in particular membrane fatty acids composition. In fact there is a growing evidence of a strong correlation between erythrocyte fatty acid membrane profile and preservation/degeneration of brain functions in aging and in neurodegenerative diseases [Kim M, et al. 2010], [Tan ZS et al. 2012]. Moreover erythrocyte membrane ω6/ω3 balance has been related to inflammation markers [Calder PC 2011].

c) a possible correlation between biochemical and cinical features;

d) therapeutic suggestions (based on biochemical alterations) aimed at reducing some of the symptoms; the examined parameters are also potentially useful peripheral biomarkers of ASD.

To our best knowledge, this is the first study which evaluates, at the same time, such wide range of different but strongly related biological biomarkers, in a group of ASD children that underwent a rigorous clinical characterization.

3.2 Materials and Methods

Table 2: research groups involved in the study		
FIRST STUDY		
Subjects	**Laboratory**	**Evalualtions**
Au: 21 **TD: 20**	**Marini M, University of Bologna**	Urinary **HEL, isoprostane,** 8-oxo-dG
	Marini M. **University of Bologna**	Plasma carbonyl groups
	Marini M. **University of Bologna**	Erythrocyte SOD and catalase activities
	Ferreri C. CNR Bologna	Erytrocyte membrane Lipidomics
	Mazzanti L. Università of Ancona	Erythrocyte Na+, K+-ATPase activity (NKA)
	Mazzanti L. University of Ancona	TBARS, sialic acid
	Mazzanti L. University of Ancona	Erythrocyte membrane fluidity
	Manfredini S. Unversity of Ferrara	ORAC

SECOND STUDY		
Sujects	**Laboratory**	**Evaluations**
Au: 12 **TD: 12**	**Marini M. University of Bologna**	Cholesterol erythrocyte membrane
	Marini M. University of Bologna	Erythrocyte membrane sphingolipids
	Marini M. + Manara (Rizzoli, Unibo)	Annexin V (phosphatidylserine)
	Marini M. University of Bologna	Hydrophilic heads of phospholipids (erytrocyte membranes)
	Ferreri C. CNR Bologna	Erytrocyte membrane Lipidomics
	Marini M. + Prof.ssa Farruggia (Unibo)	Glutathionylation in red blood cells and white blood cells
	Marini M. University of Bologna	Glutathionylation Subunits of Na K ATPase (erythrocyte membranes)
	Marini M.University of Bologna	Evaluation of Subunits of Na K ATPase (erythrocyte membranes)
	Marini M. University of Bologna	Evaluation of mRNA Subunits of Na K ATPase (erythrocyte membranes)
	Mazzanti L. University o Ancona	Lymphocyte Na+, K+-ATPase activity (NKA)
	Mazzanti L. University of Ancona	Lymphocyte membrane fluidity
	De Franceschi L. University of Verona	Peroxiredoxin I
	De Franceschi L. University of Verona	Peroxiredoxin II
	De Franceschi L. University Verona	Catalase (erythrocyte)
	Marini M. University of Bologna	Erythrocyte carbonyl groups
Au 29 **TD:28**	**Fedrizzi G. (Istituto Zooprofilattico Bologna)**	Evaluatin of a broad spectrum of metals (plasma and urine)
	Gabbianelli R. (Camerio), Dimingues V.(Porto)	Permethrin metabolites (urine)
	Naila Rabbani (Warwick University)	Protein glycation (plasma and urine)

3.2.1 Ethics Statement

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human patients were approved by Local Ethical Committee **First Study**: Azienda USL Bologna, CE 10020- n.30, 06/04/2010 prot 45424/10-03. **Second Study**: Prot. N.642/CE Bologna, 01 July 2013 and Prot. N.1198/CE Bologna, 23 December 2013. Written consent was obtained from all parents and also from children through pictures and simplified information.

3.2.2 Subject

The study was performed in two steps.

First Study. A first group of 48 children was approached as part of the first case–control study. Of these, 25 had a diagnosis of Autism (Au) and 23 were classified as Typically Developing (TD) children. Of these, 21 were recruited for inclusion in the study from the autism group (4 Female and 17 Male), and 20 in the TD group (6 F and 14 M). Reasons for rejection included: taking fish oil supplements (two subjects in the Au group), taking vitamins and/or other substance known to have antioxidant properties (two subjects in Au group and 3 subjects in TD group).

Au group mean age was 6.8 years (SD = 2.23 years, median = 6 years, range 5–12 years); in TD group mean age was 7.6 years (SD = 1.96 years, median = 7 years, range 5–12 years). Both the non-parametric comparison of the average age in the two groups and the comparison by gender (chi-square test), were not significant, confirming the comparability between cases and controls.

Second Study. A second group of 24 children was approached as part of the second case–control study. Of these, 12 had a diagnosis of Autism (9 M and 3 F) and 12 were age and gender matched Typically Developing children. Au group mean age was 7.8 years (SD= 1.9 years, median = 7.4 years, range 6-10 years).

All the patients were admitted to Child Neuropsychiatric Unit of the Maggiore Hospital of Bologna (Neurological Sciences Institute IRCCS-Bologna), for assessment by a comprehensive diagnostic-neurological workup and regular follow-ups. None of the autistic patients had active epilepsy at the time of blood and urine sampling. One patient experienced a first and (at the moment of writing this paper) single benign rolandic seizure six months after the blood and urine collection

(this patient was the only one with a normal intellectual level). Any medical and neurological comorbidity was excluded by electroencephalography (recorded during awake and sleep), cerebral magnetic resonance imaging, standard clinical and neurological examination, neurometabolic and genetic investigations (including 550 band karyotype, and molecular assay for Fragile X and MECP2). No infective or inflammatory disease was detected at the time of blood collection. No subject underwent any surgery intervention in the four months prior to blood and urine collection.

Autism diagnosis was made according to the Diagnostic and Statistical Manual of Mental Disorders IV (DSM IV TR [American Psychiatric Association (2000)]) criteria, Autism Diagnostic Observation Schedule (ADOS) [Lord C et al. 1999] and Childhood Autism Rating Scale (CARS) [Schopler E. Et al. 1988] by two clinicians (a child neuro-psychiatrist and a child psychologist) experienced in the field of autism. Developmental and cognitive levels were assessed by Psychoeducational Profile-3 (PEP-3) [Schopler E. Et al. 2005] and Leiter International Performance Scale–Revised (Leiter-R) [Roid GM, Miller LJ (1997)]. Parents were questioned regarding the age of onset of early autistic signs. Demographic and clinical features of Au groups are summarized in Table 3 and Table 4. Control group children were healthy TD children, recruited in the local community, with no sign of cognitive, learning and psychiatric involvement, as clinically and anamnestically determined by three experienced clinicians. All TD were attending mainstream school and had not been subjected to stressful events. Dietary habits were assessed by a Food Questionnaire. All patients and controls were on a typical Mediterranean diet.

Tab 3: Demographic and clinical features of the autistic children group (First Study)
(from Ghezzo A. et al. 2013, modified)

No.	Gender	Age (months)	Age of onset (months)	Cognitive/ developmental impairment	ADOS score	CARS score	CARS activity level item score	CARS stereotypes (body use) item score
1	M	66	≤12	Moderate	18	40.5	2.5	3.5
2	M	74	≤12	Severe	21	42	2.5	2.5
3	M	64	≤12	Moderate	16	35	2.5	2
4	M	103	13-18	Moderate	19	44.5	3	3
5	M	61	≤12	Severe	21	46	3.5	4
6	M	71	13-18	Severe	22	41	2	3
7	M	142	≤12	Severe	22	44.5	2.5	3
8	M	131	13-18	Severe	16	38	3	3
9	M	66	13-18	Moderate	22	40.5	3	3
10	F	66	13-18	Borderline IQ	15	41.5	2.5	2
11	M	74	13-18	Severe	22	42.5	3	2.5
12	F	66	13-18	Severe	22	43.5	3.5	3.5
13	M	66	13-18	Mild	14	34	2	2
14	M	89	19-24	Mild	19	40	3	3
15	M	102	13-18	Mild	22	36.5	2	2.5
16	F	110	25-30	Moderate	15	47.5	3.5	3
17	M	79	13-18	Moderate	19	37	2.5	3
18	M	144	13-18	Severe	20	39	3	3.5
19	M	80	≤12	Normal IQ	19	36.5	2.5	2
20	M	79	≤12	Severe	21	40.5	2.5	3
21	F	65	≤12	Mild	17	31.5	2	2

Tab 4: Demographic and clinical features of the autistic children group (Second Study)

No	Gender	Age (Months)	Cognitive/ developmental impairment	Ados Score	Cars total score	Cars activity level item score	Cars stereotypies (body use) item score	CARS verbal language (expressive) item score	CARS non verbal language item score	CARS total number of items whose score was ≥3
1	m	68	Severe	20	41,5	2,5	3	3	3	11
2	m	94	Severe	21	46	3,5	4	4	4	11
3	f	68	Normal IQ	20	43,5	2,5	3	3,5	3	10
4	m	93	Severe	20	48,5	3	4	3,5	3	12
5	m	82	Moderate	19	42	3	3	3	3	9
6	m	115	Severe	19	39,5	3	3	3	3	7
7	m	74	Severe	19	41	2,5	3	3	3	9
8	m	99	Moderate	16	35	2,5	2	3	3	3
9	m	85	Moderate	22	41	2,5	2	3	3	7
10	f	74	Severe	17	38	2	2,5	3	3	7
11	f	124	Normal IQ	21	33,5	2	2	2,5	2,5	4
12	m	129	Normal IQ	19	39	2	2,50	3	3	7

3.2.3 Biochemical Evaluations

Blood samples, obtained from Au and TD children, were collected in Na2-EDTA (~9 mL) and heparin (~5 mL) vacutainers. Some hematological parameters were carried out by routine laboratory techniques. One ml Na2-EDTA whole blood was set apart for lipidomics evaluation. The remaining blood was centrifuged (10 min. at 1000×g) in order to separate the plasma, which was frozen at −80°C in 1 mL eppendorf sterile tubes. Na2-EDTA and heparinised plasma was used for a radical absorbance capacity (ORAC) test and protein carbonyl evaluation, respectively. After diluting (1:1) the cell suspension with sterile Phosphate Buffered Saline (PBS), mononuclear white blood cells were separated from red cells by Ficoll (Histopaque 1077, Sigma, St.Louis, MO, USA) density gradient centrifugation. Cells were lysed in 1 mL Trizol® Reagent (Invitrogen, Milan, Italy) and stored at −80°C for other evaluations. In order to remove all Ficoll residue red blood cells were washed three times with PBS. Erythrocytes in Na2-EDTA were stored at 4°C and then used for the

evaluation of Na+/K+-ATPase activity (NKA) and cell membrane fluidity. Heparinised red blood cells (RBC) were used for the evaluation of superoxide dismutase (SOD) and catalase activity. In particular, for SOD activity measurement, heparinised RBC were lysed in 4 volumes of ice-cold water and then stored at −80°C. The remaining heparinised RBC were diluted 30-fold in PBS and subsequently lysed in 10 mM potassium phosphate buffer pH 7.2. Lysates were stored at −80°C and subsequently were used for catalase activity evaluation. Spot urine samples (10 mL) from Au and TD were collected. Proteinuria and creatinine determinations were carried out by laboratory techniques. The remaining urine was centrifuged at 1200 g for 10 min in order to remove insoluble materials. Five mL of clear urine were aliquoted and stored at −80°C for hexanoyl-lysine adduct (HEL) and 8-isoprostane evaluations. The remaining urine was filtered with 0,45 µm filter, supplemented with 0.05% sodium azide and stored at −80°C for 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) analysis.

Urinary 8-isoprostane.

Urinary 8-isoprostane (also known as 8-epi-PGF2α, 8-iso-PGF2α or 15-isoprostane F2t) was determined by the use of a competitive ELISA kit (Oxford Biomedical Research Inc., Oxford, MI, USA). As suggested by the manufacturer, urine samples are diluted 1:5 with a buffer provided in the kit. The 15- isoprostane F2t in the samples competes with 15-isoprostane F2t conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-isoprostane F2t coated on the microplate. A substrate was added and the absorbance was measured at 450 nm in a microplate reader. The 15-isoprostane F2t concentration was expressed in ng per milligram of creatinine.

Urinary hexanoyl-lysine adduct.

Hexanoyl-lysine adduct (HEL) concentration was measured by a competitive ELISA kit (JaICA, Fukuroi, Shizuoka, Japan) in unfiltered urine of autistic and control children. According to the manufacturer's instructions, urine samples were diluted five times with PBS. Some urine samples containing proteins were treated with 14 mg/mL alpha-chymotrypsin in PBS (pH 7.4) and incubated at 37°C O.N. Samples were filtered using ultra filters with cut-off molecular weight 10 kDa (Amicon Ultra, Millipore, Cork, Ireland). The absorbance was measured at 450 nm using a

microplate reader. The HEL concentration was expressed in nmol per milligram of creatinine (nmol/mg creatinine).

Urinary 8-oxo-dG.

Urinary 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) was measured using the HT 8-oxo-dG ELISA Kit (Trevigen Inc. Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, filtered urine was diluted 1:20 with a buffer provided by the kit and added to a plate pre-bounded with 8-oxo-dG. Bound and sample 8-oxo-dG compete for binding to the anti-8-oxo-dG which was then added to the plate; the antibody fraction captured by the immobilized 8-oxo-dG in the plate was then detected by means of a HRP-conjugated secondary antibody. The assay was developed with tetramethylbenzidine substrate (TMB) and the absorbance was measured in a microplate reader at 450 nm. The 8-oxo-dG concentration was expressed in ng per milligram of creatinine.

Protein carbonyl determination.

Protein carbonyls were determined in plasma samples using the Protein Carbonyl ELISA kit (Enzo Life Sciences Inc. Farmingdale, NY, USA) following the manufacturer's instructions. Plasma (5 µL) was derivatized with dinitrophenylhyidrazine (DNPH); derivatized proteins were then adsorbed to an ELISA plate. The adsorbed protein was then probed with biotinylated anti-DNP antibody followed by streptavidin-linked horseradish peroxidase. The absorbance was read at 450 nm using a spectrophotometer plate reader (Victor II, Pelkin-Elmer, Waltham, MA, USA). Plasma samples were assayed in duplicate, and protein carbonyl concentration was expressed as nanomoles of carbonyl groups per milligram of protein in the sample (nmol/mg).

Plasma radical absorbance capacity (ORAC).

The ORAC assay was carried out on a Fluoroskan FL® ascent (Thermo Fisher Scientific, Inc. Waltham, MA, USA) with fluorescent filters (excitation wavelength: 485 nm; emission filter: 538 nm). following a previously published procedure [Pessina F. Et al. 2004]. Briefly, in the final assay mixture (0.2 mL total volume), fluorescein sodium salt (85 nM) was used as a target of free radical attack with 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) as a peroxyl radical generator. Trolox, a water-soluble analogue of vitamin E, was used as a standard control and calibration curves were determined for 10, 20, 30, 40, 50 µM solution. Fluorescence measurements, carried

out at 37°C, were recorded at 5 min intervals, up to 30 min after the addition of AAPH. The ORAC values, calculated as difference of the areas under the quenching curves of fluoresceine between the blank and the sample, were expressed as Trolox equivalents (TE), pH = 7.4. All the assays were performed with three replicates.

Superoxide dismutase (SOD) activity.

SOD activity was determined in erythrocyte lysates by a competitive colorimetric inhibition assay, as previously described [Peskin AV, Winterbourn CC (2000)], (Abruzzo PM et al. 2010). This method is based on water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2 ,4-disulfophenyl)-2H-tetrazolium,monosodium salt) (Dojindo Laboratories Co., Kumamoto, Japan), which produces a water-soluble formazan dye upon reduction with the superoxide anion generated by xanthine and xanthine oxidase (Sigma-Aldrich, St. Louis, MO, USA). SOD activity reduces the superoxide concentration and inhibits formazan formation. A SOD standard curve was obtained; different dilutions of erythrocyte lysates were assayed in order to find a sample dilution that falls within the range of standard curve linearity. Samples or standards (10 µL) were incubated for 20 min at 37°C with 100 µL reaction mixture containing 500 µM WST-1 and 75 µM xanthine in 50 mM CHES (2-N-(Cyclohexylamino) ethanesulphonic acid, pH 8.0. Finally, 10 µL Xanthine Oxidase (350 mU/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added. Formazan formation was measured at 450 nm using a 96-well plate reader (Victor2 Multilabel Counter, Perkin-Elmer, Waltham, MA, USA). SOD concentration, expressed in units per milligram of hemoglobin, was determined using the SOD standard curve.

Catalase activity.

Catalase activity was determined in erythrocyte lysates using a method described by Ou and Wolff [Ou P, Wolff SP (1996)] based on the specific reaction of FOX-1 reagent (250 µM ammonium ferrous sulfate, 100 µM xylenol orange, 0,1 M sorbitol, 25 mM H_2SO_4) with H_2O_2 to yield a color complex having absorption maximum at 560 nm. The catalase causes decomposition of H_2O_2 such that residual H_2O_2 is inversely proportional to the activity of the catalase. One milliliter of erythrocyte lysates was incubated for 4 min. with 100 µL of 2.2 mM H_2O_2. Subsequently, 50 µL aliquots of the incubation mixtures were removed and rapidly mixed with 950 µL of FOX-1 reagent

in eppendorf tubes, which were then incubated at room temperature for 30 min. Absorbance was measured at 560 nm. Catalase concentration was expressed in units per milligram of hemoglobin.

Erythrocyte plasma membrane fluidity.

Erythrocytes plasma membrane fluidity was studied by determining the fluorescence anisotropy (reciprocal of fluidity) of two probes, TMA-DPH (1-(4-trimethylammoniophenyl)-6-phenyl-1,3,5-hexatriene),and DPH (1-6-phenyl-1,3,5-hexatriene); used to evaluate membrane fluidity of the outer and the inner leaflet of cell membrane, respectively [Fiorini R, Curatola G (1991). The fluorescent probes were purchased from Molecular Probes Inc (Eugene, OR, USA). The incubation with TMA-DPH and DPH was performed as described by Sheridan and Block [Sheridan NP, Block ER (1988)]. Briefly, 3 µl of TMA-DPH and DPH (10−3 mol/L) were incubated for 5 min and 45 min respectively, at room temperature (23°C) with 2 ml of erythrocyte membranes (final concentration of 100 µg/mL) in 50 mmol/L Tris-HCl buffer solution, pH 7.4. Fluorescence intensities (100 readings each) of the vertical and horizontal components of the emitted light were measured on a Perkin-Elmer MPF-66 spectrofluorometer equipped with two glass prism polarizers (excitation wavelength 365 nm, emission wavelength 430 nm). Sample temperature was maintained at 37°C using an external bath circulator (Haake F3). Steady-state fluorescence anisotropy (r) of TMA-DPH and DPH was calculated using the equation

$$r = (IvG - Ih)/(Iv + 2Ih)$$

where G is an instrument factor correcting for unequal detection of vertically (Iv) and horizontally (Ih) polarized light.

Na+/K+-ATPase activity.

Na+/K+-activated Mg2+-dependent ATPase activity was determined in cell membranes by the Kitao method [Kitao T, Hattori K (1983)]. ATPase activity was assayed by incubating 1 mL of erythrocyte plasma membrane after sonication (three bursts, 15 s each) at 37°C in a reaction medium containing MgCl2 (5 mmol/L), NaCl (140 mmol/L), KCl (14 mmol/L) in 40 mmol/L Tris-HCl, pH 7.7. The ATPase reaction was initiated with the addition of 3 mmol/L Na2ATP and stopped 20 min later by the addition of 1 mL of 15% trichloracetic acid. The tubes were then centrifuged at 1100 g for 10 min and the inorganic phosphate (Pi) hydrolysed from the reaction was measured in

the supernatant by a colourimetric assay using a KH2PO4 standard [Bradford M, 1976]. ATPase activity, assayed in the presence of 10 mmol/L ouabain, was subtracted from the total Mg2+-dependent ATPase activity to calculate the activity of Na+/K+-ATPase. Protein concentration was determined as described by Bradford [Bradford M 1976], using serum albumin as a standard. The interassay variation was 5.3%, while the intra-assay variation was 8.1%.

Lipoperoxide levels (TBARs) measurement.

Lipoperoxide levels were evaluated using Cayman's thiobarbituric acid reactive substances (TBARs) assay kit. The product of fatty acid peroxidation, malondialdehyde (MDA), reacts with thiobarbituric acid (TBA) to yield a product that is measured fluorometrically. Membranes (100 mg of membrane proteins) were centrifuged at 3000 g for 15 min after the addition of 30% trichloroacetic acid, and 0.5 mL of the resulting supernatant was mixed with 1.1 mL of TBA reagent (equal volumes of 0.67% TBA aqueous solution and glacial acetic acid; v/v). The reaction mixture was heated for 60 min at 95°C in a sand bath. After cooling to room temperature, 5 mL of n-butanol was added and the mixture was shaken vigorously for 2 min. Thereafter, samples were centrifuged at 4000 g for 15 min, then 150 µL from each vial were loaded to the plate for fluorometric assay and the fluorescence of samples and standards was read at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. The lipid peroxide level (Lp) was expressed in terms of MDA content (µM), using 1,1',3,3'-tetramethoxypropane as a standard.

Sialic acid.

Sialic acid content of RBC membranes was determined by the periodate thiobarbituric acid method of Denny et al. [Denny PC, et al. 1983]. Briefly, membranes (1 mg membrane proteins/mL) were first hydrolyzed in 0.05-mol/L H2SO4 in a final volume of 0.1 mL for 1 hour at 80°C to release SA [Warren L (1959)]. Standards and samples were both incubated with (assay samples) or without (correction samples) 0.25 mL periodate solution (0.025 mol/L periodic acid in 0.25 mol/L HCl) at 37°C for 30 minutes [Sobenin IA, et al. 1988]. After reduction of excess periodate with 0.25 mL 0.32 mol/L sodium thiosulfate, the reaction was completed by addition of 1.25 mL 0.1-mol/L thiobarbituric acid. The samples were heated at 100°C for 15 minutes and then cooled to room temperature. The product was extracted with acidic butanol and colorimetrically assayed with a

spectrophotometer at 549 nm. The readings of correction samples were subtracted from those of assay samples, thus corrected readings were obtained.

Protein content was determined by Bradford method to normalize the sialic acid content using BSA as standard [Bradford M, 1976].

Erythrocyte membrane lipidomic analysis.

The erythrocyte fatty acid membrane profile analysis was carried out as previously described, using the erythrocyte membrane pellet obtained by standard methods [Viviani anselmi c et al. 2010]. For this study, selection of the erythrocyte fraction was made by modification of a literature procedure for the selection of aged erythrocytes (red blood cell age >3 months), with cells selected for high density and small diameter compared to the average erythrocyte population [van der Vegt SG et al. 1985].

One mL of whole blood was first centrifuged at 2000 g for 5 min to eliminate the plasma, and a second round of centrifugation was then carried out at 4000 g at 4°C for 5 min in order to yield a stratification by cell density. The bottom layer (2.5 mm from the bottom of tube) consisted of erythrocyte cells, which were evaluated for their diameter using a Scepter™ 2.0 Cell Counter (Merck Millipore, Milan, Italy) to characterize the cell selection from each blood sample. The results were also compared with the cell population obtained from standard density gradient separation [Rennie CM et al. 1979], [Corash LM et al. 1974]. Briefly, lipids were extracted from erythrocyte membranes according to the method of Bligh and Dyer [Bligh EG, Dyer WJ (1959]. The phospholipid fraction was controlled by TLC as previously described [Viviani Anselmi C et al. 2010], then treated with KOH/MeOH solution (0.5 M) for 10 min at room temperature and under stirring [Kramer JKG et al. 1997]. Fatty acid methyl esters (FAME) were extracted using n-hexane; the hexane phase was collected and dried with anhydrous Na2SO4. After filtration, the solvent was eliminated by evaporation using a rotating evaporator, and the thin white film of the FAME was subsequently dissolved in a small volume of n-hexane. Approximately 1 µL of this solution was injected into the GC. A Varian CP-3800 gas chromatograph, with a flame ionization detector and an Rtx-2330 column (90% biscyanopropyl-10% phenylcyanopropyl polysiloxane capillary column; 60 m, 0.25 mm i.d., 0.20 µm film thickness) was used for the analysis. Temperature was held at 165°C held for the initial 3 min, followed by an increase of 1°C/min up to 195°C, held for 40 min,

followed by a second increase of 10°C/min up to 250°C, held for 5 min. The carrier gas was helium, held at a constant pressure of 29 psi. Methyl esters were identified by comparison with the retention times of commercially available standards or trans fatty acid references, obtained as described elsewhere [Ferreri C et al. 2005].

Second study. Separation of blood components through Ficoll

For each subjects enrolled in this second study were collected about 8 mL of blood in EDTA. The whole blood was centrifuged at 480 g for 10 minutes to allow the separation of plasma from the cellular component of the blood. The plasma was aliquoted into 1.5 mL eppendorf tubes and stored at -80 ° C for subsequent studies. The blood, devoid of plasma was diluted 1: 2 with phosphate saline buffer (PBS 1X) and then the solution was layered on a Ficoll (Histopaque-Sigma), previously aliquoted into two tubes of 15 mL. Afterwards was carried out a second centrifugation at 260 g for 35 minutes in order to separate the white blood cells from erythrocytes. At the end of the centrifuge, the blood is separated into the following components: plasma, a layer of mononuclear cells, the Ficoll and finally granulocytes and erythrocytes.

With the help of a pasteur pipette, taking care not to aspirate the Ficoll, the ring of mononuclear cells present in the two tubes was collected and transferred into two tubes of 15 mL. In order to eliminate any traces of Ficoll, the blood mononuclear cells were resuspended in PBS (10 ml) and subsequently centrifuged at 230 g for 10 minutes. Three washes were performed; after the last wash, the PBS 1X was aspirated and the cell pellet on the bottom of the two tubes was put together. A part of the white blood cells has been stored at -80 ° C and one part was used for the analysis of protein glutathionylation. Red blood cells were subsequently processed; in particular the red blood cells of one of the two tubes were used to isolate erythrocytes membranes (see next paragraph), while the remaining erythrocytes were were resuspended in PBS 1X (10 ml) and subsequently centrifuged at 1400 rmp for 10 minutes. Three washes were performed; after the last wash the red blood cells have been used to evaluate the distribution of phosphatidylserine in the erythrocyte membrane, the glutathionylation of proteins and for the analysis of the morphology of red blood cells by scanning microscopy (SEM) and transmission (TEM).

Preparation of erythrocyte membranes

The red blood cells were washed three times with 1X PBS (10 ml) supplemented with 5 mM glucose and, subsequently, were centrifuged at 1500 xg for 10 minutes. After the last washing, 1 mL of red blood cells was lysed with 15 mL of lysis buffer consisting of 5 mM phosphate buffer at pH 8 and 1 mM EDTA. For optimal lysis, the sample was mixed vigorously and subsequently was centrifuged at 27,500 g for 20 minutes at 4 ° C using a chilled ultracentrifuge and paying attention to balance the test pieces. At the end of the centrifuge, the supernatant rich hemoglobin has been cleared; the pellet containing the membranes, has been washed to remove traces of hemoglobin. The membranes, in fact, were resuspended in 15 mL of lysis buffer and centrifuged as before; 4 washes were performed at the end of which was obtained a pellet of colorless membranes (haemoglobin-free). Al pellets are added 5 µL of protease inhibitors for each mL of the obtained membranes. These were stored at -80 ° C and subsequently were used to measure the amount of cholesterol and to evaluate the protein expression of the subunits of the Na-K ATPase.

Assessment of the levels of cholesterol in the membranes of erythrocytes

The quantification of cholesterol in the membranes of red blood cells was carried out by a colorimetric assay. It was determined the concentration of total cholesterol, consisting of the sum of the concentration of free cholesterol and cholesteryl ester. For this reason, in the assay was used an enzyme, cholesterol esterase, which hydrolyze cholesterol esters to cholesterol. The assay is based on the oxidation of cholesterol by the enzyme cholesterol oxidase; this reaction produces H2O2 (hydrogen peroxide) which reacts with a colorimetric probe. The course of the colorimetric reaction is followed by measuring the absorbance at 570nm. A calibration curve was obtained by making serial dilutions of a standard of cholesterol at a known concentration. The amount of cholesterol present in the samples of red cell membrane is obtained by interpolating the absorbance value in the standard curve.

For the preparation of the standard we diluted 20 µL of cholesterol standard (2 µg/µL) with 140 µg of Assay Buffer cholesterol obtaining a solution with a cholesterol concentration of 0.25 µg/µL. Were added 0, 4, 8, 12, 16, and 20 µL of the solution of cholesterol standard in a 96-well plate in order to have 0, 1, 2, 3, 4, and 5 µg of cholesterol standard per well. It was finally added to each well Assay Buffer until to get a volume of 50 µL. For the preparation of samples were used 5 µL of

erythrocyte membranes which have been brought to a final volume of 50 µL with Assay Buffer. The amount of membranes (5 uL) was determined in a preliminary experiment in which, for a given sample, we used scalar amounts of membranes in order to find the volume of membranes whose amount of cholesterol fell within the linearity range of the standard curve. All samples and standards were prepared in duplicate. Once the standards and the samples were plated in each well a reaction mix was added, consisting of 44 µL of Assay Buffer, 2 µL of probe, 2 µL of enzyme mix (containing cholesterol oxidase) and 2 µL of Cholesterol esterase. The plate was incubated for 60 minutes at 37 ° C in the dark. After incubation, the absorbance was measured at 570 nm using a plate reader (Victor II, Perkin-Elmer). The amount of cholesterol was calculated by subtracting the value of the absorbance of the blank to all the absorbance values obtained. For both the standard and the samples the average of duplicates has been calculated. The standard curve was built by reporting on the abscissa the quantity, in mg, of cholesterol standards, and the absorbance on the ordinate axis. The absorbance values of each sample were interpolated in the standard curve, thus obtaining the amount of cholesterol in the unknown sample expressed in µg. The latter was divided by the volume in the well (50 µL) and multiplied by the dilution factor. This concentration value, expressed in µg/µL, was normalized to the protein concentration determined by Bradford method (Bradford 1976).

Evaluation of glutathionylation in red blood cells and white blood cells

To measure the amount of protein glutathionylated in samples of white blood cells and red blood cells a colorimetric assay has been used (S-Glutathionylated -Detection Kit, Cayman). The assay is based on the detection of glutathionylated proteins by the addition of specific reagents which determine: 1) the blocking of the free thiol groups (-SH) present in the protein; 2) the enzymatic cleavage of glutathionylation sites with formation of new thiol groups; 3) recognition of the new thiol groups by colorimetric reaction .

In particular, the assay is based on the modification of free thiol groups (-SH) of proteins by the addition of a reagent, the PSSG Blocking Reagent, supplied by the kit. In this way, the free thiol groups are protected from subsequent step of enzymatic reduction. In fact, the addition of another component of the kit, PSSG Reduction Reagent, allows the enzymatic cleavage of all the glutathionylated cysteine residues, thereby generating new free thiol groups. To detect the latter, the PSSG Labeling Reagent is added, which allows the biotinylation of thiol groups that will be

quantified by using the binding of an antibody streaptavidina FITC conjugate. The fluorescence signal will be analyzed by flow cytometry. The assay, in addition to the test sample (test tobe B), involves the preparation of a negative control (test tube A) and a positive control (test tube C). In particular, PSSG Reduction Reagent is not added in the test tube A, thus preventing the formation of new free thiol groups that can react with biotin. The preparation of the specimen A, without the reducing agent, allows us to obtain the minimum of the staining. The test tube C, however does not include adding PSSG Blocking Reagent, thus making accessible all the thiol groups of proteins the binding to biotin; therefore, this control allows to get the most of the staining. The white blood cells of autistic and control subjects were resuspended in 1.5 mL of 1X PBS and transferred to 3 eppendorf tubes in order to have a negative control (tube A), a positive control (test tube C) and the sample to be tested (test tube B). White blood cells were centrifuged at 300 g for 5 min. The supernatant was cleared and the pellet was fixed with 100 µL of 3.7% formaldehyde in cold PBS for 10 minutes at room temperature. Subsequently, the cells were washed with PBS by centrifuging the tubes at 200g for 5 min. To block free thiol groups of proteins, the PSSG Blocking Reagent has been prepared, by dissolving the powder in 100 µL of N, N-dimethylformamide (DMF); this solution was then diluted by adding it to 9.9 mL of Assay Buffer. One hundred microliters of Blocking Reagent were added to the tubes A and B, while in the tube C were added 100 µL of Assay Buffer. The samples were incubated for 30 minutes at room temperature. After the indicated time, the Blocking Reagent was eliminated by centrifuging the tubes at 200 g for 5 minutes; subsequently, the samples are washed twice with 100 µL of Assay Buffer. The same procedure was performed for the red blood cells, however, making some changes concerning the fixation and washes. In fact, the red blood cells were fixed in 100 µL of 0.025% glutaraldehyde in PBS for one hour at room temperature. All washes were performed with 1X PBS instead of Assay buffer. These protocol changes were necessary because of the extreme fragility of red blood cells that were undergoing lysis in the presence of formaldehyde and Assay buffer. Once deleted the trace of the Blocking Reagent, to each sample B and C of red blood cells or white were added 100 µL of PSSG Reduction Reagent, prepared by adding 1.1 mL of sterile water to the powder of the kit, whereas in the test tube A were added 100 µL of Assay Buffer. The samples are incubated for 15 minutes at 37 ° C. After incubation, the tubes were centrifuged at 200 g for 5 minuiti. After the supernatant aspiration, tubes A B C were washed twice with 100 µL Assay Buffer (white blood cells) or PBS 1X (red blood cells). One hundred microliters of Labeling Reagent, prepared by

dissolving the powder of the kit in 10 µL of DMF and adding this solution to 1990 µL of Assay Buffer, was added to all samples A, B and C. The tubes were incubated for 1 hour at room temperature. After incubation 3 washes were performed with Assay Buffer (white blood cells) or PBS 1X (red blood cells) centrifuging the tubes at 300 g for 5 minutes. Finally, 100 µL of PSSG Detection Reagent II (FITC) prepared by diluting the stock solution in 1 mL of assay buffer, were added to the pellet of white blood cells or red blood cells; the samples are incubated for 1 hour. Next, for each sample were carried out two washes with 100 µL of Assay Buffer (white blood cells) or PBS 1X (red blood cells) by centrifugation at 300 g for 5 min. After the last wash we resuspended samples of red blood cells in 500 µL of 1X PBS, while the white blood cells in 400 µL of assay buffer. The levels of glutathionylation protein were measured by flow cytometry; the reading parameters of the instrument have been set with a sample of white blood cells and a sample of red blood cells.

Evaluation of Phosphatidylserine

The annexin V is a calcium-dependent protein which has a high affinity for phosphatidylserine (PS), and it is used as a probe to detect the phosphatidylserine (PS) present on the cell surface, an event that occurs in apoptosis as well as in other forms of cell death. In fact, in physiological conditions, the PS is a phospholipid distributed more on the cytosolic side of the membrane. Any differences in the distribution of PS on the cell surface at baseline or after stress were detected by using the Annexin-V-FLUOS staining kit (Roche) in red blood cells.

For the preparation of samples were used 5 µL of red blood cells diluted in 10 mL of PBS 1X. One milliliter of this suspension was transferred into three 1.5 mL eppendorf: the tube 1 is the control (CTR), which contains the red blood cells not labeled with annexin V-propidium iodide; the tube 2 is the sample containing red blood cells labeled with annexin V-propidium iodide; the red blood cells of the test tube 3 are incubated with hypertonic solution of NaCl 9%, in order to damage the cells, and subsequently, were labeled with annexin V-propidium iodide.

Once prepared the three test tubes with the suspension of red blood cells, the tube 3 was centrifuged for 5 min at 3.0 rpm; the supernatant was aspirated and the pellet of red blood cells was resuspended in 1 ml of 9% NaCl solution. The sample was incubated at room temperature for

10 minutes. After incubation, we centrifuged the three tubes for 5 min at 3.0 rpm, we aspirated supernatant and resuspended the pellet of the tubes 2 and 3 in 100 μL of solution containing annexin V and propidium iodide, while the tube 1 was resuspended in 100 μL of buffer provided by the kit. Finally, the samples were incubated for 15 min at room temperature. After incubation we transferred 100 μL samples in tubes suitable for cytofluorimetric analysis, where previously they had been aliquoted 500 μL of Buffer kit. It was then carried out the reading of the samples by flow cytometry. The number of annexin positive cells in autistic patients and in control subjects were analyzed by statistical tests.

Statistics

All experiments were carried out in duplicate or triplicate and were usually repeated three times.

To compare Au and TD groups, normality tests were applied to all numeric variables, following which appropriate parametric tests (ANOVA, Student's t for independent data) or the nonparametric equivalent (Wilcoxon-Mann-Whitney) were used. Non-parametric correlation (Spearman's rho) was used to correlate clinical features and biochemical data in the Au group (non-parametric ANOVA for cognitive/developmental level). Differences were considered significant at $p<0.05$.

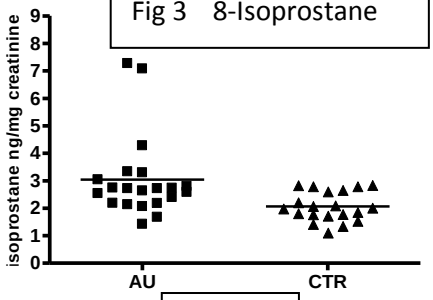
To account for multiple testing we used the Benjamini and Hochberg false discovery rate (FDR) [Benjamini Y, Hochberg Y (1995)]. Statistical analysis was performed using SAS v. 9.2.

3.3 Results

This study focused on three issues: oxidative stress markers, erythrocyte (membrane functional and structural features, erythrocyte morphology), Na K ATPase activity (erythrocyte and lymphocyte membrane).

Oxidative stress markers in urine and plasma and antioxidant enzymes activities in erythrocytes.

Fig 3 8-Isoprostane

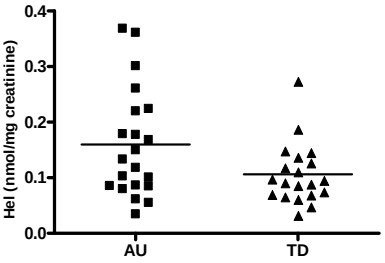


p<0.05

Peroxidation of arachidonic acid causes membranes to release 8-isoprostane, a prostaglandin-F2-like compound.

Oxidized arachidonic acid or other omega-6 fatty acids, such as linoleic acid, may also react with protein lysine residues, yielding HEL.

Fig 4 Hexanoyl-Lysine Adduct

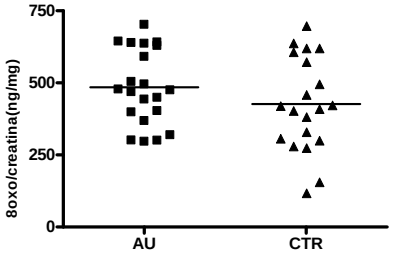


p<0.05

Both 8-isoprostane ($p<0.01$; pFDR = 0.0278) and HEL ($p<0.05$; pFDR = 0.076) were found in higher amount in the urine of Au than in the urine of TD children (+47% and +45%, respectively).

However, the amount of 8-oxo-dG, derived from the oxidation of nucleic acid bases by free radicals, did not significantly differ between the two groups.

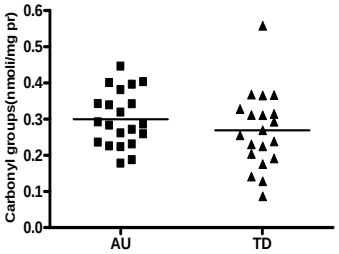
Fig. 5 8-hydroxy-2'-deoxyguanosine



p=ns

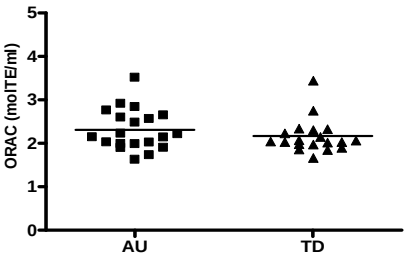
Plasma levels of carbonyl groups (an oxidative modification of proteins) and plasma radical absorbance capacity (a measure of the antioxidant capacity, which is reduced by free radicals) did not differ between the two groups.

Fig. 6 Plasma Carbonyl Groups



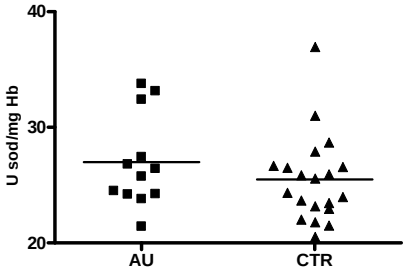
p=ns

Fig. 7 Plasma ORAC



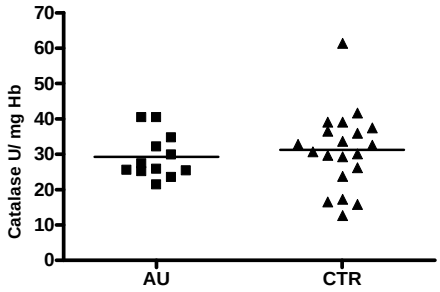
p=ns

Fig. 7 SOD



p=ns

Fig 8 CATALASE



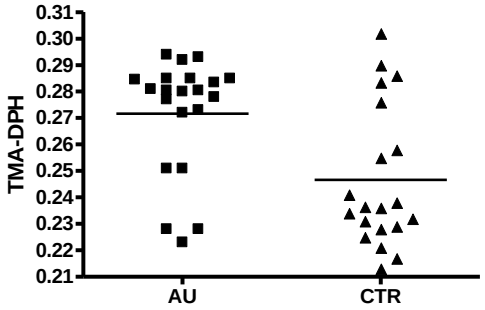
p=ns

Similarly, neither SOD nor catalase enzymatic activity measured in erythrocytes were found to differ between the two groups. The evaluation of erythrocyte catalase activity was repeated in the second study by Prof De Franceschi with a different method, which confirmed the absence of significant differences between AU and TD children.

Moreover, the expression of the chaperon HSP17 was evaluated by WB in erythrocytes and found to be the same in the two groups of children.

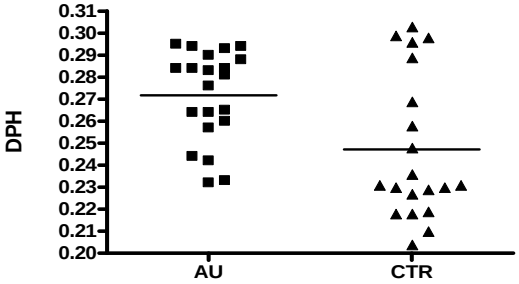
TMA-DPH and DPH are two probes used to evaluate membrane fluidity of the outer and the inner leaflet of cell membrane, respectively. Taking into account that TMA-DPH and DPH fluorescence anisotropy is inversely related to the fluidity of the microenvironment where the probe is located, it was found that erythrocyte membrane fluidity was decreased in Au with respect to TD. The decrease reached the statistical significance ($p<0.05$) for both the outer and inner membrane (pFDR = 0.0368, pFDR = 0.0469, respectively).

Fig 9 TMA-DPH



P<0.05

Fig 10 DPH

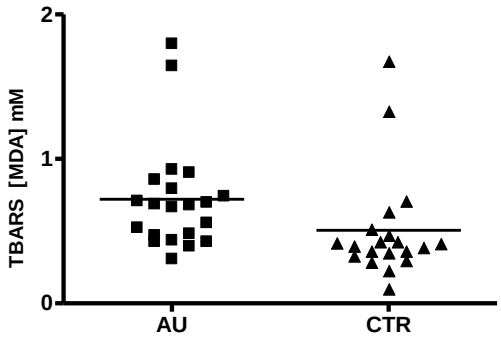


P<0.05

The lymphocyte membrane fluidity did not differ between Au and TD children (prof Mazzanti, data not shown).

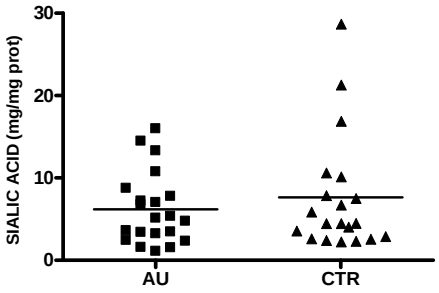
TBARS assay measures MDA generated from the decomposition of primary and secondary lipid peroxidation products. TBARS were found to be significantly higher (+41%) in the erythrocyte membrane from Au children in comparison with those from TD children ($p<0.01$; pFDR = 0.0125).

Fig. 11 TBARS



p<0.05

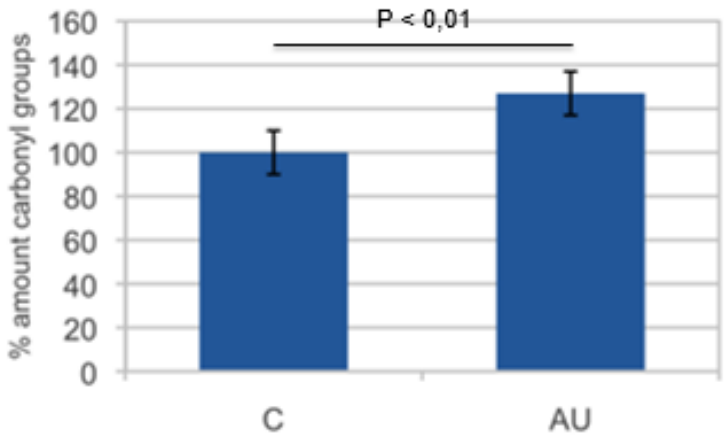
Fig 12 Sialic acid



p=ns

The presence of **carbonyl groups** was evaluated by WB, following DNP derivatization. Carbonyl groups were found to be increased in erythrocytes from AU children.

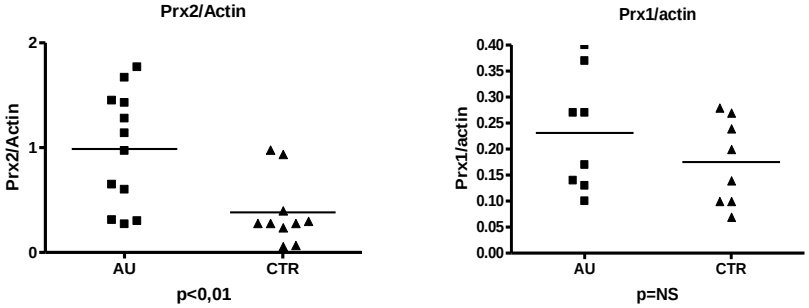
Fig.13 Carbonyl Groups (erythrocyte)



Peroxiredoxin II and Peroxiredoxin I

Peroxiredoxin II and Peroxiredoxin I were evaluated by Prof De Franceschi (University of Verona). Peroxiredoxin II levels were found to be elevated ($p<0.01$) in Au group when compared to TD children (Au: mean 0.99, SD 0.55, range 0.27-1.77; TD: mean 0.38, SD 0.32, range 0.06-0.98). Peroxiredoxin I did not differ significantly between the two groups.

Fig. 14: Prx2/Actin and Prx1/actin



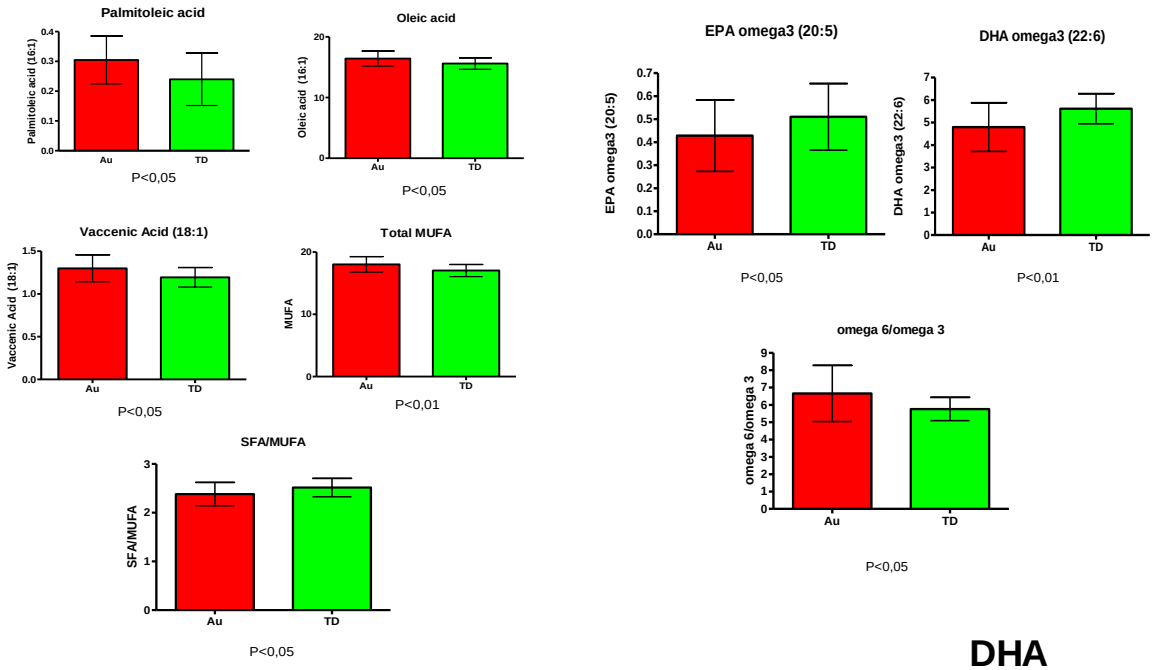
Fatty acids composition of erythrocyte membrane

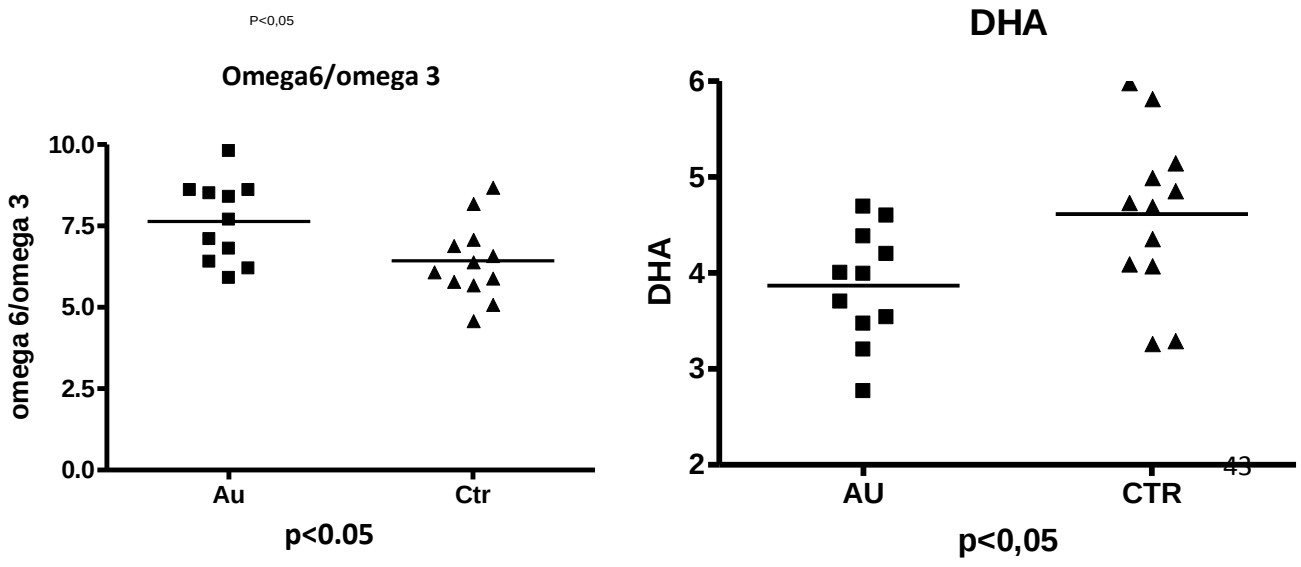
The percentage of oleic, palmitoleic and vaccenic acids and, in general, total MUFA were increased in Au with respect to TD children. This caused also a decrease in SFA/MUFA ratio in Au with respect to TD children ($p<0.05$; pFDR = 0.07329).

The relative amount of the different PUFA was also altered, since EPA and DHA-ω3 acids were decreased in Au children (−16%, $p<0.05$, pFDR = 0.10308 and −14%, $p<0.01$, pFDR = 0.0722, respectively), causing an increase in ω6/ω3 ratio (+16%, $p<0.05$, pFDR = 0.07329).

The fatty acid composition of the erythrocyte membrane has been evaluated also in the group of children evaluated in the second study. The results confirm the decrease of DHA and a significant increase of the ω6/ω3 ratio in AU children.

Fig 15: Fatty acids erythrocyte membrane

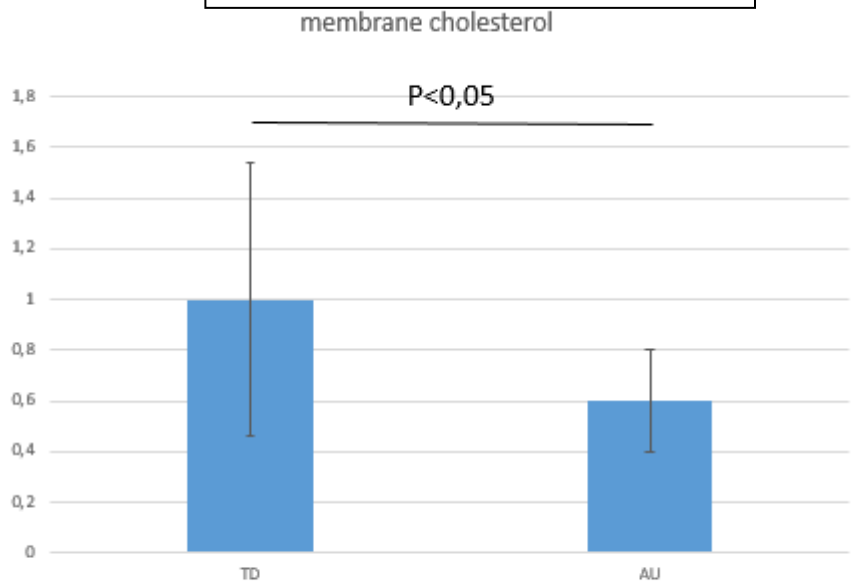




Assessment of the levels of cholesterol in the membranes of erythrocytes

Autistic children showed significantly lower cholesterol levels than children with typical development. These data are in line with those reported by Schengrund (Schengrund et al., 2011). As a whole, these data contribute to point out the presence of impairment in the membrane hydrophobic double layer. On the other hand, circulating fatty acids were found to be in the normal range.

Fig 16: erythrocyte membrane cholestrol



Evaluation of glutathionylation in red blood cells and white blood cells

The data obtained have shown that the glutathionylation of proteins in red blood cells and white blood cells did not show significant differences in between autistic children than in control subjects.

Fig. 17: Glutathionylation of protein in red blood cells

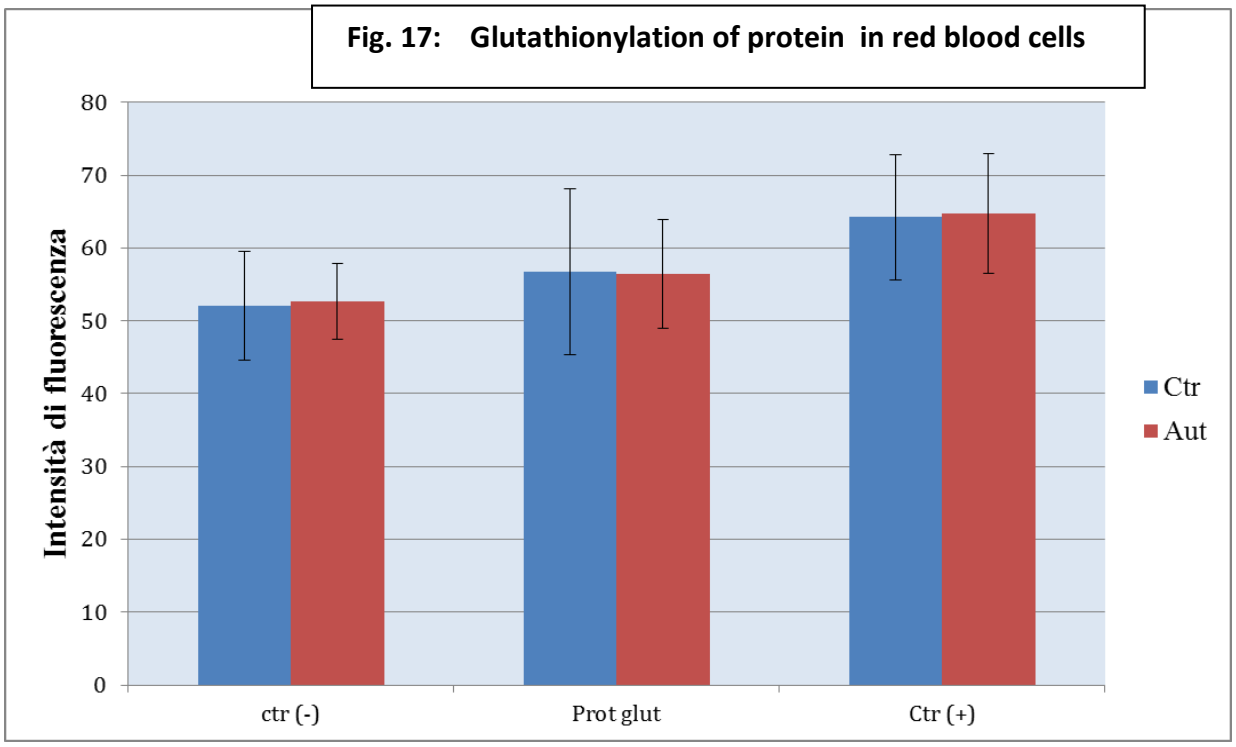
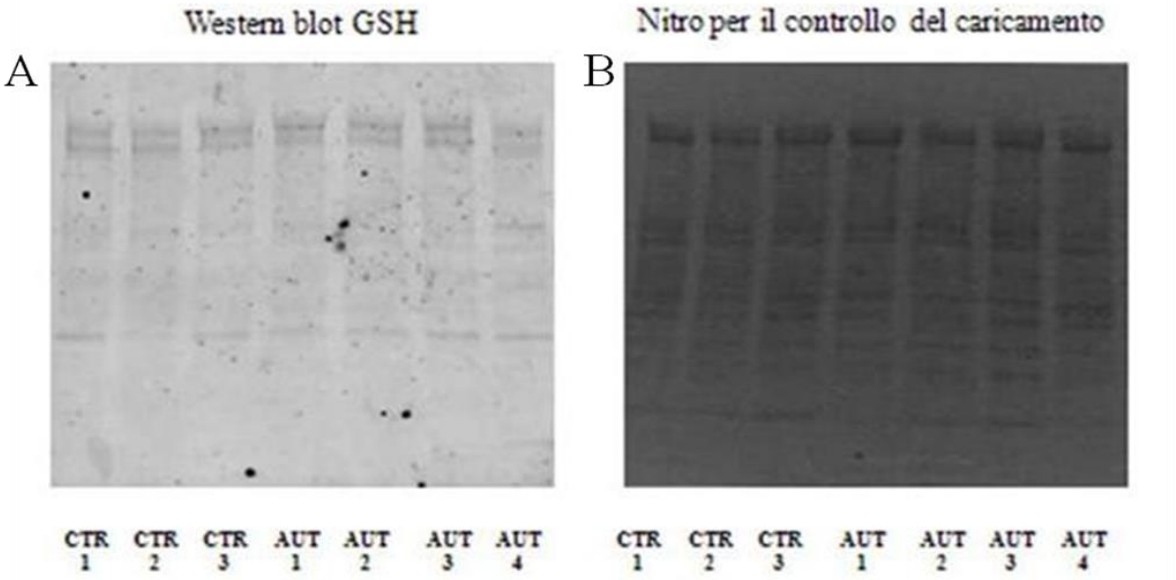


Fig. 18: Glutathionylation o protein in red blood cells



Analysis of levels of protein glutathionylation performed by Western Blot in the proteins extracted from erythrocyte membranes of autistic subjects (AUT) and control subjects (CTR).

A: Western Blot performed with the Antibody anti-GSH.

B: the total protein that are transferred on nitrocellulose and are detected by UV

Evaluation of Phosphatidylserine

In untreated erythrocytes of autistic children we showed a trend of decrease of Annexin-positive cells when compared to controls. The decrease is evident when the red cells are treated with a hypertonic solution (NaCl 9%) (see figures below).

Fig. 18a: Annex V+ cells

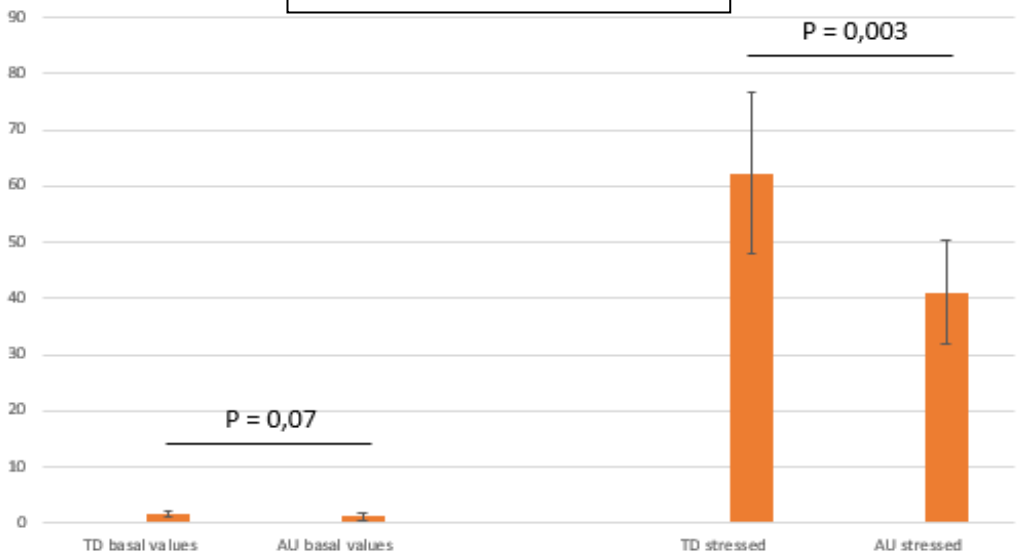
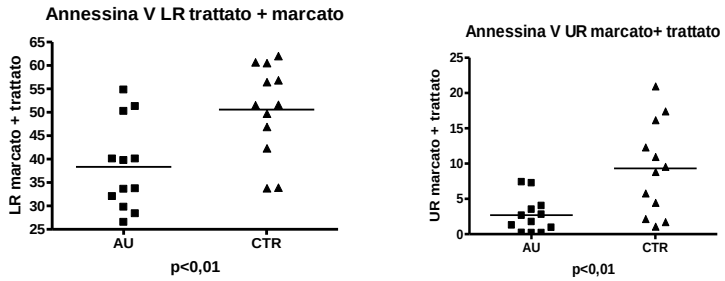


Fig. 18b: Annex V+ cells



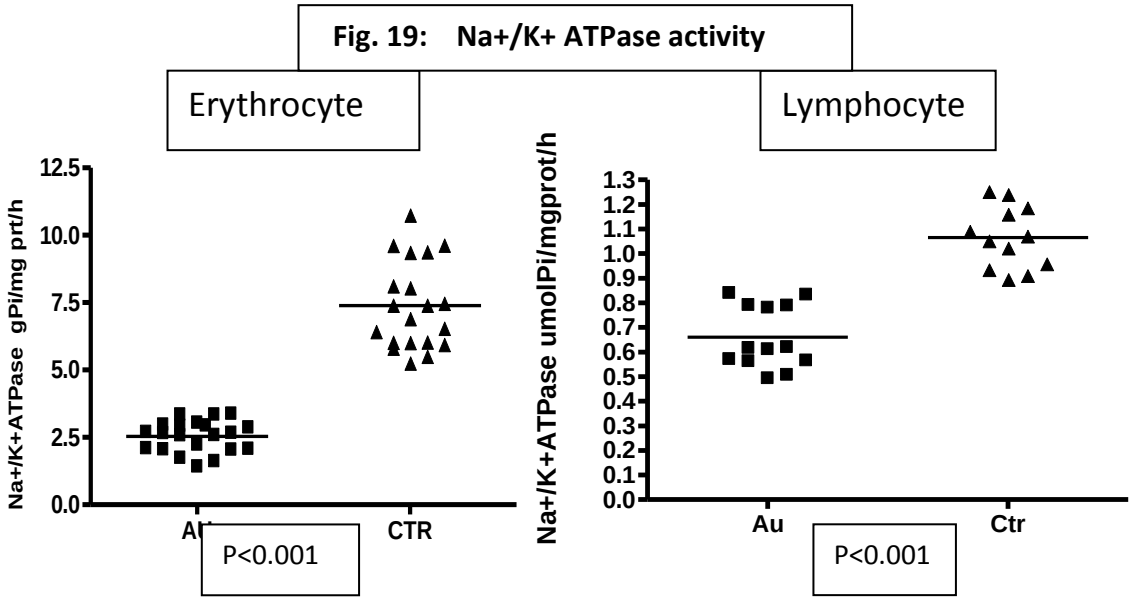
Erythrocyte Na+/K+-ATPase activity (Prof Laura Mazzanti lab, Ancona)

The activity of Na+/K+-ATPase, an active ion transporter localized in the plasma membrane, was markedly decreased (−66%) in Au in comparison with TD (p<0.0001; pFDR<0.0001), **with no overlapping values** between Au and TD (Au min. 1.41, max. 3.38; TD min. 5.27, max. 10.75).

Lymphocyte Na+/K+-ATPase activity (Prof Laura Mazzanti lab, Ancona)

The activity of lymphocyte membrane Na+/K+-ATPase was markedly decreased (−40%) in Au in comparison with TD (p<0.0001), **with no overlapping values** between Au and TD (Au min. 0.49 , max. 0.840, mean 0.646, SD 0.12 ; TD min. 0.895, max. 1.253, mean 1,065, SD 0.12).

Fig. 19: Na+/K+ ATPase activity



While NKA activity was found to be significantly decreased in leukocytes from AU children, similarly to what was observed in erythrocytes, no difference in leukocyte membrane fluidity was found between the two groups (data not shown), at variance with what observed in erythrocytes. This suggests that membrane fluidity does not affect NKA activity

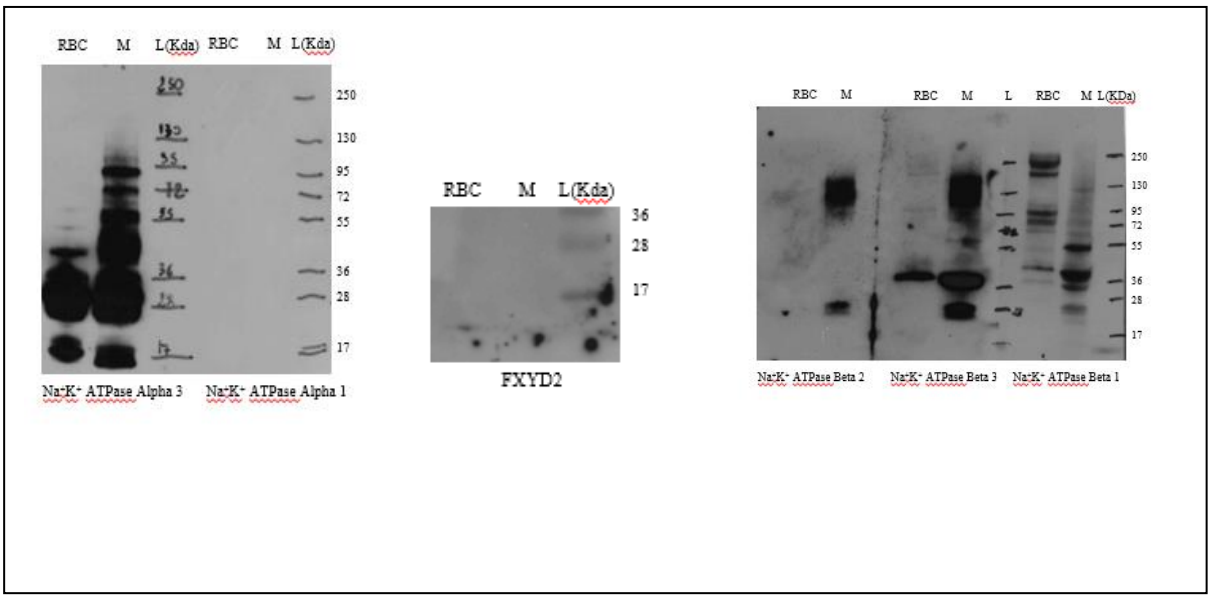
Evaluation of erythrocyte NKA subunits by Western Blotting

We attempted to evaluate erythrocyte NKA subunits by Western Blotting, by means of the primary antibodies, which had been used in a published paper (Hoffman JF, et al. 2002). Secondary antibodies were purchased from Pierce. Pre-cast gel (Mini-PROTEAN TGX Stain Free Gel, BioRad), 4-15% acrylamide were used. Following transfer, membranes were visualized in order to evaluate equal loading. The figure below shows WBs developed from two samples used as positive controls in preliminary experiments, i.e. normal erythrocytes and skeletal muscle. Apart from subunit α2, which is not expected to be present in erythrocytes, the other subunits were expected to be found as single protein bands. However, results were disappointing. In fact, the anti-subunit α3 antibody marks a high number of aspecific proteins; the anti-subunit α1 antibody does not recognize any protein; the anti- subunit β2 antibody reacts very weakly with erythrocytes; the anti-subunit β3 antibody is apparently the only one reacting with a single protein in erythrocytes; the anti-subunit β1 antibody reacts with multiple aspecific proteins; finally, the antibody directed against the regulatory subunit Fxyd1 does not recognize any protein.

We now purchased new anti-subunit α antibodies from Santa Cruz, hoping that they will work better and we'll complete the evaluation of all samples.

Fig. 19: Western Blot

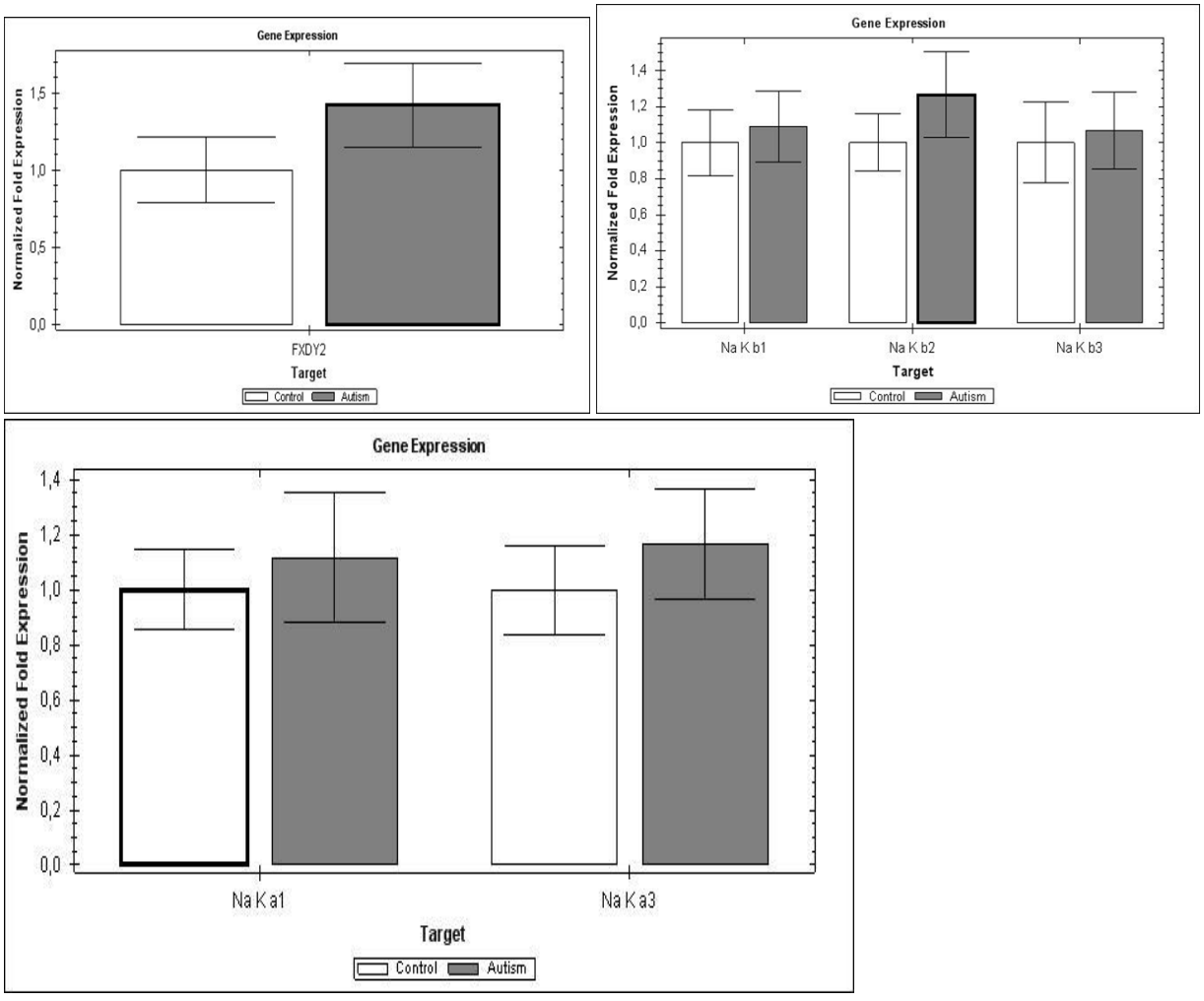
+



Gene expression of NKA subunits in leukocytes

We then evaluated the gene expression of NKA subunits in leukocytes, which are nucleated and synthesize RNA, at variance with erythrocytes. RNA was extracted from leukocytes from five AU and five TD children; RNA was quality assessed, reverse transcribed and evaluated by qRT-PCR using custom-designed primers. The RNAs coding for all NKA subunits were evaluated and their amount reported to those of housekeeping genes G6PDH and actin. Subunit α2 RNA was not detectable. Histograms in Figure below show the absence of significant differences between AU and TD children in the amount of RNAs coding for the other subunits. The evaluation will be repeated in leukocyte RNA from at least 5 more AU and 5 more TD children.

Fig. 20: gene expression sub units Na+/K+ ATPase



Erytrocyte Beta Actin levels

Erythrocyte Beta Actin levels were independently evaluated by De Franceschi (Verona) and by our group: the results showed no differences between Autistic and control group.

Analysis of the levels of Beta Actin was performed by Western blot in the proteins extracted from erythrocyte membranes of autistic subjects (AUT) than in control subjects (CTR).

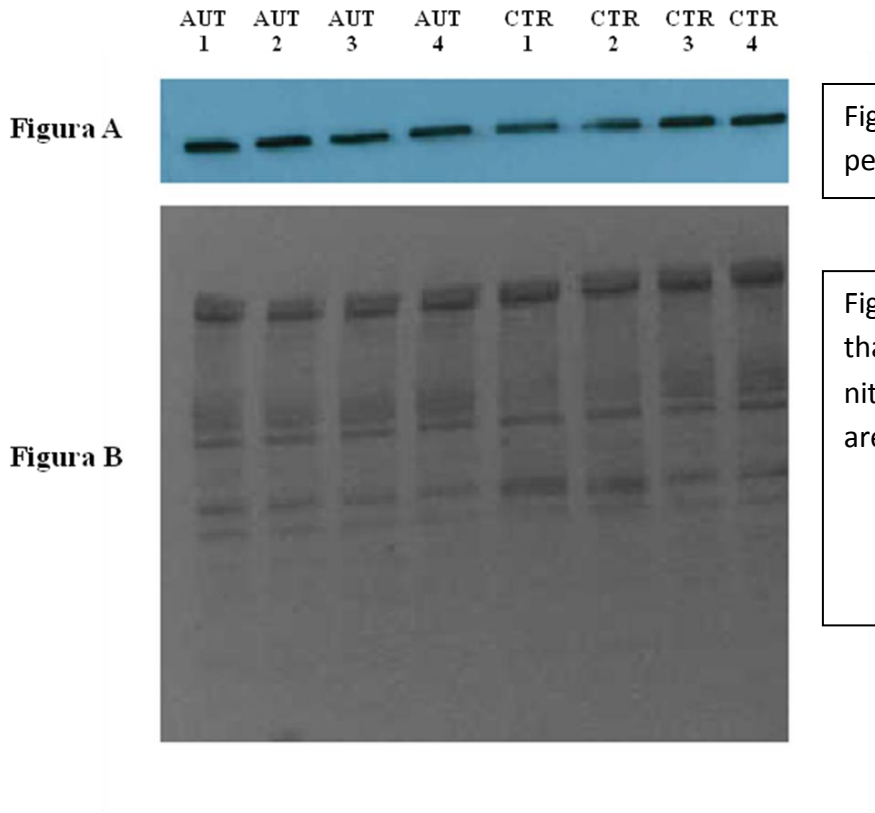


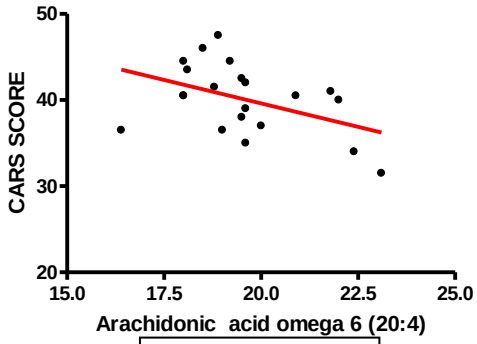
Fig 21 A Western blot performed with the anti-beta

Fig 21 B The total protein that are transferred on nitrocellulose and that are detected by UV

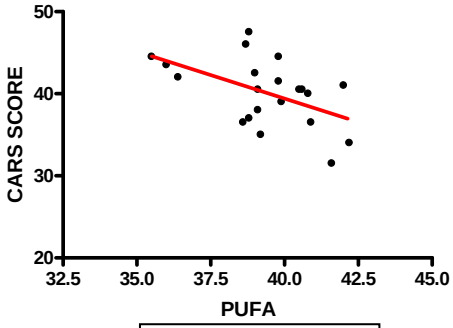
Correlation between Au Clinical Features and Biochemical Data

Non-parametric correlation (Spearman's rho) was used to correlate clinical features and biochemical data in the Au group.

CARS global scores were inversely related with ω6 arachidonic acid ($p<0.05$; pFDR = 0.31104) and PUFA ($p<0.05$; pFDR = 0.18450).

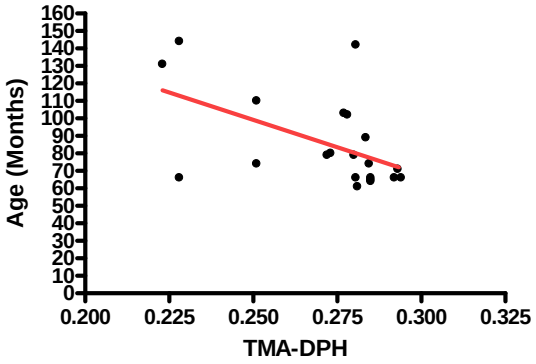


P<0.05



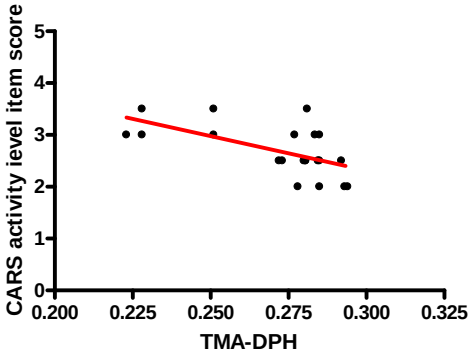
P<0.05

TMA-DPH was correlated with age ($p<0.01$ pFDR = 0.2376).



CARS body use item scores (stereotypes) were not significantly related to any biochemical marker.

CARS activity level item scores (**hyperactivity**) were negatively correlated with TMA-DPH ($p<0.01$; pFDR = 0.03720), oleic acid ($p<0.05$; pFDR = 0.15035), ω6 arachidonic acid ($p<0.05$; pFDR = 0.15035), MUFA ($p<0.05$; pFDR = 0.11728) and PUFA ($p<0.01$; pFDR = 0.03720), and directly correlated with SFA ($p<0.001$; pFDR = 0.00930), palmitic acid ($p<0.01$; pFDR = 0.03720), SFA/MUFA ($p<0.001$ pFDR = 0.03720).



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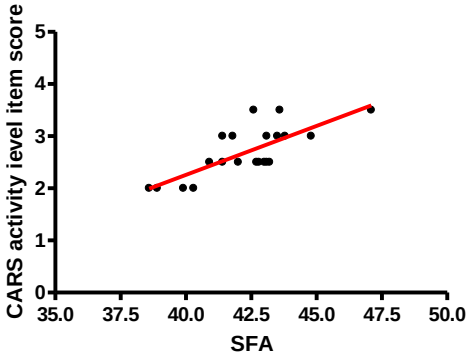

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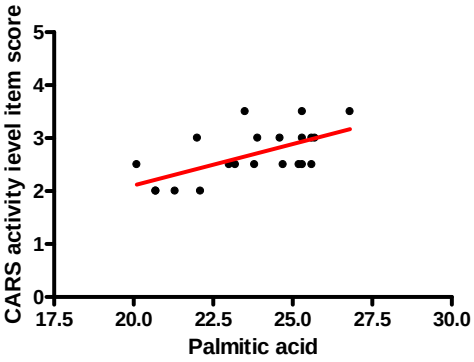

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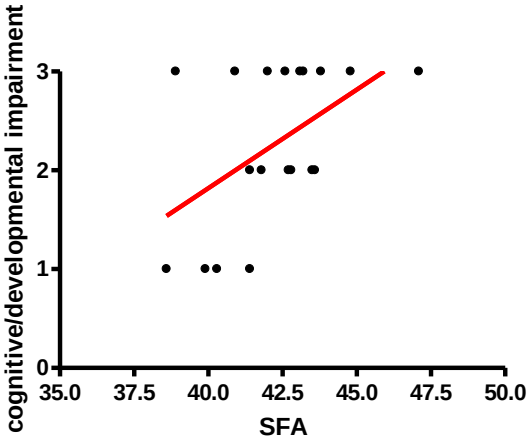
When only cognitive/developmental impaired Au children (n: 19) were considered, the non parametric ANOVA revealed that the level of cognitive/developmental impairment was inversely related with ω6 arachidonic acid ($p<0.05$; pFDR = 0.33199), and directly related with 8-isoprostane ($p<0.05$; pFDR = 0.33199), total SFA ($p<0.05$; pFDR = 0.33199) and palmitic acid ($p<0.05$; pFDR = 0.33199), while cognitive impairment and total PUFA showed only a trend of inverse correlation ($p = 0.0553$; pFDR = 0.33199).



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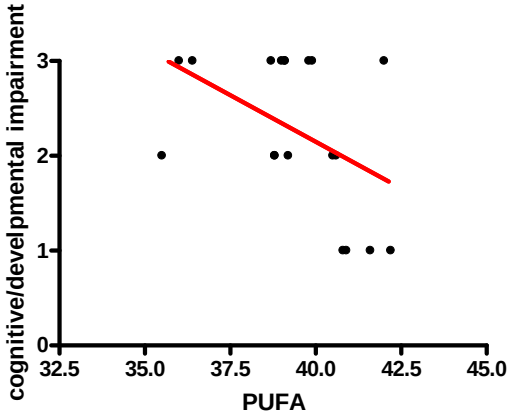

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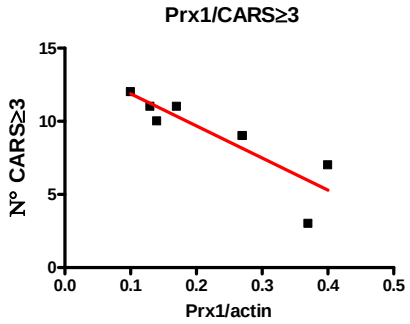
Moreover Peroxiredoxin I levels, evaluated in 8 Au children, inversely correlate with clinical features, in particular with numbers of CARS items where score is ≥3 (expression of severity of the symptoms) and with ADOS score.



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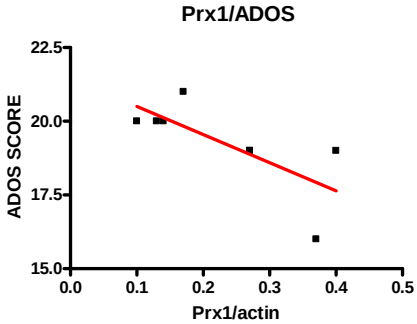
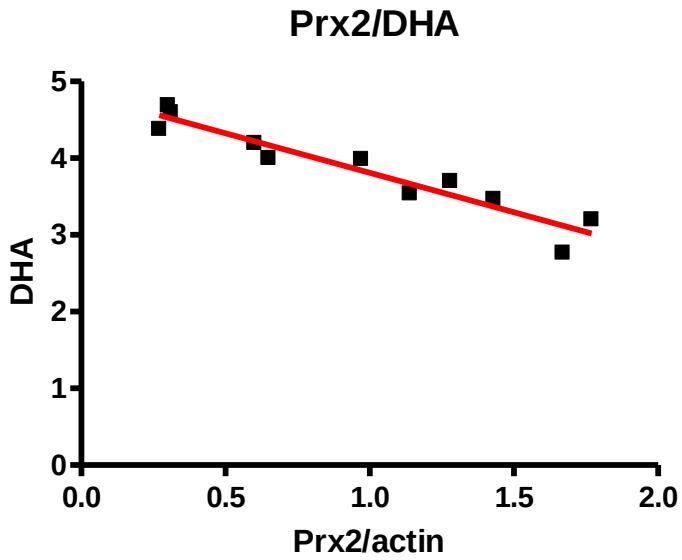


P<0.05

Correlations between biochemical features

The correlation between the evaluated biochemical parameters was beyond the scope of our work. Below I show the graphs of some correlations that were significant. These data need to be confirmed and elaborated in subsequent studies. In particular Peroxiredoxin II values are highly ($p<0.001$) and inversely correlated with DHA content of erythrocyte membrane (but, interestingly, only in autistic group); consequently they are directly correlated with omega6/omega3 ratio and inversely with omega 3 index (defined as EPA+DHA).



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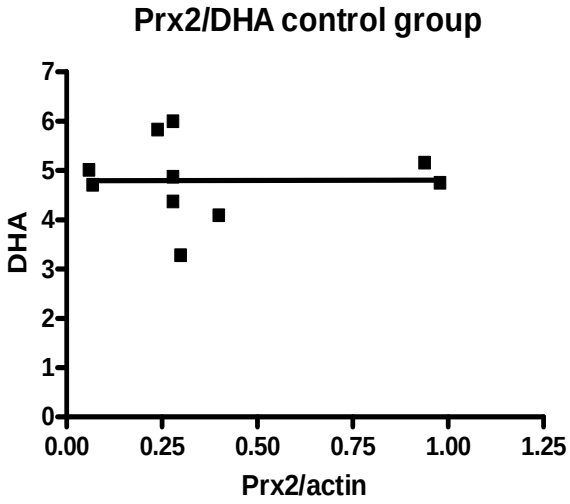

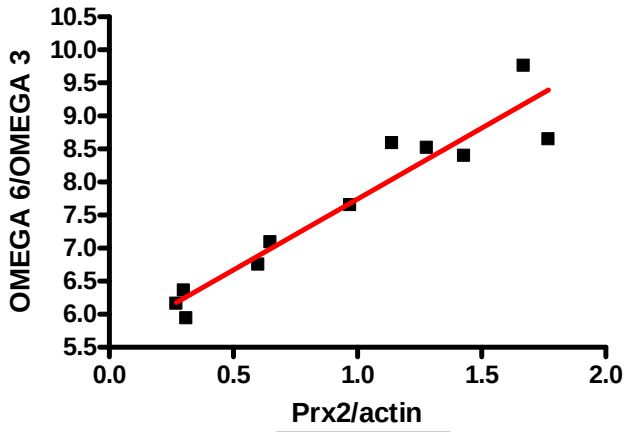

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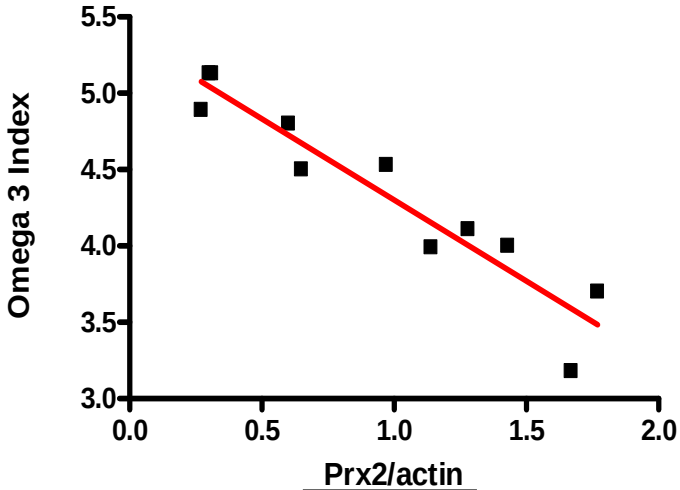

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3.4 Discussion

The present project was aimed at increasing the knowledge of the biological basis of Autism focusing on three issues: oxidative stress, erythrocyte membrane features and morphology, Na+/K+ATPase activity of erythrocyte and lymphocyte membranes.

There is increasing evidence that autistic patients show excessive ROS production and several studies reported the presence of different peripheral biomarkers of oxidative stress [Frustaci et al., 2012; Geier DA and Geier MR, 2008; Bradstreet JJ, et al. 2010]. However, the great heterogeneity of the syndrome makes it difficult to assess whether this finding is occasional and whether it is restricted to a sub-group of patients. Moreover, not all oxidative stress markers appear to be altered in patients, and it is still unknown whether oxidative stress, if really present, is secondary to a generic inflammatory status or due to genetic alterations still to be recognized. In addition, most researchers addressing this problem have a tendency to evaluate few markers at a time, thus making it very difficult to compare data obtained in different patient's subgroups [Meguid NA, et al., 2011; Pecorelli A et al., 2012].

To our best knowledge, this is the first study, which evaluated, at the same time, a wide range of different but strongly related biological biomarkers in a group of Au children that underwent a rigorous clinical characterization.

A far as concern the **oxidative stress**, we found, in Au children compared to TD children, a significant increase in TBARS, 8-isoprostane and HEL, which are markers of lipid peroxidation. The increase in carbonyl groups in erythrocyte membrane proteins of AU children highlights a direct result of oxidative injury. No significant differences were found in the oxidative biomarkers 8-oxo-dG and ORAC. This finding suggests that the oxidative stress-related phenomena are localized mainly at the cell surface. Systemic involvement is suggested by detection of these markers both in urine and in erythrocytes. The expression of two antioxidant enzymes – namely peroxyredoxin 1 and 2 (Prx1 e Prx2), was evaluated in the erythrocyte membrane by Prof De Franceschi research group. No difference was found for Prx1, while Prx2 was found to be significantly increased in AU children. This confirms that erythrocytes from AU children are the target or the site of oxidative stress. These pro-oxidant conditions usually favor the release from the erythrocyte membrane of

microvescicles carrying Prx2, which contributes to the general anti-oxidative defenses of the body. As a confirmation of the relation between oxidative stress and Prx2, statistical analysis showed a direct correlation between Prx2 amount and ω6/ω3 ratio in AU children.

The **fatty acid composition** of the brain and neural tissues is characterized by high PUFA concentrations, which play a very important role in signal transduction [Kim HY,2007], neuro-inflammation [Orr SK, Bazinet RP, 2008] and cellular repair and survival [Bazan NG, 2005]. **Erythrocyte membrane fatty acid composition** is a very sensitive indicator of the status of different tissues and may reflect the fatty acid composition of brain [Makrides M etal., 1994]. In a number of neurodevelopmental conditions, including Attention Deficit Hyperactivity Disorder (ADHD) and dyslexia, reduced concentrations of erythrocyte membrane PUFA have been reported [Richardson AJ, Ross MA (2000)]. Moreover, a polymorphism in the gene cluster associated with the fatty acid desaturase-2 gene (FADS2) for Delta 6-desaturase (the rate-limiting step in PUFA synthesis) was described in patients with ADHD [Brookes KJ et al., 2006; Schaeffer L, et al.2006] pointing to a possible correlation between membrane fatty acid composition and hyperactivity.

In our study, a significant increase of erythrocyte membrane MUFA and of ω6/ω3 ratio (due to a decrease in EPA and DHA) was shown. These results are partially superimposable to those reported by Bell et al. [Bell JG et al., 2010]. Alteration in membrane lipid composition was not related to dietary habits, since they did not significantly differ between Au and TD, as evidenced by the Food Questionnaire. On the other hand, oxidative stress is not a likely explanation for the specific decrease of the ω3, since this would have also affected the ω6 PUFA family. The observed imbalance in ω6/ω3 ratio may lead to the proinflammatory status reported previously in ASD children [Rose S, et al. 2012], [Vargas DL et al., 2005; Rossignol DA, Frye RE (2012)]. The significant increase in MUFA may be representative of a feedback remodelling of erythrocyte membrane lipid composition. It is interesting to note that a study on adipocyte membranes showed DHA loss coexistent with MUFA increase [Pietiläinen KH et al., 2011]. The data about lipidomc profile were published in 2013 (Ghezzo et al., 2013); very interestingly, the reduction of DHA and the increase of ω6/ω3 ratio was confirmed in the second study.

It has not escaped our notice that the **erythrocyte membrane fluidity decrease** we observed cannot be directly explained on the ground of these alterations in fatty acid composition. Schengrund et al. [Schengrund CL et al., 2012] recently reported a decrease in cholesterol and a

related increase in GM1 ganglioside in erythrocyte membranes from ASD children, which could affect membrane fluidity. However, we failed to observe any change in membrane sialic acid - a component of GM1 ganglioside, in Au patients, but we confirmed the decrease of choleterol in erythrocyte membranes. The reduction of membrane fluidity was not observed in lymphocytes.

Cholesterol is an essential component of cell membranes of mammals. It is a molecule of the class of sterols which plays a significant role in the human physiology; in particular plays an important role in intracellular transport, in cell signaling, and in the maintenance of membrane fluidity, inserted between the two layers phospholipid with -OH groups oriented close to the polar heads of the phospholipids. Because of its structure has hydrophobic characteristics and is thus poorly water soluble. It is present in all tissues and in greater amounts in the brain, in the bile and in the blood. Cholesterol is synthesized mainly in the liver and is excreted in the bile, after transformation into bile acids and then into bile salts. Cholesterol circulates in the blood as free and as esterified with long chain fatty acids.

Has been shown that an altered cholesterol homeostasis can affect neuronal development and synaptogenesis leading to nervous system dysfunction (Pani A et al., 2010; Linetti A et al., 2010). In addition, recent evidence suggests that an imbalance in the homeostasis of cholesterol contributes to the development of a chronic inflammatory state. Alterations of cholesterol are also highlighted in patients with autism. In fact, in a study conducted by Tierney in 2006 was shown a decrease of cholesterol levels in the serum of patients with autism. Moreover, in a previous work conducted by Schengrund (et al., 2011) has been shown a reduction in the levels of cholesterol in erythrocyte membranes of a group of 20 autistic children compared to 20 control children. However, the authors point out that only 19% of children with autism show significantly lower levels of cholesterol than controls. Cholesterol has an important role in maintaining the fluidity of membranes. In our study autistic children showed significantly lower cholesterol levels in erythrocyte memebrane than children with typical development. These data are in line with those reported by Schengrund (Schengrund et al., 2011). The presence of low levels of cholesterol in children with autism may not only affect the fluidity of erythrocyte membranes, but may have a role in the development of the nervous system and may be associated with certain behaviors characteristic of autism. To better understand the possible effect of the altered amount of

cholesterol, we must keep in mind that it contributes to the formation of lipid rafts along with gangliosides. It will therefore be important to evaluate the concentration of these lipids in order to understand whether the structure of the lipid rafts could be altered as far as concern its structure and its funcion. In particular, it is in progress the quantification of sphingomyelin in the membranes of the erythrocytes.

The **S-glutathionylation** is a post-translational modification of proteins which consists in the reversible formation of mixed disulfide bonds between cysteine residues and glutathione (GSH). The glutathionylation is involved in a series of cellular events, such as the stabilization of extracellular proteins, the protection of proteins, the irreversible oxidation of cysteine residues, the regulation of enzyme activity and the response to oxidative stress. These data led us to evaluate, in red blood cells and white blood cells of people with autism and those with typical development, the total protein glutathionylation, as a possible marker of damage to proteins. The data obtained have shown that the glutathionylation of proteins in red blood cells and white blood cells did not show significant differences in between autistic children than in control subjects.

Annexin V is a membrane protein that recognizes **phosphatidylserine**; this is a phospholipid that under physiological conditions is found almost exclusively on the cytoplasmic side of the membrane. However, following a cellular stress, a significant part of phosphatidylserine is exposed on the extracellular side of the cell membrane. This reaction can be monitored with the use of the Annexin protein conjugated to a fluorophore (FITC). We evaluated the positivity of the erythrocytes to Annexin V in basal conditions or in response to osmotic stress in order to compare the distribution of phosphatidylserine in the erythrocyte membrane of autistic children and control. In untreated erythrocytes of autistic children we showed a trend of decrease of Annexin-positive cells when compared to controls. The decrease is evident when the red cells are treated with a hypertonic solution (NaCl 9%) . This result indicates that there may be a decrease in flippase activity and/or a decreased amount of PS itself. Further studies will be needed in order to assess the amount of phosphatidylserine in erythrocyte membranes. Noteworthy, the decrease in PS amount, if confirmed, might account for the highly frequent erythrocyte morphological abnormalities we found.

Na+/K+-ATPase (NKA) maintains intracellular gradients of ions that are essential for cellular activities. Despite the crucial role of NKA in cellular metabolism and the fact that it accounts for

approximately 30% of the total body energy consumption and for 50% brain energy consumption, very little is known about NKA in autism. In a mouse model of Angelman Syndrome, a neurodevelopmental disorder associated with autism, an intrinsic alteration of membrane properties of pyramidal neurons in hippocampal area CA1 has recently been observed [Kaphzan H,et al., 2011]. Alterations were also observed in resting membrane potential, threshold potential, and action potential amplitude correlated with significant increases in the expression of the α1 subunit of Na+/K+-ATPase [Kaphzan H,et al., 2011]. In postmortem tissues from different brain regions of autistic subjects, a specific increase in NKA in the frontal cortex and cerebellum was found. The authors suggested that such increase might be due to compensatory responses to increased intracellular calcium concentration in autism [Ji L, et al. 2009].

As far as concern the **Na+, K+ activity**, we obtained the following results: a) NKA activity is reduced not only in erythrocytes but also in leukocytes from AU children; b) at variance with erythrocytes, leukocyte membrane does not display a decrease in fluidity, a results that points to the lack of relation between the decreased NKA activity and membrane fluidity; c) subunit gene expression does not seem to differ between the two groups of children.

The very significant reduction of erythrocyte NKA in Au compared to TD is in keeping with a similar report by Kurup and Kurup [Kurup RK, Kurup PA (2003)]. There is no overlap between the range values of the two groups, both as regards erythrocyte and lymphocyte NAK activity, suggesting that this parameter might be a biomarker of autism. Future work should be addressed at understanding how sensitive and specific is the decrease of NKA as far as autism is concerned. A number of other factors may affect NKA; for example, a positive correlation between the molecular activity of Na+/K+-ATPase units and the membrane content of DHA has been shown [Turner N et al., 2003] and a reduction of NKA has also been related to oxidative stress [Rodrigo R et al., 2007], [Vignini A et al. 2009]. Changes in ATPase activities might stem from sub-conformational changes in the enzymes depending on their microenvironment, indirectly reflecting changes in surrounding lipids and in membrane fluidity [Kamboj SS, et al. 2009]. In summary, although the study awaits to be completed with new antibodies and a quantitative decrease of one or more protein subunits has not yet ruled out, two hypotheses are in our opinion the most likely: either the functional alteration of the enzyme due to the oxidation of one or more subunits or the presence of plasmatic inhibitors, such as metals and/or endogenous ouabain.

Noteworthy, **some clinical features were correlated with some** parameters of the lipidomic profile. In our study, **hyperactivity** is the clinical aspect found to be most highly related to erythrocyte membrane features. The higher the fluidity of the erythrocyte membrane and the lower the PUFA concentration, the greater was the hyperactivity level. Also, the severity of hyperactivity was directly and highly correlated with erythrocyte SFA and palmitic acid concentration. These data not only suggest that such disequilibrium in membrane fatty acid composition may be a useful tool to assess the severity of the autistic clinical picture, but also suggest possible therapeutic interventions with a tailored and balanced fatty acid intake. Two distinct double blind trials showed an improvement in hyperactivity score in autistic children treated with ω3 supplementation [Amminger GP, et al., 2007; Bent S et al., 2011]. Despite these encouraging results, a Cochrane meta-analysis stated that "to date there is no high quality evidence that omega-3 fatty acids supplementation is effective for improving core and associated symptoms of ASD" [James S et al., 2011]. Nevertheless, our data clearly show an imbalance of membrane fatty acids and their correlation with relevant clinical features, thus pointing to the importance of restoring the membrane equilibrium. However, the intake of ω3 should be accompanied by antioxidant protection. For example, since our data also show the alteration of the redox balance of Au, supplementation of PUFA in the absence of antioxidant protection might paradoxically worsen the picture, as, in oxidative milieu, PUFA undergo a peroxidation process and may become, in turn, pro-oxidant. Also, omega-6/omega-3 balance might modulate neurotransmitters of the central nervous system: increased omega-3 fatty acid concentrations in cell membranes have been shown to affect serotonin and dopamine neurotransmission, especially in the prefrontal cortex [Chalon S (2006)]. Taking into account that serotoninergic and dopaminergic systems are deeply involved in ASD [Pardo CA, Eberhart CG (2007)], [Farook MF et al. 2012], cell membrane lipid profile restoration could play a significant therapeutic role in improving some ASD features. In Au children, levels of Peroxiredoxin I (that are not statistically different in comparison to those of TD group) seems inversely related to the severity of clinical features: the hypothesis is that a lower availability of endogenous antioxidant protection may promote the oxidative stress in conditions of alterated oxidative balance, resulting in cellular damage.

Through a convention with the **University of Warwick, the analysis of glycation and of nitrosylation** of plasma and urine proteins has been carried out. Although the analyses have been

completed, data are still being elaborated. These evaluations have never been carried out in autistic subjects.

Since a number of trace elements may affect several enzyme functions, when present in concentration above the normal range, we also sent aliquots of plasma and urine to the **Istituto Zoo-profilattico della Lombardia e dell'Emilia Romagna (IZSLER)** in order to obtain the quantitative analysis of the following **metals:** As, Bi, Ca, Cd, Cr, Cu, Fe, Mg, K, Mg, Mn, Ni, Pb, Se, Na, Sb, Sn, Tl, Cs, V, Li, Zn, U, Al, Hg. In particular, our attention has been attracted by the fact that the Vanadium salt orthovanadate is both an inhibitor of NKA and of Band 3 dephosphorylation. Therefore, should the quantitative analysis of trace elements reveal a significant increase of Vanadium, a link between morphological alterations of erythrocytes, NKA activity reduction and oxidative stress would be provided.

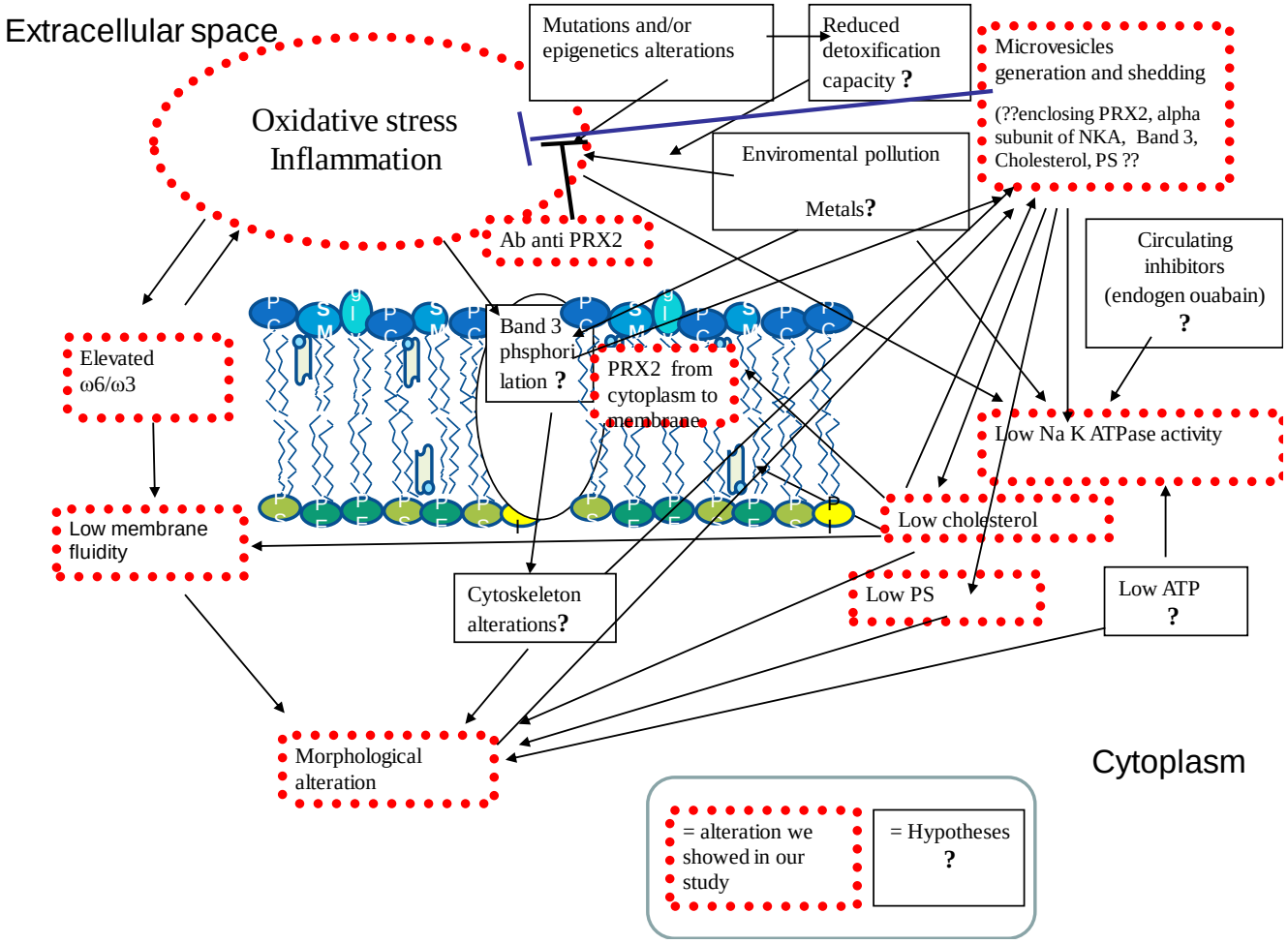
We are planning to study the transmembrane protein Band 3, a highly expressed anion exchanger, which plays a crucial role in cytoskeletal organization. When modified by oxidation, it is extensively phosphorylated by Lyn and Syk Tyr-kinases. This causes it to release cytoskeletal components and favors its clustering and its release within microvescicles (Ferru E et al., 2011).

The figures below summarize the main hypotheses emerged in our study

3.5 Conclusions and future perspective

In summary, our results support the notion that a systemic oxidative stress is present in AU patients, that alterations in functional, structural and morphological features of erythrocyte may play a role in the pathogenesis of Autism, that some of the parameter we have studied (such as erythrocyte and lymphocyte Na+ K+ ATPase activity) are strongly candidates for peripheral biomarkers of ASD, to be exploited for a more precise or an earlier diagnosis of ASD.

Our research has now many future directions: a) to collect the latest data that we lack on possible environmental factors (pollutants, metals), b) to understand the reason(s) for the impairment of the NKA, c) to deepen the study of the components of the red cell membrane and cytoskeleton, d) to test the sensitivity and specificity of altered biochemical parameters in order to use them as peripheral biomarkers, e) to identify an evidence-based pharmacological/nutraceutical intervention in the light of what has emerged from this study.


Extracellular space
Mutations and/or epigenetics alterations
Reduced detoxification capacity ?
Microvesicles generation and shedding
(??enclosing PRX2, alpha subunit of NKA, Band 3, Cholesterol, PS ??
Oxidative stress Inflammation
Enviromental pollution
Metals?
Ab anti PRX2
Circulating inhibitors (endogen ouabain) ?
Band 3 phsphori lation ?
PRX2 from cytoplasm to membrane
Elevated ω6/ω3
Low Na K ATPase activity
Low membrane fluidity
Low cholesterol
Cytoskeleton alterations?
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Morphological alteration
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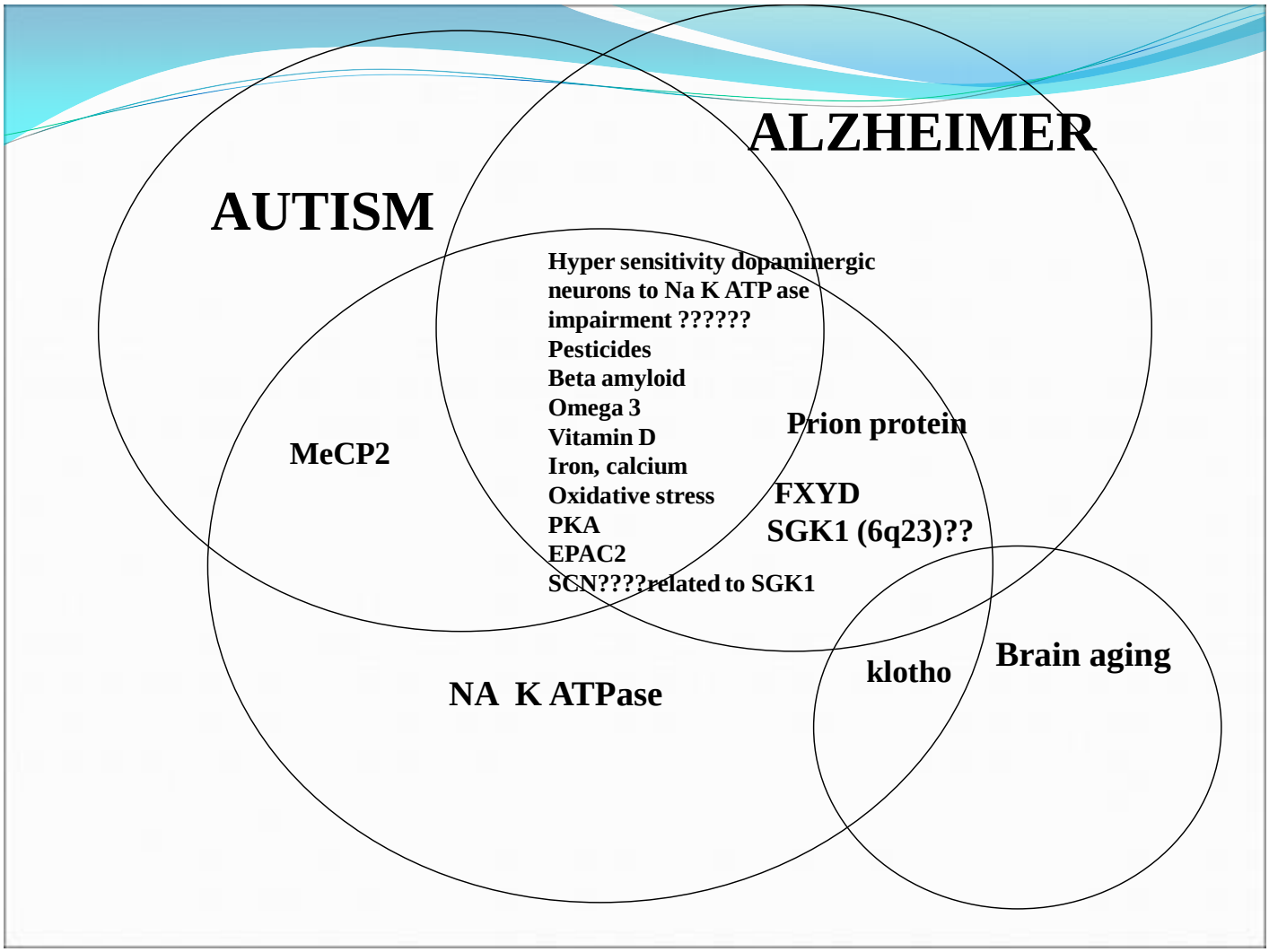
= Hypotheses ?


ALZHEIMER
AUTISM
Hyper sensitivity dopaminergic
neurons to Na K ATP ase
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Pesticides
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Omega 3
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Oxidative stress
PKA
EPAC2
SCN????related to SGK1
Prion protein
FXYD
SGK1 (6q23)??
MeCP2
klotho
Brain aging
NA K ATPase

Acknowledgments

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A special thanks to all the people who have been with me in my training. In order...of appearance: my parents and my brothers, my friends, my family (expecially my son Enrico and and his classmates, who took part in this research).

Now I have a white beard and I look back with great nostalgia when I graduated, many...many...many years ago: without the aid provided my brother Roberto I would not have ever made! Thanks Robi!

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Supplementary material

Table 1S Erythrocyte membrane features and molecules, oxidative stress markers in urine and plasma, oxidative stress-related markers in erythrocytes, First study (form Ghezzo A et al. 2013)

	Mean values (± St. Dev)		**p (p calculated)**	**P (FDR)**
	Au (N=21)	**TD (N=20)**		
Erythrocyte membrane features and molecules				
Na+/K+ATPasi µgPi/mg prt/h*	2.54 (± 0.58)	7.39 (± 1.62)	<.0001 (<0.0001)	<.0001
TMA-DPH*	0.27 (± 0.02)	0.25 (± 0.03)	<0.05 (0.0123)	0.0368
DPH*	0.27 (± 0.02)	0.25 (± 0.03)	<0.05 (0.0196)	0.0469
TBARS*	0.72 (± 0.38)	0.51 (± 0.37)	<0.01 (0.0021)	0.0125
Sialic Acid*	6.19 (±4.36)	7.63 (±7.08)	NS (0.7248)	0.7248
Oxidative stress markers in urine				
Isoprostane ng/mg creat.*	3.04 (±1.50)	2.07 (±0.54)	<0.01 (0.0069)	0.0278
Hexanoyl-lysine adduct°	0.16 (±0.09)	0.11 (±0.05)	<0.05 (0.0380)	0.0760
8-Oxo-guanosine°	484.80 (±130.07)	426.46 (±163.64)	NS (0.2127)	0.365
Oxidative stress markers in plasma				
Carbonyl Groups°	0.30 (±0.08)	0.27 (±0.11)	NS (0.2509)	0.3763
Radical Absorbance Capacity*	2.47 (±0.86)	2.36 (±0.94)	NS (0.4573)	0.5487
Oxidative stress-related markers in erythrocytes				
SOD activity* (Au N=12)	26.10 (±4.02)	25.49 (±3.78)	NS (0.2960)	0.3947
Catalase activity° (Au N=12)	29.28 (±6.34)	31.25 (±11.06)	NS (0.5783)	0.6309

*Non parametric Wicoxon-Mann-Whithey test applied

° Parametric ANOVA test applied

Table 2S Erythrocyte membrane Fatty Acid profile (From Ghezzo A. et al. 2013)

Test	AD (n: 21)	TD (n: 20)	P	False Discovery Rate
	Means values ± SD	Means values ± SD		p-value
DHA omega 3 (22:6)*	4.8 ± 1.08	5.61 ± 0.67	**0.0065**	0.07220
Total Monounsaturated Fatty Acid (MUFA)*	18.03 ± 1.25	17.03 ± 0.98	**0.0076**	0.07220
Vaccenic acid (18:1)°	1.3 ± 0.16	1.19 ± 0.11	**0.0220**	0.08297
Oleic acid (18:1)*	16.42 ± 1.25	15.6 ± 0.94	**0.0228**	0.08297
SFA / MUFA°	2.38 ± 0.24	2.5 ± 0.19	**0.0232**	0.08297
Palmitoleic acid (16:1)°	0.3 ± 0.08	0.24 ± 0.09	**0.0262**	0.08297
EPA omega 3 (20:5)°	0.43 ± 0.15	0.51 ± 0.14	**0.0434**	0.11780
omega6 /omega3°	6.66 ± 1.62	5.76 ± 0.67	0.0600	0.14250
Total Polyunsaturated Fatty (PUFA)°	39.39 ± 1.8	40.18 ± 1.74	0.1173	0.24763
Trans 18:1°	0.11 ± 0.05	0.14 ± 0.07	0.1863	0.35397
Stearic acid (18:0)*	18.58 ± 1.04	18.78 ± 0.88	0.2709	0.46792
Eicosatrienoic acid omega 6 (20:3)°	2.25 ± 0.45	2.13 ± 0.34	0.3714	0.58805
Total Saturated Fatty Acid (SFA)*	0.66 ± 0.07	0.67± 0.08	0.6012	0.76738
Linoleic omega 6 (18:2)°	0.23 ± 0.08	0.23 ± 0.06	0.6012	0.76738
Total TRANS°	0.12 ± 0.06	0.11 ± 0.03	0.6180	0.76738
Trans-ARA°	42.31 ± 1.96	42.6 ± 1.53	0.6756	0.76738
Arachidonic acid omega 6 (20:4)*	19.57 ± 1.67	19.38 ± 1.13	0.6866	0.76738
EFA deficiency°	23.73 ± 1.94	23.81 ± 1.48	0.7961	0.84033
Palmitic acid (16:0)*	12.2 ± 0.96	12.66 ± 1.34	0.8810	0.88100

° Wilcoxon – Mann - Whitney test

*t Student test