

## Increased Seroprevalence of Chronic Toxoplasmosis in Autistic Children: Special Reference to the Pathophysiology of IFN- $\gamma$ and NO Overproduction

Joseph Prandota, Noha Abdel Fattah Elleboudy, Khadiga Ahmed Ismail, Osama Kamal Zaki, Hanan Hussein Shehata

Joseph Prandota, Faculty of Health Sciences, Wroclaw Medical University, Wroclaw, Poland

Noha Abdel Fattah Elleboudy, Khadiga Ahmed Ismail, Department of Parasitology, Ain-Shams University, Cairo, Egypt

Khadiga Ahmed Ismail, Faculty of Applied Sciences, Taif University, Turbah, Saudi Arabia

Osama Kamal Zaki, Medical Genetics Unit, Ain-Shams University, Cairo, Egypt

Hanan Hussein Shehata, Department of Biochemistry, Faculty of Medicine, Ain-Shams University, Cairo, Egypt

Correspondence to: Joseph Prandota, Faculty of Health Sciences, Wroclaw Medical University, Wroclaw, Poland.

Email: [jozef.prandota@umed.wroc.pl](mailto:jozef.prandota@umed.wroc.pl)

Telephone: +48-713484210

Fax: + 48-713432086

Received: June 9, 2015

Revised: July 13, 2015

Accepted: July 16, 2015

Published online: September 1, 2015

### ABSTRACT

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental disorders with a significant rate of increase over the last years. *T. gondii* is a ubiquitous intracellular pathogen affecting approximately 30-50% of human population, with special preference to the central nervous system (CNS). Accumulating evidence suggest that latent chronic toxoplasmosis play a role in triggering and development of many psychiatric and neurological disorders, but so far there is no clear epidemiological link with its prevalence in ASD. The aim of the study was therefore to estimate the seroprevalence of chronic toxoplasmosis among autistic children, determine the changes in serum levels of IFN- $\gamma$  and nitric oxide (NO) in *T. gondii*-positive and *T. gondii*-free patients, and evaluate the combined effect of both diseases on the Childhood Autism Rating

Scale (CARS) score. Forty-six children with ASD (mean age  $6.1 \pm 2.2$  years; 41 boys, 5 girls) were studied for anti-*T. gondii* IgG antibody seropositivity (ELISA kit, DRG Int), and their serum IFN- $\gamma$  (ELISA kit, Ray Biotech) and NO (one-step enzymatic assay) concentrations were measured. Chronic toxoplasmosis was found in 23.9% of the studied patients (8 males, 3 females), while among 50 age-matched control children (45 boys, 5 girls) 4% (2 boys) were *T. gondii*-positive ( $\chi^2 = 8.11$ ;  $p < 0.0043$ ). Autistic children with toxoplasmosis had markedly increased both serum IFN- $\gamma$  and NO levels (11- and 5-fold median peak increases, respectively) compared with *T. gondii*-free participants. A statistically significant positive correlation was found between the serum IFN- $\gamma$  and NO levels ( $r = 0.79$ ,  $p < 0.001$ ). These molecular disturbances may exert harmful effects on further development of the CNS in the infected individuals, although no marked difference in the CARS score was detected, probably because of young age and immune function maturation of the participants. The obtained results strongly suggest that latent chronic *T. gondii* infection have an important impact on triggering and development of ASD, at least in a subset of autistic children, and this requires some modification(s) of its diagnostic procedures and treatment regimens.

© 2015 ACT. All rights reserved.

**Key words:** Autism spectrum disorders; autistic children; *T. gondii* infection; chronic toxoplasmosis; seroprevalence IgG; interferon-gamma; nitric oxide; oxidative stress

Prandota J, Elleboudy NAF, Ismail KA, Zaki OK, Shehata HH. Increased Seroprevalence of Chronic Toxoplasmosis in Autistic Children: Special Reference to the Pathophysiology of IFN- $\gamma$  and NO Overproduction. *International Journal of Neurology Research* 2015; 1(3): 102-122 Available from: URL: <http://www.ghrnet.org/index.php/ijnr/article/view/1241>

## INTRODUCTION

### ASD

This clinical entity is a complex multifactorial neurodevelopmental disorder with genetic, immunological, and environmental contributions<sup>[1]</sup>. It is characterized by reduced sociability, abnormalities in verbal and nonverbal communication, restricted stereotyped interests and repetitive behaviors<sup>[2]</sup>. Manifestations onset starts in the first 3 yrs of life<sup>[3]</sup>. The prevalence of ASD among children aged 8 years has risen in the U.S. dramatically by approximately 78% during 2002-2008, and for 2010, the overall prevalence of ASD among The Autism and Developmental Disabilities Monitoring Network sites was 14.7 per 1000 children (one in 68)<sup>[4-6]</sup>. Approximately one in 42 boys and one in 189 girls were found to have this disorder, and the median age of earliest ASD diagnosis was 53 months<sup>[5]</sup>. Recently, Blumberg *et al*<sup>[7]</sup> suggested that much of the prevalence increase from 2007 to 2011-2012 for school-age children was the result of diagnoses of children with previously unrecognized ASD. In the UK, prevalence of parent-reported data collected in 2008-2009 for 14,043 children was even higher compared to earlier UK and U.S. estimates and showed that 1.7% of children were reported as having ASD (95 % CI 1.4-2.0) at mean age 7.2 yrs (SD = 0.2; range = 6.3-8.2 yrs)<sup>[7a]</sup>. A statistically positive association found between autism prevalence and childhood vaccination uptake across the U.S. population seems to play an important role because the higher the proportion of children receiving recommended vaccinations, the higher was the prevalence of autism or speech and language disorders (a 1% increase in vaccination was associated with an additional 680 children having these abnormalities)<sup>[8]</sup>. Moreover, a highly significant correlation was demonstrated between the increasing number of vaccine doses and increasing infant mortality rates ( $r = 0.992$ ;  $P < 0.0009$ ), especially that the childhood immunization schedule in the USA specified 26 vaccine doses for infants during the first year of life (most in the world)<sup>[9]</sup>.

Despite decades of intensive research, the pathogenesis of autism still remains unknown. Several studies proposed infectious, cytokine, and autoimmune-related etiologies. It was suggested that chronic viral and bacterial infections and immunological abnormalities associated with ASD may contribute to the manifestations and severity of the disease<sup>[10]</sup>. A number of environmental factors and associated clinical abnormalities, as well as immune irregularities have been linked to pathophysiology of ASD and neuroglial activation/neuroinflammation in the brain of patients with autism<sup>[6,11-13]</sup>. Markedly lower subpopulation of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes together with imbalance between Th1 and Th2 type cytokines skewing more towards Th2 arm have been demonstrated in children with ASD<sup>[6,14,15]</sup>. The serum immunoglobulin levels were also found to be disturbed because total protein was significantly increased in autistic children, including higher albumin and gamma globulin concentrations, as well as increased serum IgG, IgG2 and IgG4 levels that probably was associated with an enhanced susceptibility to infections<sup>[16]</sup>. Both postmortem and neuroimaging investigations in patients with ASD showed abnormalities in various brain regions including the frontal cortex, cerebellum, hippocampus, the amygdaloid nucleus and cerebello-thalamo-cortical pathways<sup>[17]</sup>.

Accumulating evidence indicates that in schizophrenia and autism, inflammatory cytokines including IL-1 $\beta$  and IL-6 have a role in onset and progress of neuropsychiatric symptoms probably via perinatal inflammation<sup>[18-20]</sup>. These findings may be in line with the enhancement of intracellular replication of *T. gondii* by IL-6 and

reversal of IFN- $\gamma$ -mediated toxoplasmicidal activity, as well as with the markedly higher prevalence of anti-*Toxoplasma* antibodies in patients with autoimmune diseases<sup>[21-24]</sup>. It appeared that antibody against IL-6 reduced inflammation and numbers of cysts in the brains of mice with toxoplasmic encephalitis, while the antibody directed against IFN- $\gamma$  had an opposite effect<sup>[22]</sup>. In turn, Shapira *et al*<sup>[24]</sup> demonstrated that serum anti-*T. gondii* IgG antibodies were detected in 42% (637/1514) of patients with autoimmune diseases versus 29% (127/437) of controls ( $P < 0.0001$ ). Potential pathomechanisms responsible for development of several neuropsychiatric diseases and changes in behavior also include an important role of tryptophan metabolism and its metabolites, such as for example melatonin<sup>[25]</sup>, and hypothalamic-pituitary-adrenal axis function, both known to be affected during chronic *T. gondii* infection<sup>[26]</sup>.

### *T. gondii*

This intracellular pathogen believed to be a global threat<sup>[27-30]</sup>, chronically infects approximately 30-50% of the human population, and ophthalmoimmunologists<sup>[31]</sup> suggested that even some 6 billion people are chronically infected with the parasite. Unrecognized ingestion of *T. gondii* oocysts leads to congenital toxoplasmosis and causes epidemics in North America<sup>[32]</sup>. Recent study by Flegr *et al*<sup>[30]</sup> showed that the seroprevalence of toxoplasmosis correlated with various specific disease burden and therefore may be a neglected triggering factor responsible for development of several clinical entities. Extensive *T. gondii* host/pathogen interactome enrichment involving approximately 3000 host genes or proteins was found in nine psychiatric or neurological disturbances<sup>[10]</sup>, and it was demonstrated that the protozoan ingested and digested host cytosolic proteins using cathepsin L and other proteases within its endolysosomal system<sup>[33]</sup>.

Evans *et al*<sup>[29]</sup> found that in rats *T. gondii* cysts were randomly distributed throughout the 53 analyzed forebrain regions with individual variation in cysts localization, beginning 3 weeks post-infection. This can explain individual differences in the effects of the parasite on behavior of the animals, especially that the immune response to cysts was striking. In the brain of mice, persistent infection with the parasite caused ventricular dilatation, inflammation, neuronal injury, and altered cellular functions associated with neurological and behavioral abnormalities<sup>[34,35]</sup>. Localization of the parasite cysts in different regions of murine brain and brain cells along with time and the number of cysts after inoculation were presented in Tables 1-4<sup>[36-38]</sup>. The development of behavioral changes was paralleled by the preferential persistence of cysts in defined anatomic structures of the brain, depending on the host, strain of the pathogen, its virulence, and route of inoculation<sup>[39-49]</sup>.

*T. gondii* tachyzoites may invade different type of brain cells including neurons, astrocytes, microglial cells, and Purkinje cells in cerebellum. Intracellular tachyzoites manipulate signaling pathways and several signs for transduction mechanisms involved in apoptosis, immune cell maturation, and antimicrobial effector functions<sup>[50]</sup>. Wang *et al*<sup>[51]</sup> found that the parasite induced apoptosis of neural stem cells via endoplasmic reticulum stress pathway. It was demonstrated that in neurons infected by *T. gondii* not only parasitic cysts but also the host cell cytoplasm and some axons were stained positive for the parasite antigens, thus supporting the notion that it may interfere with neuronal function<sup>[39,49]</sup>.

Among infections, toxoplasmosis has had so far no clear epidemiological link with its prevalence among autistic children established except for the few recent reviews suggesting that the manifestations, biochemical disturbances and brain morphological

findings in autism are associated with *T. gondii* infection<sup>[52-54]</sup>. The aim of study was therefore to determine the seroprevalence of chronic toxoplasmosis in children with ASD admitted to the Medical Genetics Unit, Pediatric Hospital, Ain Shams University Hospital, and to estimate its effect on changes in serum IFN- $\gamma$  and NO levels, and the Childhood Autism Rating Scale (CARS) values.

**Table 1** Preferential localization of *T. gondii* ME49 strain cysts in different regions of murine brain at 2 and 6 months post inoculation (acc. to Melzer *et al.*<sup>[36]</sup>; with own modification)

Brain region	Two months after inoculation <sup>1</sup>	Six months after inoculation <sup>2</sup>
Cerebral cortex	34	57
Hippocampus	10	25
Thalamus	5	-
Hypothalamus	6	3
Amygdala	25	9
Caudate putamen	12	6
Cerebellum	8	-

<sup>1</sup>Total number of cysts observed=67; <sup>2</sup>Total number of cysts observed=32.

**Table 2** The number of cysts load in brain tissue and congenital transmission rate from the offspring of mice (acc. to Wang *et al.*<sup>[37]</sup>; with own modification).

Group of mice	Number of cysts in brain	Congenital <i>T. gondii</i> transmission rate (%)
Earlier stage infection	224 $\pm$ 59 <sup>1,2</sup> (n = 18)	94.74
Intermediate stage infection	202 $\pm$ 44 <sup>1</sup> (n = 19)	90.48
Late stage infection	134 $\pm$ 31 (n = 22)	91.67

Each group of mice was infected with 5 cysts of *T. gondii* by oral inoculation on the 5<sup>th</sup>, 10, and 15<sup>th</sup> day after gestation. Results are expressed as the mean number of cysts collected from each group  $\pm$  SD, generated by using one-way ANOVA: <sup>1</sup>*p* < 0.01 *vs* late stage infection and <sup>2</sup>*p* > 0.05 *vs* intermediate stage infection group. The congenital *T. gondii* transmission rate is shown at the age of 12 weeks after birth in the offspring from the infected group.

**Table 3** Infection rates of different cell types from embryonal rat cortices (E15) after in vitro infection with *T. gondii* tachyzoites (acc. to Lüder *et al.*<sup>[38]</sup>; with own modification).

Cell type	mAb for host cell identification	Frequency of cell type <sup>1</sup>	Rate of <i>T. gondii</i> infection <sup>1</sup>
Neurons	Anti-Nf 200 kDa	88.0 $\pm$ 1.3%	9.5 $\pm$ 1.1%
Astrocytes	Anti-GFAP	7.9 $\pm$ 2.1%	9.7 $\pm$ 3.3%
Microglia	Anti-CD71	4.1 $\pm$ 0.9%	31.5 $\pm$ 5.9%

<sup>1</sup>Determined 48 hrs post infection by double immunofluorescence (at least 100 parasitophorous vacuoles were examined for each determination). Data represent mean  $\pm$  SD from three independent experiments. mAb, monoclonal antibody. GFAP, glial filament acidic protei.

**Table 4** Replication and morphology of *T. gondii* in different cell types from embryonal rat cortices (E15) (acc. to Lüder *et al.*<sup>[38]</sup>; with own modification).

Intracellular replication (% PV) <sup>1</sup>	Neurons	Astrocytes	Microglia
1-2 parasites/PV	66 $\pm$ 2.6	67.2 $\pm$ 5.0	93.1 $\pm$ 4.5
4-8 parasites/PV	30 $\pm$ 2	26.2 $\pm$ 1.2	8.9 $\pm$ 4.5
16-32 parasites/PV	4.3 $\pm$ 1.5	5.3 $\pm$ 2.9	0
> 32 parasites/PV	0	1.3 $\pm$ 1.2	0
Morphology of <i>T. gondii</i>	Normal	Normal	Often degenerated

<sup>1</sup>Determined 48 hrs post infection by double immunofluorescence (at least 100 PV were examined for each determination). Data represent mean  $\pm$  SD from three independent experiments. PV, parasitophorous vacuole.

## PATIENTS AND METHODS

The present study was performed in 46 autistic children who were admitted to the Medical Genetics Unit, Pediatric Hospital, Ain Shams

University Hospital, Cairo, Egypt, from January 2010 to January 2013. The diagnosis of autism was established by CARS and 50 age-matched healthy children were included as the control group. This investigation has been carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study was approved by the Research Ethics Committee, Faculty of Medicine, Ain Shams University, and informed consents were obtained from parents of all patients.

Five milliliters of blood were taken under sterile conditions after overnight fasting from all participants. The sera were then separated and stored at -20°C until the analysis for anti-*T. gondii* IgG antibodies and serum INF- $\gamma$  and NO levels determination.

### Estimation of serum anti-*T. gondii* IgG antibodies

All serum samples were tested for IgG anti-*T. gondii* antibodies using commercially available enzyme linked immunosorbent assay (ELISA) kit (DRG International, Inc., USA). All reagents and controls were supplied by the manufacturer. Serum samples, negative control, positive control and calibrators were diluted (1:40). Then, 100  $\mu$ l of diluted sera, controls and calibrators were dispensed into the appropriated wells of microtiter plate coated with purified *T. gondii* antigen and incubated at 37°C for 30 min. Horseradish peroxidase-conjugated antibody (100  $\mu$ l) was then added to react with the bound antibody. Substrate (100  $\mu$ l) for peroxidase was added and the reaction was stopped by stop solution (100  $\mu$ l). The intensity of the color was measured at 450 nm in an ELISA reader. The mean of duplicated cut-off calibrator value (32 IU/mL), positive control, negative control and serum samples were calculated. *T. gondii* index of each determination was calculated by dividing the mean value of each sample by the calibrator mean value. A sample was considered positive for IgG when a *T. gondii* index was equal or greater than 1.0 (> 32 IU/mL). A negative reaction corresponded to *T. gondii* index less than 0.90 ( $\leq$  32 IU/mL), a positive reaction to *T. gondii* index of 1.00 or greater (> 32 IU/mL), and an equivocal result corresponded to *T. gondii* index between 0.91-0.99.

### Detection of serum INF- $\gamma$ levels

All serum samples were tested for serum INF- $\gamma$  level using commercially available enzyme linked immunosorbent assay (ELISA) kit Bio (Cat#: ELH-IFN gamma-001) Ray Biotech, Inc. Serum samples and standards were diluted, then 100  $\mu$ l of diluted sera and standards were dispensed into the appropriated wells of microtiter plate and incubated at room temperature for 2.5 hrs or overnight. Biotin antibody (100  $\mu$ l) was then added to each well incubated at room temperature for 1 h. Streptavidin solution (100  $\mu$ l) was next added to each well and incubated at room temperature for 45 min. 100  $\mu$ l of TMB one-step substrate reagent was then added to each well incubated for 30 min, and 50  $\mu$ l of stop solution were added to finish the reaction. The intensity of the color was measured at 450 nm immediately in an ELISA reader, and the concentration was calculated on the standard curve (in pg/mL).

### Determination of serum NO levels

Serum nitrate concentration as a stable end-product of nitric oxide was measured by an endpoint one-step enzymatic assay using nitrate reductase as described by Bories & Bories<sup>[55]</sup>. The concomitant reduction of nitrate to nitrite by NADPH was monitored by the oxidation of the coenzyme and the decrease in absorbance at 340 nm. The NO concentrations were measured in  $\mu$ M. The method was linear from 5 to 200  $\mu$ M nitrate in serum. The median (range) concentration

in serum of 20 healthy individuals was 16 (0-42)  $\mu\text{M}$ <sup>[55]</sup>.

### Study of the CARS

The CARS is a widely used test for screening and diagnosis of autism. The scale consists of 15 items assessing the severity of behaviors associated with autism. Total scores can range from 15 to 60 according to the severity. The items in the scale include: relationship to people, imitation, emotional response, body use, object use, adaptation to change, visual response, listening response, perceptive response and usage, fear or anxiety, verbal communication, non-verbal communication, activity level, consistency and level of intellectual response, and finally the general impressions<sup>[56]</sup>.

### Statistical analysis

The obtained data were coded, tabulated and introduced to a PC using the Statistical Package for Social Science (SPSS) for Windows version 17. The Chi-square was used to analyze the frequency of anti-*T. gondii* IgG antibodies in the study groups. The serum IFN- $\gamma$  and NO levels in the studied patients have been expressed as a nonparametric variable and were compared between the groups using the Mann-Whitney test to establish statistically significant differences at  $p < 0.05$  or less. Results were presented as means  $\pm$  SD.

## RESULTS

Our study demonstrated that 11 (23.9%) out of 46 autistic children (41 males, 5 females) were positive for serum anti-*T. gondii* IgG antibodies as compared with 2 patients (4%) with toxoplasmosis found among 50 control children ( $\chi^2 = 8.1143$ ;  $p < 0.004392$ ). The *T. gondii* positive group of autistic patients was significantly older than the seronegative group (mean age 7.56 vs 4.69 yrs, respectively;  $P < 0.05$ ) (Table 5), and among toxoplasmosis positive participants there were 8 (73%) boys and 3 (27%) girls (Table 6).

Autistic children with toxoplasmosis had markedly increased both serum IFN- $\gamma$  and NO concentrations (11- and 5-fold increases, respectively) as compared with the values obtained in patients without toxoplasmosis, and a highly statistically significant positive correlation was found between serum IFN- $\gamma$  and NO levels ( $r = 0.79$ ,  $p < 0.001$ ) (Tables 7 and 8; Figure 1).

The CARS scores showed no marked difference between the autistic children with and without toxoplasmosis (Table 9).

## DISCUSSION

The study for the first time showed that 23.9% of the studied autistic children had chronic toxoplasmosis compared with 4% of the age-matched controls ( $P < 0.00439$ ). This finding strongly support recent suggestions of Prandota<sup>[52-54]</sup> that *T. gondii* play an important role in the pathogenesis and clinical course of autism spectrum disorders, at least in a subset of individuals with this clinical entity.

### Increased serum IFN- $\gamma$ levels in autistic children with toxoplasmosis

*T. gondii*-positive autistic children had significantly increased serum IFN- $\gamma$  concentrations as compared with *Toxoplasma*-free patients (Table 5). IFN- $\gamma$ , a classic T cell cytokine produced also in the brain, influences more than 200 genes<sup>[56a]</sup>, and its overproduction play an important role in development of neurodegeneration<sup>[56b,56c]</sup>.

**ASD.** Children with autism have peripheral and central inflammation<sup>[2]</sup>. Neuroglial activation and neuroinflammation processes<sup>[12]</sup> with significantly increased plasma proinflammatory

**Table 5** Age of autistic children with and without toxoplasmosis.

Study group	Age (yrs)	T-test	P value
<i>T. gondii</i> (+)	7.56 $\pm$ 2.24	3.3	< 0.05
<i>T. gondii</i> -negative	4.69 $\pm$ 2.19		

Results are means  $\pm$  SD. Statistically significant difference at  $p < 0.05$ .

**Table 6** Sex distribution in autistic children with and without toxoplasmosis.

Sex	Study group		Total	$\chi^2$	P value
	<i>T. gondii</i> (+)	<i>T. gondii</i> negative			
Boys	No of pts	8	33	41	
	%	19.5	80.5		
Girls	No of pts	3	2	5	4 < 0.05
	%	60	40		
Total	No of pts	11	35	46	
	%	23.9	76.1		

**Table 7** Serum IFN- $\gamma$  concentrations in autistic children with and without toxoplasmosis.

Study group	Serum IFN- $\gamma$ levels (pg/mL)	Mann-Whitney test	Asymptomatic significance (2- tailed)	95% CI <sup>1</sup>
Toxo(+)	Median			
	Percentiles			
	25	2135.65		
	75	2375.96		
Toxo-negative	Median	194.36	8.000	0.000 <sup>2</sup> HS L .000
	Percentiles			U .030
	25	158.44		
	50	291.86		

<sup>1</sup> CI, Confidence Interval. <sup>2</sup> HS, highly significant difference between the groups. L and U, lower and upper limits of significant values.

**Table 8** Serum NO concentrations in autistic children with and without toxoplasmosis.

Study group	Serum NO levels ( $\mu\text{M}$ )	Mann-Whitney test	Asymptomatic significance (2- tailed)	95% CI <sup>1</sup>
Toxo(+)	Median			
	Percentiles			
	25	33.83		
	75	57.10		
Toxo-negative	Median	8.20	10.000	0.000 <sup>2</sup> HS L .000
	Percentiles			U .030
	25	6.83		
	50	10.08		

<sup>1</sup> CI, Confidence Interval. <sup>2</sup> HS, highly significant difference between the groups. L and U, lower and upper limits of significant values.

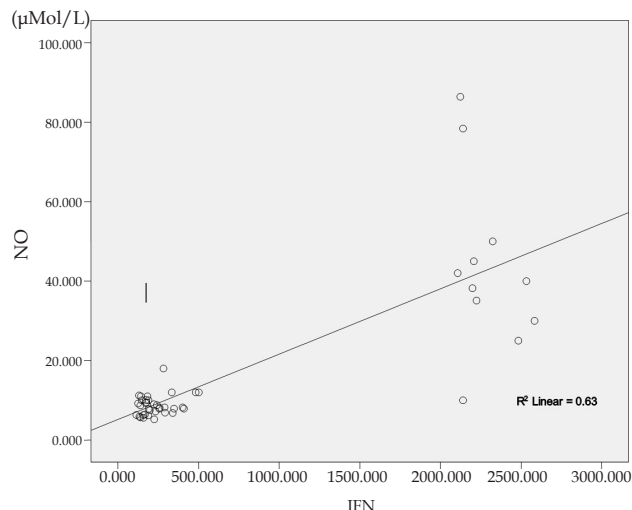
**Table 9** CARS score in autistic children with and without toxoplasmosis.

Study group	CARS score (mean $\pm$ SD)	T-test value	P value
<i>T. gondii</i> (+)	32.90 $\pm$ 7.02	1.02	> 0.05
<i>T. gondii</i> -negative	30.69 $\pm$ 4.36		

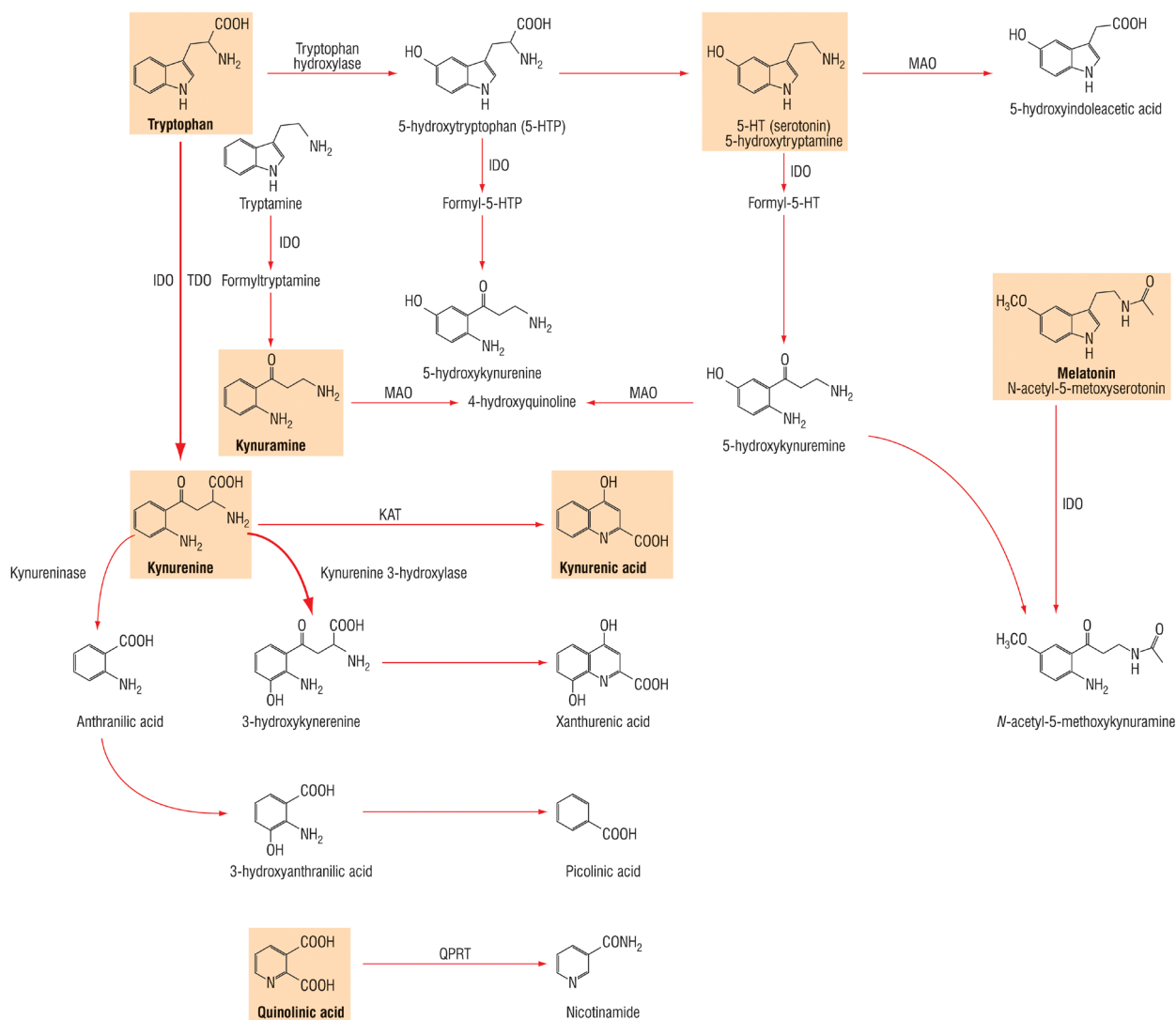
and anti-inflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , IL-1RA, IL-5, IL-6, IL-8, IL-12(p40), IL-12(p70), IL-13, IL-17, GRO-a) and evidence of altered T cell responses (in 2-5 yrs old children with autism, following phytohemagglutinin (PHA) stimulation of peripheral mononuclear cells, the frequency of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells were markedly reduced), disturbed innate and adaptive immune responses in the brain, cerebrospinal fluid, and other tissues, have been reported<sup>[3,13,57-63]</sup>. Natural killer (NK) cells function also was found to be decreased<sup>[64]</sup>. Enhanced generation of proinflammatory cytokines was associated with impaired behavioral outcome, while production of Th2 type of interleukins resulted in better cognitive and adaptive function<sup>[63]</sup>.



Potential roles for altered tryptophan metabolism and its metabolites such as serotonin (increased in the brain, blood, serum, platelets, and urine of autistic patients), melatonin (decreased in serum), kynurenine pathway metabolites, in the etiology of autism and schizophrenia, also have been taken into consideration<sup>[25,65-74]</sup> (Figures 2 and 3). Recently, Schwartz<sup>[75]</sup> even suggested that aberrant tryptophan metabolism may be considered as the unifying biochemical basis for development of ASD. In mice, tryptophan 2,3-dioxygenase (TDO) was found to be a molecular key biomodulator of physiological neurogenesis and anxiety-related behavior<sup>[76,77]</sup>. In this context, decreased tryptophan metabolism found in patients with ASD<sup>[72]</sup> may reflect a defense reaction of the host since IFN- $\gamma$  blocked growth of the parasite by inducing the host cells to degrade tryptophan<sup>[78]</sup>. This is in line with the finding that tryptophan loading induced oxidative stress because its metabolites generated free radicals<sup>[79]</sup>, known to affect intensity of pathological oxidative damage and redox signalling. Finally, it should be noted that the individuals with schizophrenia had either IgG or IgM class antibodies reactive to *T. gondii* proteins<sup>[19,80,81]</sup>.

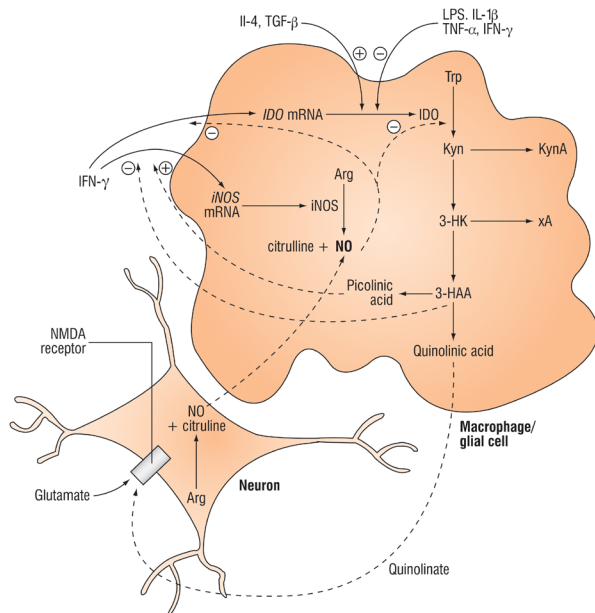


**Figure 1** Relationship between serum IFN- $\gamma$  (pg/mL) and NO concentrations in the studied autistic children.



**Figure 2** Various pathways of the essential amino acid tryptophan metabolism. About 99% of the dietary tryptophan is metabolized along the kynurenine pathway (red arrows). Alternative pathways are the conversion of tryptophan to 5-hydroxytryptamine (5-HT) and then to melatonin, or to tryptamine and then to the kynuramines (or kynurenamines). N1-acetyl-5-methoxykynuramine is a metabolite deriving from melatonin by mechanisms involving free radicals, exhibits potent antioxidant properties exceeding those of its direct precursor N1-acetyl-N2-formyl-5-methoxykynuramine, a biogenic amine generated through either an enzymatic or a chemical reaction (free radicals) pathway. 3-HAO, 3-hydroxyanthranilate oxidase; IDO, indoleamine 2,3-dioxygenase; KAT, Kynurenine aminotransferase; MAO, monoamine oxidase; QPRT, quinolinic-acid phosphoribosyl transferase; TDO, tryptophan 2,3-dioxygenase (acc. to Stone & Darlington<sup>[74]</sup>; with own modification)

***T. gondii***. The parasite infects a variety of host brain and other tissue cells, and IFN- $\gamma$ -mediated immune responses control the parasite in both phagocytic and non-phagocytic cells through at least 6 different mechanisms depending on the types of cells responding to this cytokine. Such effector functions involving several neurobiologic



**Figure 3** Interrelationships between indoleamine 2,3-dioxygenase (IDO) and nitric oxide synthase (NOS) in macrophages or glial cells, and the potential interactions with neurons by means of N-methyl-D-aspartate (NMDA)-receptor-induced nitric oxide (NO) formation. Arg, arginine; 3-HAA, 3-hydroxyanthranilic acid; 3-HK, 3-hydroxykynurenine; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; Kyn, kynurenine; KynA, kynurenic acid; LPS, lipopolysaccharide; mRNA, messenger RNA; iNOS, inducible nitric oxide synthase; TGF- $\beta$ , transforming growth factor- $\beta$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; Trp, tryptophan; xA, xanthurenic acid. The broken lines represent possible reactions (acc. to Stone & Darlington<sup>[74]</sup>, with own modification).

biomechanisms affecting *T. gondii* survival in the host cells include: (1) mechanisms mediated by an IFN- $\gamma$  responsive gene family. Several of these proteins, including IGTP (IFN- $\gamma$ -regulated gene), may be involved in the processing and trafficking of cytokines and/or antigens. IGTP is an essential mediator of specialized antimicrobial activities of IFN- $\gamma$ ; (2) production of NO by inducible NO synthase (iNOS); (3) production of various cytokines (TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , etc.); (4) tryptophan degradation by the enzyme indoleamine 2,3-dioxygenase (IDO) and TDO; (5) limiting the availability of intracellular iron to the parasite; (6) production of reactive oxygen species (ROS), reactive nitrogen intermediates (RNI)<sup>[82-84]</sup>. Several molecular, biochemical and immune host defense mechanisms associated with *T. gondii* infection were presented in Tables 10 and 11<sup>[44,85]</sup>.

The IFN- $\gamma$  cytokine is central in resistance to *T. gondii* at both acute and chronic stages of infection, as demonstrated by cytokine depletion, cytokine repletion, and gene knockout studies<sup>[117-120]</sup>. Cell sources of IFN- $\gamma$  include: NK cells<sup>[121-125]</sup> (promotes iNOS expression<sup>[126]</sup>), CD4<sup>+</sup> T cells<sup>[127]</sup> (promotes p47 GTPase-mediated killing of *T. gondii*<sup>[128,129]</sup>), and CD8<sup>+</sup> T cells<sup>[127]</sup> (promotes tryptophan degradation<sup>[78,114,130,131]</sup>). Virulence of the parasite was associated with distinct dendritic cell (DC) responses and reduced numbers of activated CD8<sup>+</sup> T cells<sup>[132]</sup>. Although IFN- $\gamma$  may play multiple roles in resistance to the pathogen, macrophage activation is generally believed to be the critical effector function<sup>[120,133,134]</sup> because activation of these cells resulted in iNOS gene induction and synthesis of moderate levels of RNI generated during degradation of arginine into citrulline by iNOS<sup>[85,120]</sup>. Synthesis of RNI was also highly enhanced by certain microbial products, as well as by triggering macrophage TNF- $\alpha$  synthesis<sup>[85,120]</sup>. Infection with *T. gondii* stimulated also tryptophan metabolism along the kynurenine pathway, which contains several neuroactive metabolites, including 3-hydroxykynurenine, quinolinic acid and kynurenic acid<sup>[78,114,130,131]</sup>, and cytokines mediated regulation of IFN- $\gamma$ -induced L-tryptophan biodegrading enzyme, IDO<sup>[135]</sup> (Figure 2).

**Table 10** Several defense mechanisms that allow *T. gondii* to escape the effects of an active host immune response, as well as various endogenous and exogenous mediators/ substances that may interfere with the host defense against the parasite (acc. to James<sup>[85]</sup>, with own modification).

Inhibition of:	Through induction of (and/or use/interference with):	References
T <sub>H</sub> 1-related IFN- $\gamma$ production	IL-4, IL-10, NO (negative effects on antigen presentation and cell proliferation, PGE <sub>2</sub> <sup>1</sup> (decreases IL-2 and IFN- $\gamma$ production)	[86-88]
IFN- $\gamma$ -mediated NO generation	IL-10, TGF- $\beta$ <sup>2</sup> , IL-4 ( $\pm$ ) <sup>3</sup> , IL-13 ( $\pm$ ) <sup>3</sup> , NO (inactivation of NOS and IDO), 3-HAA <sup>4</sup> , different drugs, foods and food additives (these substances cause production of NO via the conversion of L-arginine to citrulline and NO by NOS, which results in suppressing NOS activity), MAO inhibitors (interference with dietary tryptophan-kynuramine-5-hydroxykynuremine metabolic pathway). Iron down-regulates macrophage NO production stimulated by IFN- $\gamma$ plus LPS, although, on the other hand, iron deprivation is one of the mechanisms mediating anti- <i>Toxoplasma</i> activity. These abnormalities are consistent with the impaired iron homeostasis in the periaqueductal gray matter reported in the patients with episodic migraine and chronic daily headache. PGE <sub>2</sub> and LTB <sub>4</sub> inhibit NOS type 2 expression, iNOS synthesis and expression, and NO production in hepatocytes and macrophages. And vice versa, NO mediates IL-1-induced PGE <sub>2</sub> production by vascular smooth muscle cells.	[74, 86, 89-96]
NO antiparasitic effects	NO may serve as a molecular trigger of the parasite stage conversion, either by selecting for parasites that spontaneously convert to preferential use of NO-insensitive anaerobic metabolism or by actively forcing the parasite to adopt this energy-generating mechanism to survive.	[85, 97]

<sup>1</sup> PGE<sub>2</sub> produced by human monocytes during *T. gondii* infection suppresses T<sub>H</sub>1 cytokine production and induce T<sub>H</sub>2 cytokines, such as IL-10<sup>[98-100]</sup>, and decreases NO production by activated microglia<sup>[100,101]</sup>. PGE<sub>2</sub> (as well as PGE<sub>1</sub> and PGE<sub>2a</sub>) are potent activators of the N-acetyltransferase enzyme, which synthesizes melatonin from 5-HT and stimulates melatonin release from the pineal gland, and conversely, melatonin and its kynuramine metabolites are potent inhibitors of prostaglandin synthesis<sup>[74,102]</sup>. IDO, indoleamine 2,3-dioxygenase, the first enzyme of the kynurenine pathway, the main pathway for tryptophan metabolism, is powerfully induced by IFN- $\gamma$ ; inhibition of NOS consequently results in an exaggerated output of kynurenines.

<sup>2</sup> The anti-inflammatory cytokines TGF- $\beta$  and IL-4 suppress IDO activity in human monocytes and fibroblasts<sup>[78]</sup>, which is consistent with IDO metabolic pathway being a significant contributor to the pro-inflammatory system<sup>[74]</sup>. Enhanced IDO activity was found in vivo in diseases that were associated with chronic stimulation of T<sub>H</sub>1-mediated immunity, such as the CNS *T. gondii* infection<sup>[103,104]</sup>. Metabolites of tryptophan, L-kynurenine, picolinic acid and quinolinic acid, were found to inhibit proliferation of CD4<sup>+</sup>, CD8<sup>+</sup> T lymphocytes and NK cells, thus affecting immune response of the host to the parasite<sup>[104]</sup>. NO can directly inactivate NOS<sup>[105]</sup>.

<sup>3</sup> IL-13 exerted dual effects, i.e. a decreased and an enhanced NO production by LPS-stimulated GMC-stimulated factor-derived bone marrow macrophages.

<sup>4</sup> 3-HAA (3-hydroxyanthranilic acid), a metabolite of dietary tryptophan metabolic pathway, inhibits NOS enzyme activity, the expression of NOS mRNA, and activation of the inflammatory transcription factor NF- $\kappa$ B<sup>[106]</sup>. It must be added that tryptophan metabolites, such as quinolinic acid, 3-hydroxykynurenine, and 3-HAA are known to have the ability to generate free radicals, which may exert an antitoxoplasmic effect<sup>[79]</sup>.

It should be noted that *T. gondii* disproportionately infected DCs and macrophages, and both these infected cell types displayed an activated phenotype characterized by enhanced levels of CD86 compared to cells that had phagocytosed the pathogen<sup>[125]</sup>. DC were required for optimal CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses, and the phagocytosis of heat-killed or invasion-blocked parasites was not sufficient to induce T cell responses<sup>[125]</sup>.

In summary, several cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , and NO, protect brain cells and neurons from infection, and part of this protection involving killing, inhibition of growth and intracellular multiplication of the parasite, may occur also through the promotion of cyst formation, however particularly complex interplay of these molecular compounds released by all the cells provide protective mechanism involved in the response to *T. gondii* infection. On the other hand, IFN- $\gamma$  and some other cytokines produced by glial cells, neurons, T lymphocytes or NK cells also play an important role in development of neurodegeneration and oxidative stress.

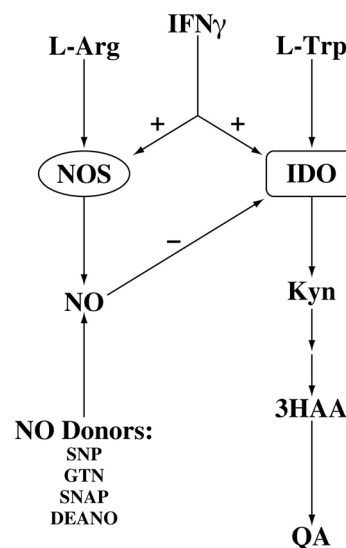
#### Increased serum NO concentrations in autistic children with toxoplasmosis

Our study showed that autistic children with toxoplasmosis had markedly increased serum NO levels as compared with *Toxoplasma*-free participants. This is in agreement with literature data reporting that patients with autism have significantly increased NO production<sup>[136-139]</sup>. Higher plasma nitrite (NO<sub>2</sub><sup>-</sup>)/nitrate (NO<sub>3</sub><sup>-</sup>) levels (total NO, recovery from plasma approximately 87%, with 17,6% of the total level as nonstable nitrite<sup>[140]</sup>) were also found in children with autism compared with controls<sup>[136]</sup>. Higher NO-derived reactive nitrogen oxides (NOx) concentrations demonstrated in red blood cells of autistic patients compared to age- and sex-matched normal controls, along with enzymatic evidence of NO-related oxidative stress<sup>[137]</sup>, were associated with mitochondrial dysfunction<sup>[141]</sup>. The induction of a high-output inducible enzyme NOS (iNOS) is triggered primarily by IFN- $\gamma$ , in combination with TNF- $\alpha$  and IL-1 $\beta$ , or endotoxin<sup>[138,142,143]</sup> (Figure 4). It must be noted that autistic children showed markedly enhanced production of the cytokines IFN- $\gamma$ , TNF- $\alpha$  and IL-1 $\beta$  compared to controls<sup>[144,145]</sup>.

*In vivo*, concentrations of NO approach 80  $\mu$ M<sup>[146]</sup>, therefore locally the levels of NO may be orders of magnitude greater<sup>[147]</sup>. High levels of NO (about 1 mM) and more prolonged exposure can reversibly or irreversibly damage the mitochondrial iron-sulphur centers<sup>[148]</sup>. In the central nervous system, NO at physiological concentrations acts as an intracellular messenger, but at higher concentrations it can initiate a neurotoxic cascade leading to cell death<sup>[149-152]</sup>. NO has been implicated in a wide range of pathological processes in the brain and other tissues, including neurodegenerative diseases, stroke, and ischemia<sup>[153,154]</sup>. It was suggested that a number of the physiological and pathological effects of NO may be mediated

by suppression of mitochondrial functions, particularly by inhibition of cytochrome oxidase. Brown and his group<sup>[155,156]</sup> showed that astrocytes and macrophages activated by cytokines and endotoxin to express the inducible NO synthase produced up to 1  $\mu$ M NO and inhibited their own cellular respiration and that of cocultured cells via the NO inhibition of cytochrome oxidase. Moreover, NO caused glutamate release from synaptosomes via inhibition of synaptosomal respiration<sup>[42]</sup>, and killed cerebellar granule neurons by excitotoxic mechanisms<sup>[151]</sup> (Table 12).

Prolonged exposure of cells to NO might cause cell death by opening a mitochondrial permeability transition pore and subsequent activation of caspases<sup>[10]</sup>, and the mode of cell death as proapoptotic or pronecrotic<sup>[158]</sup> (Table 13) seemed to be determined by cellular level of ATP<sup>[151]</sup>. (Nb. autophagy is physiologically essential for mitochondrial clearance in mature T lymphocytes, because it contributes to the elimination of intracellular pathogens as well as the major histocompatibility complex class II molecules cross-presentation of endogenous antigens<sup>[159]</sup>). Excess of NO caused increased intestinal permeability ("leaky gut")<sup>[160,161]</sup>, prevalent in autism<sup>[162]</sup>, since this biomolecule mediated IFN- $\gamma$ -induced hyperpermeability in cultured human intestinal epithelial



**Figure 4** Possible model for NO-mediated regulation of IDO in IFN- $\gamma$ -primed mononuclear phagocytes. NOS, nitric-oxide synthase; IDO, indoleamine 2,3-dioxygenase; L-Arg, L-arginine; L-Trp, L-tryptophan; IFN- $\gamma$ , interferon- $\gamma$ ; NO, nitric oxide; Kyn, kynurenine; 3-HAA, 3-hydroxyanthranilic acid; QA, quinolinic acid; SNP, sodium nitroprusside; GTN, glyceryl trinitrate; SNAP, S-nitroso-N-acetylpenicillamine; DEANO, diethylaminodinitric oxide. SNP, DEANO, and SNAP release NO extracellularly, while GTN is thought to release NO intracellularly (acc. to Thomas, Mohr, & Stocker<sup>[89]</sup>).

**Table 11** Efficiency of *T. gondii* infection and cytokine/NO release in primary cultures of brain cells (acc. to Fagard *et al.*<sup>[44]</sup>, with own modification).

Parameters	Astrocytes	Microglial cells	Neurons	References
Relative efficiency of infection	100 <sup>1</sup>	50 <sup>1</sup>	5-15 <sup>1</sup>	107-110
Cytokine release	IFN- $\gamma$ , IL-1, IL-6, GM-CSF, NO	IFN- $\gamma$ , IL-10, IL-6, TNF- $\alpha$ , NO	TNF- $\alpha$ ?, NO, glutamate	111-113
Cyst formation	Yes	Limited	Yes	110, 113
Size of cysts	Large (~50 $\mu$ m)	Small (~10 $\mu$ m)	Small (~10 $\mu$ m)	111
Size of brain cells	100 $\mu$ m (harbor several dozen tachyzoites)	5-10 $\mu$ m	10-15 $\mu$ m <sup>2</sup> (contain only few tachyzoites)	110
Effect of IFN- $\gamma$ and TNF- $\alpha$	Encystation	Parasite killing	?	107, 112, 114
Inducible NOS	Low	High	Yes <sup>3</sup>	115, 116

<sup>1</sup> Lüder *et al.*<sup>[38]</sup> found that in rats only 30% of microglial cells were infected with *T. gondii*, whereas 10% of neurons and astrocytes were invaded. Besides, parasites showed low replication rates, with only one or two degenerated parasites in 93% of the parasitophorous vacuole. <sup>2</sup> Cerebellar granular neurons and pyramidal hippocampal neurons (when the size of a cell doubles, its volume increases eight fold). It must be noted that *T. gondii* size is 2-4  $\mu$ m. <sup>3</sup> Not documented for *T. gondii* infection. GM-CSF, granulocyte-macrophage colony-stimulating factor.

**Table 12** Possible consequences on neurons of cytokines and biomolecules secreted upon *T. gondii* infection (acc. to Fagard *et al*<sup>[44]</sup>; with own modification)

Cell type	Secreted biomolecules	Neurotic
Astrocyte	IL-6	-
	GM-CSF	-
	TNF- $\alpha$	$\pm$
	IL-1 $\beta$	-
	arachidonic acid	+
Macrophage	IL-12	-
	NO	$\pm$
Microglial cells	RNI	+
	NO	$\pm$
	H <sub>2</sub> O <sub>2</sub>	+
	IFN- $\gamma$	-
	Glutamate	+
Neuron	NO	$\pm$
	TNF- $\alpha$	$\pm$
	Glutamate	+
Natural killer cell	IFN- $\gamma$	-
	PAF	+
T cell	IL-4	-
	IFN- $\gamma$	-
	IL-10	-

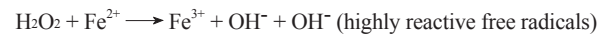
RNI, reactive oxygen intermediates; PAF, platelet-activating factor; GM-CSF, granulocyte-macrophage colony stimulating factor. It must be noted that *T. gondii* infection caused a significant increase in dopamine metabolism in neural cells, which may lead to psychobehavioral changes in individuals with toxoplasmosis<sup>[47]</sup>. Dopamine concentrations were 14% higher in the brain of mice with chronic infections than in controls<sup>[40]</sup>, and this neurotransmitter dysfunction has been associated with a variety of neurological disorders including schizophrenia, attention deficit hyperactivity, tic disorders, Tourette's syndrome, and dyskinesias. In addition, induction of indoleamine 2,3-dioxygenase expression and decreased levels of tryptophan and increased formation of kynurenine were found in the brain, lungs and serum of mice infected with the pathogen<sup>[45]</sup>. Moreover, dopamine stimulated tachyzoite proliferation in human fibroblast and primary neonatal rat astrocyte cell cultures<sup>[48]</sup>, thus further enhancing harmful effects of the parasite on the brain function. In addition, chronic latent *T. gondii* infection is associated with various cytokines overproduction and it was postulated that cytokines may induce changes in mood and behavior leading to depressive illness in man<sup>[46,157]</sup>. Nitric oxide caused glutamate release from brain synaptosomes<sup>[42]</sup>. Interestingly, lithium chloride (a drug used in psychiatric diseases) potentiated astroglial nitric oxide synthase type-2 mRNA expression, and therefore treatment with this medicament could exacerbate inflammatory responses in the brain<sup>[43]</sup>.

**Table 13** The induction of necrosis or apoptosis by NO donors or inhibitors of mitochondrial respiration in PC12 cells (acc. to Bal-Price *et al*<sup>[51]</sup>; with own modification).

Treatment	Necrotic cells (%)	Apoptotic cells (%)
Control	13.00 $\pm$ 1.40	0.10 $\pm$ 0.10
SNAP	83.80 $\pm$ 4.30	0.56 $\pm$ 0.10
SNAP + glucose	19.05 $\pm$ 1.80	19.45 $\pm$ 1.40
SNAP + glucose + VAD	14.80 $\pm$ 2.30	0.09 $\pm$ 0.1
NOC-18	97.80 $\pm$ 0.80	0.10 $\pm$ 0.01
NOC-18 + glucose	17.30 $\pm$ 1.80	25.50 $\pm$ 2.4
NOC-18 + glucose + VAD	8.30 $\pm$ 1.10	0.50 $\pm$ 0.10
Myxothiazol	92.50 $\pm$ 3.40	0.00
Myxothiazol + glucose	11.20 $\pm$ 3.60	10.38 $\pm$ 2.20
Azide	97.23 $\pm$ 1.50	0.00
Azide + glucose	15.20 $\pm$ 3.10	8.23 $\pm$ 2.80

SNAP, S-nitroso-N-acetylpenicillamine; NOC-18, DETA NONOate; VAD, a broad-spectrum caspase inhibitor. In all studies, medium was changed to glucose-free 1 day before experiments, and to fresh medium (with or without 20 mM glucose) containing NO donors (1 mM SNAP or 1 mM NOC-18) or mitochondrial inhibitors (2  $\mu$ M myxothiazole or 2 mM azide) for 24 h. After this time the cell death was analyzed by tryptan blue or propidium iodide staining (necrotic cells) or by using chromatin dye Hoechst 33342 (apoptotic cells).

monolayers<sup>[160,163]</sup>. NO as a regulator of mast cell activation and mast cell-mediated inflammation, may participate in development of this pathology<sup>[164]</sup>. Because NO directly impairs the intestinal barrier function<sup>[161]</sup> this abnormality may be responsible for the iron, calcium and zinc deficiency reported in individuals with Pica behavior<sup>[165-167]</sup>. Because NOS2-derived NO (NOS type 2 is induced by cytokines, LPS, and endotoxin, is independent of calcium, and is prototypically expressed in inflammatory macrophages), controls pathogens by restricting their access to micronutrients<sup>[168,169]</sup>, Pica behavior may therefore represent the host response to the pathomechanism that participate in defense against *T. gondii* infection. It should be noted that NO also enhanced hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-mediated microvascular endothelial permeability, and this NO effect was concentration dependent<sup>[146]</sup>. McQuaid *et al*<sup>[170]</sup> demonstrated that the permeability produced by H<sub>2</sub>O<sub>2</sub> could be exacerbated when relatively high levels of NO (100  $\mu$ M sodium nitroprusside) were present, and this endothelial dysfunction may be associated with generation of singlet oxygen from H<sub>2</sub>O<sub>2</sub><sup>[171]</sup>. NO did not affect H<sub>2</sub>O<sub>2</sub> metabolism by endothelial cells but markedly depleted intracellular glutathione<sup>[146]</sup>. The mechanism in which NO and H<sub>2</sub>O<sub>2</sub> cooperated to promote increased microvascular permeability probably involved the cell-surface bound iron (Fe<sup>3+</sup>)-catalyzed formation of a potent oxidant such as hydroxyl radical (OH $\cdot$ ) generated from decomposition of H<sub>2</sub>O<sub>2</sub> in the following reactions<sup>[146,172-174]</sup>:



and by NO-mediated depletion of intracellular glutathione<sup>[174]</sup>. One cannot exclude that NO also intercepted iron on route to ferritin and indirectly facilitated removal of iron from the protein in a glutathione-dependent manner<sup>[175]</sup>. Okayama *et al*<sup>[174]</sup> proposed that physiologic conditions that can substantially elevate locally H<sub>2</sub>O<sub>2</sub> and NO might then result in a potential widening of the intercellular spaces only in junctions of the microcirculation cells exposed to such an oxidant stress, especially when relatively high levels of NO were present<sup>[146]</sup>. It must be added that limiting the availability of iron could represent a broad antimicrobial mechanism through which the activated enterocytes exert control over intracellular pathogens, such as *T. gondii*<sup>[176]</sup>. This finding may be supported by the fact that iron down-regulated NO synthesis and release by macrophages<sup>[90,177,178]</sup>. Interestingly, it was demonstrated that macrophages loaded with iron lose their ability to kill intracellular pathogens via NO formation<sup>[178]</sup>, and this may be in line with finding of the significant association between neonatal iron overload and an increase in neurodevelopmental impairment among infants with iron overload compared to infants with normal iron status (64% vs 41%,  $p = 0.05$ )<sup>[179]</sup>.

Tachyzoites of *T. gondii* stimulate production of IL-12<sup>[180-183]</sup> and this proinflammatory cytokine activates NK cells and T cells to produce IFN- $\gamma$  and it is this early-produced IFN- $\gamma$  that is crucial for host resistance<sup>[114,117,184-186]</sup>. IFN- $\gamma$  and TNF- $\alpha$  act synergistically to mediate killing of tachyzoites by macrophages<sup>[123,185,187]</sup>. The combination of these two cytokines results in a greatly enhanced production of free oxygen radicals and NO, both of which can effect parasite killing<sup>[123,185,187]</sup> (Figure 4), although NO and its metabolites appear to be the primary effectors. NO is produced as a result of iNOS activation, which is dependent on activation of NF $\kappa$ B<sup>[183,188]</sup>. It must be noted that Gomez-Marín<sup>[189]</sup> obtained evidence of NO generation not only in the host cells, but also in *T. gondii* (similarly like in some other protozoans<sup>[190,191]</sup>, which has its own constitutive calcium-dependent NOS producing 2-6  $\mu$ M of nitrites that could be essential in intracellular signaling. The NO defensive mechanism,



where levels of nitrites can reach 120  $\mu\text{M}$  or more, is probably toxic for human and mice tissues<sup>[189]</sup>.

In summary, while NO at physiological levels is beneficial for the host, especially that it induces antitoxoplasmic activity, at higher and toxic concentrations it can be implicated in development of many pathological processes, including inhibition of mitochondrial and cellular respiration, leading to oxidative stress, neurodegeneration, and damage of other tissues.

#### **Excessive NO production associated with chronic *T. gondii* infection cause down-regulation of various forms of cytochrome P450 enzyme activities in the liver, brain, and other tissues**

NO reacts with various molecules, including superoxide, iron, thiol compounds and various hemoproteins, such as CYP450 isoforms at nearly diffusion-limited rates, and these proteins might be the primary targets<sup>[192,193]</sup>. NO donors decreased substantially in a concentration-dependent manner catalytic activities of CYP450 isoenzymes in isolated perfused rat livers with the one-half-life of maximum inhibition being in the order of  $2\text{C11} > 2\text{B1/2} > 2\text{E1} = 3\text{A2} > 1\text{A1/2}$ . NO may regulate enzymatic activity of transglutaminases, Ca-dependent enzymes, and their biologic effects, via S-nitrosylation of their crucial thiol groups (e.g. coagulation factor XIII)<sup>[194-197]</sup>. The use of NOS inhibitors *in vitro* and *In vivo* has been reported to attenuate declines in CYP450 activities, protein and mRNA levels produced by inflammatory stimuli<sup>[198]</sup>. For example, administration of N<sup>o</sup>-L-arginine methyl ester to rats treated with phenobarbital and LPS blocked the down-regulation of CYP2B1/2 activity, mRNA and protein<sup>[199]</sup>. It must be noted that not only bacterial LPS but also *T. gondii* soluble antigen induced a subset of LPS-inducible genes in macrophages<sup>[200]</sup>.

Constitutive cytochrome P450 isoenzymes play important roles in the metabolism of physiological substrates and xenobiotics, such as steroids, fatty acids, prostaglandins, environmental pollutants and carcinogens<sup>[192,198,201]</sup>. Infection or inflammatory and/or immunological stimuli cause changes in the activities and expression levels of various forms of CYP450 enzymes in the liver, as well as extrahepatic tissues<sup>[198,201]</sup>. Inflammatory mediators derived from different CYP450s could either enhance (NO, CYP3A) or inhibit (CYP2C, CYP2J) inflammatory responses<sup>[201]</sup>. For example, CYP2E1 was induced in astrocytes during brain tissue inflammation and CYP1A1 was down-regulated<sup>[202]</sup>. Injection of LPS in the brain had profound effect on CYP1A, -2B, -2E1, and -3A activities in the liver<sup>[203,204]</sup>. LPS caused also release of many different cytokines, including TNF- $\alpha$ , IL-1, IL-6, IFN- $\gamma$ <sup>[198]</sup>. LPS and IFN- $\gamma$  induced excessive generation of NO in rat C6 glial cells, which differentially modulated several endogenous antioxidant enzymes, including catalase, glutathione peroxidase, CuZn- and Mn-superoxide dismutases<sup>[205]</sup>.

Cytokines administered *in vivo* or *in vitro* also have enzyme-selective effects on CYP450 expression<sup>[198,206]</sup>. The reason for down-regulation of CYP450 enzymes in the liver could be related to their ability to form NO, for example, rat CYP3A enzymes can form NO from N-hydroxyarginine<sup>[207]</sup>, and inhibition of CYP3A activity in hepatocytes inhibits LPS and cytokine-stimulated production of NO and citrulline by more than 90% without affecting N-hydroxyarginine formation<sup>[208]</sup>. LPS-induced NO formation in animals was potentiated by dexamethasone induction of CYP3A, and the potentiation was inhibited by troleandomycin, an antibiotic inhibitor of CYP3A enzymes<sup>[209]</sup>. NO stimulates TNF- $\alpha$  production, and inhibition of NO generation blocked TNF- $\alpha$  release<sup>[210]</sup>. Thus, all these biomolecular metabolic alterations may be a basis of NO-induced cytotoxicity in disease states associated with excessive NO production.

## **OXIDATIVE STRESS AS AN IMPORTANT DETRIMENTAL PATHOMECHANISM IN AUTISTIC CHILDREN WITH TOXOPLASMOSIS ASSOCIATED WITH NO OVERPRODUCTION**

### **ASD**

Oxidative stress is an important mechanism in autism and direct markers for enhanced lipoxidation were reported by several authors<sup>[71,136,211,212]</sup>. There is evidence that children with autism have mitochondrial dysfunction, mtDNA overreplication, and mtDNA deletions, which may be, at least in part, an underlying pathophysiological mechanism in a subset of individuals with ASD<sup>[141,213-216]</sup>. It must be noted that brain cells and cerebrovascular endothelium require high energy demands and have many mitochondria. Neuronal synapses also are areas of high energy consumption, and mitochondria are concentrated in the dendritic and axonal termini where they play an important role in ATP production, calcium homeostasis and synaptic plasticity<sup>[216]</sup>. Zhang *et al.*<sup>[217]</sup> suggested that mtDNA could activate toll-like receptors (TLR) on immune or glial cells, finally resulting in a release of several proinflammatory cytokines in the brain of autistic patients<sup>[61]</sup>. It should be added that mtDNA components may be released also from viable neutrophils<sup>[218]</sup>, and from activated tissue mast cells, a rich source of neurotensin<sup>[217-219]</sup>. Finally, mitochondria has been long recognized for their important role in cellular defense against microbial infection<sup>[220]</sup>, and this is well in line with the intracellular *T. gondii* infection.

In one study, which eliminated dietary and medicinal cofounders, red-cell thiobarbituric reactive substances (TBARS, a measure of lipoxidation) were twice higher in autistic children than in age-matched controls<sup>[136,211,212]</sup>. It was also demonstrated that serum lipid peroxides and urinary isoprostanes were markedly higher in children with ASD<sup>[211,221]</sup>. Patients with autism also have decreased plasma total GSH and increased oxidized GSH levels<sup>[71,211,222-224]</sup>.

Inflammatory reactions are believed to be an important contributor to neuronal damage in neurodegenerative diseases such as AD, Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis<sup>[225]</sup>. Enhanced NO production play an important role in these diseases, including Down syndrome<sup>[226]</sup>. Excess of NO leads to increased formation of its metabolites and reactive nitrogen species (RNI) which have antimicrobial activity. High levels of NO (> 1 mMol) and/or peroxynitrite (ONOO<sup>-</sup>), nitrogen dioxide (NO<sub>2</sub>) and dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>) formed by reaction of NO with superoxide (O<sup>-</sup>), strong oxidizing and nitrating agents, can cause lipid peroxidation and generation of nitrated lipid adducts, inactivation of sodium channels, interaction with metals which have redox potential such as iron and copper, irreversible inhibition of mitochondrial respiration and damage to a variety of mitochondrial components (e.g. mitochondrial DNA, the ATP synthase, creatine kinase, superoxide dismutase, the mitochondrial membrane, induces mitochondrial swelling, uncoupling, depolarization, calcium release and permeability transition)<sup>[227-229]</sup>. Apparent hydroxyl-radical production by ONOO<sup>-</sup> resulted in endothelial injury from NO and superoxide<sup>[230]</sup>. Peroxynitrite can be generated in the brain by microglial cells activated by proinflammatory cytokines or beta-amyloid peptide and by neurons in three situations: depletion of L-arginine or tetrahydrobiopterin, mitochondrial dysfunction, and hyperactivity of glutamate neurotransmission<sup>[224,231]</sup>. Mitochondrial disease and dysfunction (abnormal lactate, pyruvate, ubiquinone, AspAT, low carnitine levels) have been increasingly recognized in

a subset of individuals with autism<sup>[213,232,233]</sup>, and plasma glutamate levels were augmented in these patients<sup>[234]</sup>. The decreased serum levels of carnitine, coupled with significant elevation of alanine and ammonia concentrations in autistic persons<sup>[71]</sup>, supported the notion about mitochondrial dysfunction in this clinical entity<sup>[216,235]</sup>. Also, it should be noted that low serum uric acid level and hyperuricosuria commonly found in autistic individuals<sup>[236,237]</sup> may be explained by a neuroprotective role of uric acid as a putative scavenger of peroxynitrite<sup>[168]</sup>, thus reflecting a self-defense of the host against oxidative stress.

*T. gondii*. In the sera of 37 IgG-seropositive patients with *T. gondii* infection, Karaman *et al.*<sup>[238]</sup> demonstrated significantly increased malondialdehyde (MDA) and NO concentrations, and a decrease in glutathione activity as compared with healthy controls (Table 14). It was also found markedly higher MDA levels ( $P < 0.001$ ) paralleled with significantly decreased concentrations of glutathione peroxidase ( $P < 0.0188$ ) and tocopherol fractions (alpha, gamma and lambda) ( $P < 0.001$ ) in *T. gondii* seropositive than in seronegative blood donors<sup>[239,240]</sup>. These significant alterations in redox status between the two groups of blood donors indicate that chronic *T. gondii* infection is associated with oxidative stress because MDA is arising from the lipid peroxidation and is an indicator of oxidative stress, glutathione defends cells against oxidative damage by ROS and peroxidase, and tocopherol is an antioxidant<sup>[240,241]</sup>. Clancy *et al.*<sup>[242]</sup> also found that NO reacted with intracellular glutathione and activated the hexose monophosphate shunt in human neutrophils. The increased NO concentrations can be associated with the stimulation of the cell-mediated immune system in these individuals reflecting a defense of the host against the infection with the parasite. This may be supported by the finding that NO is a major effector molecule of macrophage cytotoxicity against *T. gondii*, and its production by macrophages is catalyzed by a cytokine-inducible form of the NO synthase positively controlled by TNF- $\alpha$ , IFN- $\gamma$ , IL-2, and negatively controlled mainly by IL-10, IL-4, TGF- $\beta$ <sup>[243,244]</sup>. Other cell types, such as endothelial cells and hepatocytes, display a similar capacity for NO generation in response to cytokine stimulation<sup>[244]</sup>.

In mice, *T. gondii* infection caused a significantly increased formation of reactive nitrogen radicals probably due to elevated serum NO concentrations, and a significantly higher serum kynurenine/tryptophan ratio compared with control animals ( $P < 0.05$ )<sup>[245]</sup>. The authors suggested that increased free radical toxicity may cause elevation in tissue MDA in *T. gondii*-infected mice, while unchanged serum MDA might indicate the increased oxidative stress due to the parasite infection restricted to intracellular area.

In summary, oxidative stress in autistic patients may be at least in part due to the concomitant latent chronic toxoplasmosis, especially that *T. gondii* infestation often is regarded as a global threat.

**Table 14** Serum glutathione, malondialdehyde, and NO concentrations in *T. gondii*-seropositive patients and healthy controls (acc. to Karaman *et al.*<sup>[238]</sup>; with own modification).

Bioparameter	Group	No of participants	Serum levels (mean $\pm$ SD)	P values
Glutathione	Patients	37	3.96 $\pm$ 0.10	0.001
	Controls	40	10.37 $\pm$ 0.13	
Malondialdehyde	Patients	37	41.32 $\pm$ 2.05	0.001
	Controls	40	9.18 $\pm$ 1.21	
NO	Patients	37	47.47 $\pm$ 1.00	0.001
	Controls	40	39.18 $\pm$ 1.29	

Serum glutathione and NO levels are expressed as  $\mu$ M, and malondialdehyde concentrations represent nM. Results statistically significant at  $p < 0.05$ .

### Unchanged CARS values found in the autistic children with and without toxoplasmosis. Possible effect of young age and maturation of the immune function

Our study performed in young children with autism did not show difference between the CARS values obtained in patients with and without toxoplasmosis (Table 7). This finding may be partly explained by a little difference in the mean age between these two groups of participants and maturation stage of their immune function. There was however approximately 5-fold increase in median serum NO levels in the *Toxoplasma*-positive vs *Toxoplasma*-free patients (Table 8), and otherwise it was demonstrated that even low physiological NO levels can cause substantial inhibition of respiration, and potentially make tissue respiration very sensitive to the oxygen tension<sup>[228,246]</sup>.

**ASD.** Studies of Ashwood *et al.*<sup>[247]</sup> showed a significantly altered adaptive cellular immune function in 2-5 years-old children with ASD following *in vitro* stimulation of their peripheral blood mononuclear cells with PHA and tetanus. The production of IL-13, TNF- $\alpha$ , and GM-CSF were about 2 to 7-fold increased in ASD cultures compared to controls following PHA stimulation. In contrast, there was greater than 30% decrease in IL-12p40 production after this immunostimulant administration<sup>[247]</sup>. Following stimulation, the frequency of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing activation markers CD134 and CD25 were markedly reduced in ASD, reflecting dysfunctional immune activation profile for T cells. Children 2 to 5 years-of-age also had significant increases in plasma levels of IL-1 $\beta$ , IL-6, IL-8, and IL-12p40 compared with controls ( $P < 0.04$ )<sup>[62]</sup>. Moreover, plasma chemokine levels MCP-1, RANTES, and eotaxin were markedly higher in children with ASD compared to controls ( $P < 0.03$ )<sup>[63]</sup>. It was noted that all these biomolecular perturbations were associated with more impaired communication and aberrant behaviors<sup>[62,63,247]</sup>. In addition, significantly higher IL-13/IL-10 and IFN- $\gamma$ /IL-10 ratios were found in children with ASD than in matched controls<sup>[15]</sup>. IL-13 is a pleiotropic cytokine that shares a receptor component and signaling pathways with IL-4<sup>[248]</sup>. IL-13 may affect T cell functions and type 1 cell differentiation indirectly<sup>[249,250]</sup> through its down-regulatory effects on the production of proinflammatory cytokines<sup>[251,252]</sup>, particularly IL-12<sup>[253]</sup>. Neonatal antigen-presenting cells showed a limited capacity to produce IL-12, due to a defect in the expression of IL-12(p35) gene<sup>[254]</sup>. In view of the importance of IL-13 in type 2 inflammatory responses and clinical allergy<sup>[255]</sup>, including eotaxin production<sup>[256]</sup>, it must be noted that the elevated plasma IL-13 levels in autistic children have been associated with markedly lower scores or less hyperactivity, as measured by the Aberrant Behavior Checklist<sup>[247]</sup>. Thus, it seems that the young age and associated maturation stage of the immune system played an important role in the lack of significant difference in the CARS values found in our autistic children with toxoplasmosis.

### *T. gondii*

The above-mentioned immune findings are important because Authier *et al.*<sup>[257]</sup> demonstrated that IL-13 pretreatment of murine peritoneal macrophages increased their anti-*T. gondii* activity induced by LPS, and the cytokine used alone triggered polarization of macrophages towards T<sub>H</sub>2 type of cytokine generation. In addition, a correlation between the increase of NO production and enhancement of the microbicidal activity of the macrophages was found<sup>[257]</sup>. It appeared that IL-13 primed iNOS expression at mRNA and protein levels induced by LPS, and thus potentiated inhibition of *T. gondii* intracellular replication<sup>[257,258]</sup>. On the other hand, Chaves *et al.*<sup>[259]</sup> reported that IL-13 and IL-4 negatively regulated the induction of

IDO mRNA expression, tryptophan catabolism, and the control of *T. gondii* replication in human fibroblasts activated with IFN- $\gamma$ . This dual *in vitro* effects of IL-13 on *T. gondii* replication in various cells may therefore be well in line with the changes in behavior and developmental functioning of children with ASD<sup>[62,63]</sup>.

## SIGNIFICANT DIFFERENCES BETWEEN AUTISTIC CHILDREN WITH AND WITHOUT TOXOPLASMOSIS ASSOCIATED WITH AGE AND SEX MAY BE AT LEAST IN PART EXPLAINED BY DIFFERENT MATURATION STAGE OF THEIR IMMUNE SYSTEM

The phenotypic analysis of cord blood and neonatal and adult peripheral blood has shown differences in T-lymphocyte subpopulations (Table 15)<sup>[260,261]</sup>. The balance of T<sub>H</sub>1 and T<sub>H</sub>2 cytokine production differs between neonates and adults. At birth, the response is skewed in favor of T<sub>H</sub>2 type of responses, due to reduced generation of regulatory cytokines. For example, compared to LPS-stimulated lymphocytes from neonates, production of IL-12 was 5-fold higher at age 5 yrs, 15-fold higher at age 12, and 50 times higher in adults<sup>[262]</sup>. The predominance of T<sub>H</sub>2 responses, even in children up to 12 years of age, decreased the efficiency of host protective responses, particularly to intracellular pathogens<sup>[261]</sup> such as *T. gondii*. Age contributed to the percentage of CD29<sup>+</sup> lymphocytes (inducers of help) in adolescent children aged 12-18 yrs ( $F = 3.25$ ,  $p < 0.002$ ), with older individuals having a significantly higher percentage; to the number ( $F = 2.31$ ,  $p < 0.03$ ) and percentage ( $F = 2.14$ ,  $p < 0.04$ ) of B cells, with younger participants having higher values; and to the percentage of NK cells ( $F = 2.34$ ,  $p < 0.03$ ), with older individuals having higher percentages<sup>[263]</sup>.

Bartlett *et al.*<sup>[263]</sup> demonstrated also differences between males and females because a markedly lower percentage (but not number) of T cells was found in males than in females ( $F = 5.85$ ,  $p < 0.0001$ ), and the number of B cells ( $F = 3.43$ ,  $p < 0.0009$ ) was higher in males, as was the percentage ( $F = 2.14$ ,  $p < 0.04$ ). In addition, there were significantly lower numbers of CD4<sup>+</sup> cells in the male adolescents than in the females ( $F = 2.24$ ,  $p < 0.03$ ), and a lower percentage of CD4<sup>+</sup> cells was demonstrated among males than among females ( $F = 5.85$ ,  $p < 0.0001$ )<sup>[263]</sup>. Moreover, the percentage but not the number of CD29<sup>+</sup> cells was lower among males than among females ( $F = 2.54$ ,  $p < 0.02$ ), and the helper-to-suppressor ratio was higher in females than in males ( $F = 2.44$ ,  $p < 0.02$ ). The study of Kang *et al.*<sup>[264]</sup> also reported sex differences in immune responses and demonstrated enhanced immune reactivity to stress in adolescents.

It seems that the neonatal innate TLR-mediated responses (distinct from those of adults) important for orchestrating immunity of the

host against *T. gondii* infection, as well as the impaired cellular responses observed during the first 2 years of life<sup>[255-269]</sup>, are at least in part, responsible for the delayed diagnosis of autism despite evidence of prenatal changes in the brain<sup>[270,271]</sup>. The mature B cell differentiation and homing patterns associated with minimal response to polysaccharide antigens (e.g. *T. gondii*) observed in young children 17-18 months of age<sup>[269]</sup> (Table 16) are in line with the prenatal and postnatal neurobehavioral alterations produced in animals exposed to antibodies from mothers of children with autism and other neurological and psychiatric diseases<sup>[269,272-275]</sup>.

The important immune responses and immune reactivity findings related to age and gender in the adolescent children<sup>[263,264]</sup> may partly explain the statistically significant differences in age, sex and seroprevalence of *Toxo*-IgG levels found in our study between autistic children with and without toxoplasmosis. This suggestion may be further supported by the fact that in mice sex-determined resistance to *T. gondii* was associated with temporal maturational differences in cytokine production<sup>[276]</sup>. In male animals, a rapid response to infection with high levels of TNF- $\alpha$  and IFN- $\gamma$  helped to control parasite multiplication, after which IL-10 production (similar in both males and females) may be important in down regulating these potentially harmful excessive inflammatory mediators. The failure of female mice to respond quickly in terms of T-cell proliferation and IFN- $\gamma$  generation compared with their male counterparts may account for their higher mortality rates and cyst burdens<sup>[276]</sup>. It should be also added that different strains of *T. gondii* induce different cytokine responses<sup>[277]</sup>, as well as stage of parasite development and generated stage-specific antigens following pathogen interconversion (tachyzoites, bradyzoites, oocyst-sporozoites) (Table 17)<sup>[278,279]</sup>, all these factors may affect intensity and specificity of immune responses in the host. It seems therefore that the increased generation of antibodies and autoantibodies directed against brain proteins in patients with autism and their families may be caused by latent chronic *T. gondii* infection<sup>[280]</sup>.

**Table 16** Age related functional characteristics of systemic B cell (antibody response in early childhood (acc. to Ogra<sup>[269]</sup>, with own modification).

Age	B cell response	
	T cell dependent	T cell independent
Birth	B cell receptor diversity. Priming for B cell memory	Absent
2 months	Effective B cell response to most antigens	Minimal or no response to polysaccharide antigens
17-18 months	Mature B cell differentiation and homing patterns <sup>2</sup>	Minimal response to polysaccharide antigens <sup>1</sup>
4-6 years	Effective response	Effective responses. Marginal B cell zones in lymph nodes

<sup>1</sup>Based on data provided by Wilson & Kollmann<sup>[267]</sup>. <sup>2</sup>It must be noted that *T. gondii*-derived HSP70 functions as a B cell mitogen<sup>[266]</sup> and mature B cells are essential for vaccination-induced resistance to virulent *T. gondii*<sup>[265]</sup>.

**Table 15** Distribution of lymphocyte subtypes in the fetus, newborn and adult (acc. to Schultz *et al.*<sup>[260]</sup>, Luebke *et al.*<sup>[261]</sup>, with own modification).

Marker	Fetus				Neonate				Adult	
	Percent	% of adult	Absolute <sup>1</sup>	% of adult	Percent	% of adult	Absolute <sup>1</sup>	% of adult	Percent	Absolute
WBC			5,154 <sup>1</sup>	89.6			13,426 <sup>1</sup>	234.1		5,750
Lymphocytes			3,700 <sup>1,2</sup>	180.3			4,263 <sup>1</sup>	207.7		2,052
CD2 <sup>+</sup>	57 <sup>1,2</sup>	69.5	1,936 <sup>2</sup>	120.5	72 <sup>1</sup>	87.8	2,971 <sup>1</sup>	185.0	82	1,606
CD3 <sup>+</sup>	52 <sup>1</sup>	67.5	1,771 <sup>2</sup>	127.3	61 <sup>1</sup>	79.2	2,579 <sup>1</sup>	185.4	77	1,391
CD4 <sup>+</sup>	39 <sup>1</sup>	78.0	1,321 <sup>2</sup>	136.6	45 <sup>1</sup>	90.0	1,897 <sup>1</sup>	196.2	50	967
CD8 <sup>+</sup>	15 <sup>1,2</sup>	62.5	499 <sup>2</sup>	107.3	18 <sup>1</sup>	75.0	874 <sup>1</sup>	188.0	24	495
CD4:CD8			2.9 <sup>1</sup>	138.1			2.3	109.5		2.1
CD19 (B cells)	18 <sup>1,2</sup>	138.5	547 <sup>1</sup>	225.1	11	84.6	429 <sup>1</sup>	176.5	13	243

<sup>1</sup>Per mm<sup>3</sup>. <sup>2</sup>Significantly different from adults. <sup>3</sup>Significantly different from neonates.



**Table 17** Identification of stage-specific antigens of *T. gondii*. Value of ELISA titers of pooled immune mouse antisera to *Toxoplasma* tachyzoites, bradyzoites, and oocyst-sporozoites<sup>1</sup> [278].

Antiserum fraction	Oocyst -sporozoites	Antibody titers for: Tachyzoites	Bradyzoites
IgG			
Bradyzoites	450	1350	12150
Oocyst-sporozoites	36450	450	1350
Tachyzoites	450	12150	1350
IgM			
Bradyzoites	1350	450	1350
Oocyst-sporozoites	1350	150	150
Tachyzoites	1350	450	450

<sup>1</sup> Parasites were used at a concentration of  $10^4$  per well. Mouse antisera were raised against the oocyst-sporozoite (feline-excreted stage), bradyzoite (chronic tissue cyst stage), and tachyzoite (invasive stage).

## LIMITATIONS OF SERODIAGNOSIS IN PATIENTS WITH TOXOPLASMOSIS

Despite major advances in the field of DNA technology, most serological tests used for diagnosis of *T. gondii* infection still employ paraformaldehyde-fixed parasite, the Sabin-Feldman dye test, or crude extracts from tachyzoites, instead of parasite recombinant antigens. At present, diagnostic tests used for estimation of *T. gondii* IgG, IgM, and IgA seropositivity are not fully sensitive and specific, and there are different methods of serum sample preservation and elaboration [280,281]. Disease state of the host, its duration and stage (e.g. developing oxidative stress and resulting protein oxidation), may also affect results of performed tests for the parasite [280,282]. Other factors, such as for example uncontrolled treatment of other diseases and improper diet may provide NO donors [86,283,284] with possible associated consequences (Figure 4). It must be also noted that accidental antiparasitic treatment may suppress production and avidity of *T. gondii*-specific antibodies [285].

Recently, a significantly lower occurrence of seropositive titers against *T. gondii* was found in patients with diabetes mellitus type 1 (T1DM) and their close family members [286] as compared with healthy controls. Similar surprising results were obtained in the preliminary study of 83 autistic children aged 1-18 yrs (mean age 6 yrs; 70 boys, 14 girls) in whom only three boys had positive serum anti-*T. gondii* IgG levels (2.5, 4 and 4 yrs old patients had 7.0, 5.94, and > 650 IU/mL IgG levels, respectively; positive value > 6 IU/mL) (unpublished results, Magdalena Cubala-Kucharska, personal information, November 2012) [287]. This unexpected finding may be at least in part explained by the suppression of cytokine IL-2 generation, decreased activation of lymphocyte B cells responsible for immunoglobulin secretion and markedly lower levels of IgG, IgA, and IgM due to *T. gondii* infection [288]. In addition, persistent and prolific primary autoimmune-induced generation of many antibodies characteristic for the patients with ASD directed against own proteins (the so called a *perpetuum mobile*-like biomachinery [280,289], may be associated with their exhausted secondary innate and adaptive immune responses directed against foreign *T. gondii* antigens [290], in which host-endoplasmic reticulum-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in *T. gondii*-infected dendritic cells [83,291]. This explanation may be at least in part responsible for the above-mentioned markedly low occurrence of specific antibodies directed against the parasite found in the patients with T1DM and their families [286], and in the autistic children [287]. Congenital toxoplasmosis may also be a triggering factor responsible for the positive relationship found between the proportion of children who received the recommended vaccines by age 2 years

and the prevalence of autism or speech/language impairment across the U.S. population [8,287].

Lefevre-Pettazzoni *et al.* [292] demonstrated delayed maturation of IgG avidity (i.e. strength of antigen binding with antibody) and its implication for the diagnosis of toxoplasmosis in pregnant women. In the hospital database, persistent low avidity was found even after a median follow-up period of 6 years. They suggested that various factors could interfere with maturation of avidity, including the assay system used, variations between individuals investigated, and the treatment administered. In contrast, Prandota *et al.* [293] studied the seroprevalence of anti-*T. gondii* IgG and IgG avidity using enzyme immunoassay Patelia Toxo IgM, IgG in 178 children with headaches and it was found that 19 children (10.67%) (8 boys, 11 girls; 8-17 yrs old, mean age 14.36 yrs) had high serum anti-*T. gondii* IgG antibody levels (mean 120.10 IU/mL, range 32.2 to > 240 IU/mL; positive value was  $\geq 9$  IU/mL). It appeared that all *Toxoplasma*-positive children had high avidity index indicating chronic infestation. Nb. in only one study performed up to the present, 61% (11/18) of the analyzed patients with autism had headaches, with migraine as most frequent type of headache [294,295]. It seems therefore that the enhanced cerebrospinal fluid production and elevated extra-axial fluid found in infants, older children and adults with ASD [296-298], as well as the recurrent headaches reported in individuals with idiopathic intracranial hypertension [282,283,299], were due to congenital or acquired latent *T. gondii* infection, especially that plexus chorioideus is the preferential brain tissue area of the parasite settlement. In addition, it should be emphasized that different strains of the parasite induced different cytokine responses [277], and depending on the stage of differentiation (tachyzoites, bradyzoites, oocyst-sporozoites) specific antigens of *T. gondii*, IgG and IgM ELISA titers showed a wide range of values (Table 9) [278]. In autistic children, fever associated with eventual comorbidities, further complicates this situation because of eventual tachyzoite-bradyzoite interconversion and change of the parasite antigens in blood [300]. Thus, the increased generation of antibodies and autoantibodies against brain proteins in autistic individuals and their families should not be surprising [280], especially that for example autistic subjects had significantly elevated levels of antibodies to heat shock protein 90 (HSP) [301], which is critical for the maintenance of proper protein synthesis and function [302]. Nb. *T. gondii* HSP90 could be linked with many essential processes of the parasite such as host cell invasion, replication and tachyzoite-bradyzoite interconversion [303,304]. In this context, it seems that the method used in our study for serum anti-*T. gondii* IgG level investigation fulfills criteria regarding high sensitivity and specificity, probably because it contains the horseradish peroxidase-conjugated antibody in the kit, similarly to the peroxidase-antiperoxidase (PAP) immunohistochemical staining technique developed by Sternberger *et al.* [305] and modified and applied successfully by Conley & Jenkins [39], especially that all lots used at that time were negative for *Toxoplasma* antibody analyzed by the Sabin-Feldman dye test [306].

These data indicate that the above-mentioned discrepancies in serum anti-*T. gondii* IgG titers obtained by other authors were caused by multiple factors, including different laboratory methods of investigation, stage-specific antigens of the parasite, its virulence, duration and intensity of infection and the disease, host age, sex, immunity, and associated comorbidities. On the other hand, it must however also be noted that recently Chapey *et al.* [307] proposed a simple test for diagnosis of congenital toxoplasmosis based on whole-blood IFN- $\gamma$  release after stimulation by crude parasitic antigens (the sensitivity and specificity of the assay were 94% and 98%, respectively).



## CONCLUSIONS

This study for the first time showed a significantly higher seroprevalence of chronic toxoplasmosis among autistic children than healthy controls. *Toxoplasma*-positive autistic children had markedly increased serum IFN- $\gamma$  and NO concentrations as compared with *Toxoplasma*-free participants. A significant correlation was found between the levels of these two biomarkers. Since these biochemical abnormalities are known to be associated with oxidative stress, latent chronic toxoplasmosis may thus exert a persistent harmful effects on development of the CNS, although the CARS values showed no marked difference between the two groups of autistic children, probably because of their young age and maturation of immune function. We suggest that test(s) for *T. gondii* infection should be performed in patients with ASD and diagnostic procedures/treatment regimens adequately modified.

## CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

## REFERENCES

- Brown AS. Epidemiologic studies of exposure to prenatal infection and risk of schizophrenia and autism. *Develop Neurobiol.* 2012;72(10):1272–1276.
- Depino AM. Peripheral and central inflammation in autism spectrum disorders. *Mol Cell Neurosci.* 2013;53:69–76.
- Goines PE, Ashwood P. Cytokine dysregulation in autism spectrum disorders (ASD): Possible role of the environment. *Neurotoxicol Teratol.* 2013;36:67–81.
- Matson JL, Kozlowski AM. The increasing prevalence of autism spectrum disorders. *Res Autism Spectr Disord.* 2011;5(1):418–425.
- Baio J. Prevalence of autism spectrum disorder among children aged 8 years – Autism and Developmental Disabilities Monitoring Network, 11 sites, United States, 2010. *MMWR.* 2014;63(2):1–21.
- Samsam M, Ahangari R, Naser SA. Pathophysiology of autism spectrum disorders: revisiting gastrointestinal involvement and immune balance. *World J Gastroenterol.* 2014;20(29):9942–9951.
- Blumberg SJ, Bramlett MD, Kogan MD, Schieve LA, Jones JR, Lu MC. Changes in prevalence of parent-reported autism spectrum disorders in school-aged U.S. children: 2007 to 2011–2012. *Nat Health Stat Rep.* 2013 Mar 20;65:1–11.
- Russell G, Rodgers LR, Ukoumunne OC, Ford T. Prevalence of parent-reported ASD and ADHD in the UK: findings from the Millennium Cohort Study. *J Autism Dev Disord.* 2014;44(1):31–40.
- Delong G. A positive association found between autism prevalence and childhood vaccination uptake across the U.S. population. *J Toxicol Environ Health.* 2011;74(14):903–916.
- Miller NZ, Goldman GS. Infant mortality rates regressed against number of vaccine doses routinely given: is there a biochemical or synergistic toxicity?. *Human Exp Toxicol.* 2011;30(9):1429.
- Carter CJ. Toxoplasmosis and polygenic disease susceptibility genes: Extensive *Toxoplasma gondii* host/pathogen interactome enrichment in nine psychiatric or neurological disorders. *J Pathog.* 2013;965046.
- Yonk LJ, Warren RP, Burger RA, Cole P, Odell JD, Warren WL, White E, Singh VK. CD4+ helper T cell depression in autism. *Immunol Lett.* 1990;25(4):341–345.
- Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. *Ann Neurol.* 2005;57(1):67–81.
- Suzuki K, Matsuzaki H, Iwata K, Kameno Y, Shimmura C, Kawai S, Yoshihara Y, Wakuda T, Takebayashi K, Takagai S, Matsumoto K, Tsuchiya KJ, Iwata Y, Nakamura K, Tsujii M, Sugiyama T, Mori N. Plasma cytokine profiles in subjects with high functioning autism spectrum disorders. *PLoS ONE* 2011;6(5): e20470.
- Gupta S, Aggarwal S, Roshanravan B, Lee T. Th1- and Th2-like cytokines in CD4+ and CD8+ T cells in autism. *J Neuroimmunol.* 1998;85(1):106–109.
- Molloy CA, Morrow AL, Meinen-Derr J, Schleifer K, Dienger K, Manning-Courtney P, Altaye M, Wills-Karp M. Elevated cytokine levels in children with autism spectrum disorder. *J Neuroimmunol.* 2006;172(1-2):198–205.
- Croonenberghs J, Wauters A, Devreese K, Verkerk R, Scharpe S, Bosmans E, Deboutte D, Maes M. Increased serum albumin, gamma globulin, immunoglobulin IgG, and IgG2 and IgG4 in autism. *Psychol Med.* 2002;32(8):1457–1463.
- Santangelo SL, Tsatsanis K. What is known about autism: genes, brain, and behavior. *Am J Pharmacogenom.* 2005;5(2):71–92.
- Benros ME, Mortensen PB, Eaton WW. Autoimmune diseases and infections as risk factors for schizophrenia. *Ann NY Acad Sci.* 2012;1262: 56–66.
- Meyer U, Feldon J, Dammann O. Schizophrenia and autism: both shared and disorder-specific pathogenesis via perinatal inflammation?. *Pediatr Res.* 2011;69(5 Pt 2):26R–33R.
- Watanabe Y, Someya T, Nawa H. Cytokine hypothesis of schizophrenia pathogenesis: evidence from human studies and animal models. *Psychiatry Clin Neurosci.* 2010;64(3):217–230.
- Beaman MH, Hunter CA, Remington JS. Enhancement of intracellular replication of *Toxoplasma gondii* by IL-6. Interactions with IFN-gamma and TNF-alpha. *J Immunol.* 1994;153(10):4583–4587.
- Suzuki Y, Yang Q, Conley FK, Abrams JS, Remington JS. Antibody against interleukin-6 reduces inflammation and numbers of cysts in brains of mice with toxoplasmic encephalitis. *Infect Immun.* 1994;62(7):2773–2778.
- Fang Y, Tan Y, Zhang Y, Li J. Effect of IL-6 on multiplication of *Toxoplasma gondii*. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi.* 1999;17(2):106–108.
- Shapira Y, Agmon-Levin N, Selmi C, Petrikova J, Barzilai O, Ram M, Bizzaro N, Valentin G, Matucci-Cerinic M, Anaya JM, Katz BS, Shoenfeld Y. Prevalence of anti-toxoplasma antibodies in patients with autoimmune diseases. *J Autoimmun.* 2012;39(1-2):112–116.
- Melke J, Goubran Botros H, Chaste P, Betancur C, Nygren G, Anckarsater H, Rastam M, Stahlberg O, Gillberg IC, Delorme R, Chabane N, Mouren-Simeoni MC, Fauchereau F, Durand CM, Chevalier F, Drouot X, Collet C, Launay JM, Gillberg C, Bourgeron T. Abnormal melatonin synthesis in autism spectrum disorders. *Mol Psychiatry* 2008;13(1):90–98.
- Henriquez SA, Brett R, Alexander J, Pratt J, Roberts CW. Neuropsychiatric disease and *Toxoplasma gondii* infection. *Neuroimmunomodulation* 2009;16(2):122–133.
- Pappas G, Roussos N, Falagas ME. *Toxoplasma* snapshots: global status of *Toxoplasma gondii* seroprevalence and implications for pregnancy and congenital toxoplasmosis. *Int J Parasitol.* 2009;39(12):1385–1394.
- Furtado JM, Smith JR, Belfort R Jr, Gattley D, Winthrop KL. Toxoplasmosis: a global threat. *J Glob Infect Dis.* 2011;3(3):281–284.
- Evans AK, Strassmann PS, Lee IP, Sapolsky RM. Patterns of *T. gondii* distribution in the forebrain associate with individual variation in predator odor avoidance and anxiety-related behavior in male Long-Evans rats. *Brain Behav Immun.* 2014;37:122–133.
- Flegr J, Prandota J, Sovickova M, Israili ZH. Toxoplasmosis – a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries. *PLoS ONE* 2014;9(3):e90203.
- Klaren VN, Kijlstra A. Toxoplasmosis, an overview with emphasis on ocular involvement. *Ocul Immunol Inflamm.* 2002;10(1):1–26.

32. Boyer K, Hill D, Mui E, Wroblewski K, Karrison T, Dubey JP, Sautter M, Noble AG, Withers S, Swisher C, Heydemann P, Hosten T, Babiarz J, Lee D, Meier P, McLeod R, Toxoplasmosis Study Group. Unrecognized ingestion of *T. gondii* oocysts leads to congenital toxoplasmosis and causes epidemics in North America. *Clin Infect Dis*. 2011;53(11):1081-1089.
33. Dou Z, McGovern OL, Di Cristina M, Carruthers VB. *Toxoplasma gondii* ingests and digests host cytosolic proteins. *MBio* 2014;5(4):e01188-14.
34. Hermes G, Ajioka JW, Kelly KA, Mui E, Roberts F, Kasza K, Mayr T, Kirisits MJ, Wollmann R, Ferguson DJ, Roberts CW, Hwang JH, Trendler T, Kennan RP, Suzuki Y, Reardon C, Hickey WF, Chen L, McLeod R. Neurological and behavioral abnormalities, ventricular dilatation, altered cellular functions, inflammation, and neuronal injury in brains of mice due to common, persistent, parasitic infection. *J Neuroinflammation* 2008;5:48.
35. Berenreiterova M, Flegr J, Kubena AA, Nemeč P. The distribution of *Toxoplasma gondii* cysts in the brain of a mouse with latent toxoplasmosis. Implications of the behavioral manipulation hypothesis. *PLoS One* 2011;6(12):e28925.
36. Melzer TC, Cranston HJ, Weiss LM, Halonen SK. Host cell preference of *Toxoplasma gondii* cysts in murine brain: A confocal study. *J Neuroparasitology* 2010;1.pii: N100505.
37. Wang T, Liu M, Gao X-J, Zhao Z-J, Chen X-G, Lun ZR. *Toxoplasma gondii*: the effects of infection at different stages of pregnancy on the offspring of mice. *Exp Parasitol*. 2011;127(1):107-112.
38. Lüder CG, Giraldo-Velasquez M, Sendtner M, Gross U. *Toxoplasma gondii* in primary rat CNS cells: differential contribution of neurons, astrocytes, and microglial cells for the intracerebral development and stage differentiation. *Exp Parasitol*. 1999;93(1):23-32.
39. Conley FK, Jenkins KA. Immunohistological study of the anatomic relationship of *Toxoplasma* antigens to the inflammatory response in the brains of mice chronically infected with *Toxoplasma gondii*. *Infect Immun*. 1981;31(3):1184-1192.
40. Stibbs HH. Changes in brain concentrations of catecholamines and indoleamines in *Toxoplasma gondii* infected mice. *Ann Trop Med Parasitol*. 1985;79(2):153-157.
41. Ferguson DJ, Hutchison WM. An ultrastructural study of the early development and tissue cyst formation of *Toxoplasma gondii* in the brains of mice. *Parasitol Res*. 1987;73(6):483-491.
42. McNaught KSP, Brown GC. Nitric oxide causes glutamate release from brain synaptosomes. *J Neurochem*. 1998;70(4):1541-1546.
43. Feinstein DL. Potentiation of astroglial nitric oxide synthase type-2 expression by lithium chloride. *J Neurochem*. 1998;71(2):883-886.
44. Fagard R, Van Tan H, Creuzet C, Pelloux H. Differential development of *Toxoplasma gondii* in neural cells. *Parasitol Today* 1999;15(12): 504-507.
45. Silva NM, Rodrigues CV, Santoro MM, Reis LFL, Alvarez-Leite JJ, Gazzinelli RT. Expression of indoleamine 2,3-dioxygenase, tryptophan degradation and kynurenine formation during in vivo infection with *Toxoplasma gondii*: induction of endogenous gamma interferon and requirement of interferon regulatory factor 1. *Infect Immun*. 2002;70(2):859-868.
46. Dunn AJ, Swiergiel AH, de Beaupaire R. Cytokines as mediators of depression: what can we learn from animal studies?. *Neurosci Biobehav Rev*. 2005;29(4-5):891-909.
47. Prandovszky E, Gaskell E, Martin H, Dubey JP, Webster JP, McConkey GA. The neurotropic parasite *Toxoplasma gondii* increases dopamine metabolism. *PLoS ONE* 2011;6(9):e23866.
48. Strobl JS, Goodwin DG, Rzigalinski BA, Lindsay DS. Dopamine stimulates propagation of *Toxoplasma gondii* tachyzoites in human fibroblast and primary neonatal rat astrocyte cell cultures. *J Parasitol*. 2012;98(6):1296-1298.
49. Haroon F, Händel U, Angenstein F, Goldschmidt J, Kreutzmann P, Lison H, Fischer KD, Scheich H, Wetzel W, Schluter D, Budinger E. *Toxoplasma gondii* actively inhibits neuronal function in chronically infected mice. *PLoS One* 2012;7(4):e35516.
50. Fond G, Capdevielle D, Macgregor A, Attal J, Larue A, Brittner M, Ducasse D, Boulenger JP. *Toxoplasma gondii* : a potential role in the genesis of psychiatric disorders. *Encephale* 2013;39(1):38-43.
51. Wang T, Zhou J, Gan X, Wang H, Ding X, Chen L, Wang Y, Du J, Shen J, Yu L. *Toxoplasma gondii* induce apoptosis of neural stem cells via endoplasmic reticulum stress pathway. *Parasitology* 2014;141(7):988-995.
52. Prandota J. Neuropathological changes and clinical features of autism spectrum disorders are similar to that reported in congenital and chronic cerebral toxoplasmosis in humans and mice. *Res Autism Spectr Disord*. 2009;4(2):103-118.
53. Prandota J. Autism spectrum disorders may be due to cerebral toxoplasmosis associated with chronic neuroinflammation causing persistent hypercytokinemia that resulted in an increased lipid peroxidation, oxidative stress, and depressed metabolism of endogenous and exogenous substances. *Res Autism Spectr Disord*. 2010;4(2):119-155.
54. Prandota J. Metabolic, immune, epigenetic, endocrine and phenotypic abnormalities found in individuals with autism spectrum disorders, Down syndrome and Alzheimer disease may be caused by congenital and/or acquired chronic cerebral toxoplasmosis. *Res Autism Spectr Disord*. 2011;5(1):14-59.
55. Bories PN, Bories C. Nitrate determination in biological fluids by an enzymatic one-step assay with nitrate reductase. *Clin Chem*. 1995;41(6):904-907.
56. Rellini E, Tortolani D, Trillo S, Carbone S, Montecchi F. Childhood Autism Rating Scale (CARS) and Autism Behavior Checklist (ABC) correspondence and conflicts with DSM-IV criteria in diagnosis of Autism. *J Autism Dev Disord*. 2004;34(6):703-708.
- 56a. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annu Rev Immunol*. 1997;15:749-795.
- 56b. Blasko I, Marx F, Steiner E, Hartmann T, Grubeck-Loebenstien B. TNFalpha plus IFNgamma induce the production of Alzheimer beta-amyloid peptides and decrease the secretion of APPs. *FASEB J*. 1999;13(1):63-68.
- 56c. Blasko I, Ransmayr G, Veerhuis R, Eikelenboom P, Grubeck-Loebenstien B. Does IFN-g play a role in neurodegeneration?. *J Neuroimmunol*. 2001;116(1):1-4.
57. Warren RP, Yonk LJ, Burger RA, Cole P, Odell JD, Warren WL, White E, Singh VK. Deficiency of suppressor-inducer (CD4+CD45RA+) T cells in autism. *Immunol Invest*. 1990;19(3):245-251.
58. Singh VK. Plasma increase of interleukin IL-12 and interferon-gamma. Pathological significance in autism. *J Neuroimmunol*. 1996;66(1-2):143-145.
59. Jyonouchi H, Sun S, Le H. Proinflammatory and regulatory cytokine production associated with innate and adaptive immune responses in children with autism spectrum disorders and developmental regression. *J Neuroimmunol*. 2001;120(1-2):170-179.
60. Chez MG, Dowling T, Patel P, Khanna P, Kominsky M. Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. *Pediatr Neurol*. 2007;36(6):361-365.
61. Li X, Chauhan A, Sheikh AM, Patil S, Chauhan V, Li XM, Brown T, Malik M. Elevated immune response in the brain of autistic patients. *J Neuroimmunol*. 2009;207(1-2): 111-116.
62. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah IN, Van de Water J. Altered T cell responses in children with autism. *Brain Behav Immun*. 2011;25(5):840-849.
63. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah IN, Van de Water J. Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. *Brain Behav Immun*. 2011;25(1):40-45.
64. Warren RP, Foster A, Margaretten NC. Reduced natural killer

- cell activity in autism. *J Am Acad Child Adolesc Psychiatry* 1987;26(3):333-335.
65. Schaechter JD, Wurtman RJ. Serotonin release varies with brain tryptophan levels. *Brain Res.* 1990;532(1-2):203-210.
  66. Rolf LH, Haarmann EY, Grottemeyer KH, Kehrer H. Serotonin and amino acid content in platelets of autistic children. *Acta Psychiatr Scand.* 1993;87(5): 312-316.
  67. D'Euphemia P, Finocchiaro R, Celli M, Viozzi L, Monteleone D, Giardini O. Low serum tryptophan to large neutral amino acids ratio in idiopathic infantile autism. *Biomed Pharmacol.* 1995;49(6):288-292.
  68. McDougle CJ, Naylor ST, Cohen DJ, Aghajanian GK, Heninger GR, Price LH. Effects of tryptophan depletion in drug-free adults with autistic disorders. *Arch General Psych.* 1996;53(11):993-1000.
  69. Kulman G, Lissoni P, Rovelli MG, Brivio F, Sequeri P. Evidence of pineal endocrine hypofunction in autistic children. *Neuro Endocrinol Lett.* 2000;21(1): 31-34.
  70. Johansson J, Landgren M, Fernell E, Vumma R, Ahlin A, Bjerkenstedt L, Venizelos N. Altered tryptophan and alanine transport in fibroblasts from boys with attention-deficit/hyperactivity disorder (ADHD): an in vitro study. *Behav Brain Funct.* 2011;7:40.
  71. Ratajczak HV. Theoretical aspects of autism: biomarkers – a review. *J Immunotoxicol.* 2011;8(1):80-94.
  72. Boccuto L, Chen CF, Pittman AR, Skinner CD, McCartney HJ, Jones K, Bochner BR, Stevenson RE, Schwartz CE. Decreased tryptophan metabolism in patients with autism spectrum disorders. *Mol Autism* 2013;4(1):16.
  73. Notarangelo FM, Wilson EH, Horning KJ, Thomas MAR, Harris TH, Fang Q, Hunter CA, Schwarcz R. Evaluation of kynurenine pathway metabolism in *Toxoplasma gondii*-infected mice: Implications for schizophrenia. *Schizophr Res.* 2014;152(1): 261-267.
  74. Stone TW, Darlington LG. Endogenous kynurenines as targets for drug discovery and development. *Nat Rev Drug Discov.* 2002;1(8):609-620.
  75. Schwartz CE. Aberrant tryptophan metabolism: the unifying biochemical basis for autism spectrum disorders?. *Biomarkers Med.* 2014;8(3):313-315.
  76. Kanai M, Funakoshi H, Takahashi Hayakawa T, Mizuno S, Matsumoto K, Nakamura T. Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice. *Mol Brain* 2009;2:8.
  77. Funakoshi H, Kanai M, Nakamura T. Modulation of tryptophan metabolism, promotion of neurogenesis and alteration of anxiety-related behavior in tryptophan 2,3-dioxygenase-deficient mice. *Int J Tryptophan Res.* 2011;4: 7-18.
  78. Pfefferkorn ER. Interferon gamma blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc Natl Acad Sci USA* 1984;81(3):908-912.
  79. Forrest CM, Mackay GM, Stoy N, Egerton M, Christofides J, Stone TW. Tryptophan loading induces oxidative stress. *Free Radic Res.* 2004;38(11): 1167-1171.
  80. Yolken RH, Bachmann S, Rouslanova I, Lillehoj E, Ford G, Torrey EF, Schroeder J. Antibodies to *Toxoplasma gondii* in individuals with first-episode schizophrenia. *Clin Infect Dis.* 2001;32(5):842-844.
  81. Emelia O, Amal RN, Ruzanna ZZ, Shahida H, Azzubair Z, Tan KS, Noor Aadila S, Siti NA, Aisah MY. Seroprevalence of anti-*Toxoplasma gondii* IgG antibody in patients with schizophrenia. *Trop Biomed.* 2012;29(1):151-159.
  82. Suzuki Y. Genes, cells and cytokines in resistance against development of toxoplasmic encephalitis. *Immunobiology* 1999;201(2):255-271.
  83. Butcher BA, Kim L, Johnson PF, Denkers EY. *Toxoplasma gondii* tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NF- $\kappa$ B. *J Immunol.* 2001;167(4):2193-2201.
  84. Suzuki Y. Immunopathogenesis of cerebral toxoplasmosis. *J Infect Dis.* 2002;186 Suppl 2:S234-S240.
  85. James SL. Role of nitric oxide in parasitic infections. *Microbiol Rev.* 1995;59(4):533-547.
  86. Toth C. Medications and substances as a cause of headache: a systematic review of the literature. *Clin Neuropharmacol.* 2003;26(3):122-136.
  87. Roland CR, Walp L, Stack RM, Flye MW. Outcome of Kupffer cell antigen presentation to a cloned murine Th1 lymphocyte depends on the inducibility of nitric oxide synthase by IFN- $\gamma$ . *J Immunol.* 1994;153(12):5453-5464.
  88. Sicher SC, Vazquez MA, Lu CY. Inhibition of macrophage Ia expression by nitric oxide. *J Immunol.* 1994;153(3):1293-1300.
  89. Thomas SR, Mohr D, Stocker R. Nitric oxide inhibits indoleamine 2,3-dioxygenase activity in interferon- $\gamma$  primed mononuclear phagocytes. *J Biol Chem.* 1994;269(20):14457-14464.
  90. Harhaji L, Vuckovic O, Miljkovic D, Stosic-Grujicic S, Trajkovic V. Iron down-regulates macrophage anti-tumour activity by blocking nitric oxide production. *Clin Exp Immunol.* 2004;137(1):109-116.
  91. Burke JM, Roberts CW, Hunter CA, Murray M, Alexander J. Temporal differences in the expression of mRNA for IL-10 and IFN- $\gamma$  in the brains and spleens of C57BL/10 mice infected with *Toxoplasma gondii*. *Parasite Immunol.* 1994;16(6):305-314.
  92. Welch KM, Nagesh V, Aurora SK, Gelman N. Periaqueductal gray matter dysfunction in migraine: cause or the burden of illness? *Headache* 2001;41(7):629-637.
  93. Harbrecht BG, Kim YM, Wirant EM, Shapiro RA, Billiar TR. PGE2 and LTB4 inhibit cytokine-stimulated nitric oxide synthase type 2 expression in isolated rat hepatocytes. *Prostaglandins* 1996;52(2):103-116.
  94. Harbrecht BG, Kim YM, Wirant EA, Simmons RL, Billiar TR. Timing of prostaglandin exposure is critical for the inhibition of LPS- or IFN- $\gamma$ -induced macrophage NO synthesis by PGE2. *J Leukoc Biol.* 1997;61(6):712-720.
  95. Griffon B, Cillard J, Chevanne M, Morel I, Cillard B, Sergent O. Macrophage-induced inhibition of nitric oxide production in primary rat hepatocyte cultures via prostaglandin E2 release. *Hepatology* 1998;28(5): 1300-1308.
  96. Inoue T, Fukuo K, Morimoto S, Koh E, Ogihara T. Nitric oxide mediates interleukin-1-induced prostaglandin E2 production by vascular smooth muscle cells. *Biochem Biophys Res Commun.* 1993;194(1):420-424.
  97. Bohne W, Heesemann J, Gross U. Reduced replication of *Toxoplasma gondii* is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. *Infect Immunol.* 1994;62(5): 1761-1767.
  98. Phipps RP, Stein SH, Roper RL. A new view of prostaglandin E regulation of the immune response. *Immunol Today* 1991;12(10):349-352.
  99. Miles EA, Aston L, Calder PC. In vitro effects of eicosanoids derived from different 20-carbon fatty acids on T helper type 1 and T helper type 2 cytokine production in human whole-blood cultures. *Clin Exp Allergy* 2003;33(5):624-632.
  100. Rozenfeld C, Martinez R, Figueiredo RT, Bozza MT, Lima FR, Pires AL, Silva PM, Bonomo A, Lannes-Vieira J, De Souza W, Moura-Neto V. Soluble factors release by *Toxoplasma gondii*-infected astrocytes down-modulate nitric oxide production by gamma interferon-activated microglia and prevent neuronal degeneration. *Infect Immun.* 2003;71(4):2047-2057.
  101. Aloisi F, De Simone R, Columba-Cabezas S, Levi G. Opposite effects of interferon- $\gamma$  and prostaglandin E2 on tumor necrosis factor and interleukin-10 production in microglia: a regulatory loop controlling microglia pro- and anti-inflammatory activities. *J Neurosci Res.* 1999;56(6): 571-580.
  102. Franchi AM, Gimeno MF, Cardinali DP, Vacas MI. Melatonin, 5-methoxytryptamine and some of their analogues as cyclo-



- oxygenase inhibitors in rat medial basal hypothalamus. *Brain Res.* 1987;405(2):384-388.
103. Widner B, Weiss G, Fuchs D. Tryptophan degradation to control T-cell responsiveness. *Immunol Today* 2000;21(5):250.
  104. Frumento G, Rotondo R, Tonetti M, Damonte G, Benatti U, Ferrara GB. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *J Exp Med.* 2002;196(4):459-468.
  105. Griscavage JM, Rogers NE, Sherman MP, Ignarro LJ. Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide. *J Immunol.* 1993;151(11):6329-6337.
  106. Sekkai D, Guitted O, Lemaire G, Tenu JP, Lepoivre M. Inhibition of nitric oxide synthase expression and activity in macrophages by 3-hydroxyanthranilic acid, a tryptophan metabolite. *Arch Biochem Biophys.* 1997;340(1):117-123.
  107. Chao CC, Anderson WR, Hu S, Gekker G, Martella A, Peterson PK. Activated microglia inhibit multiplication of *Toxoplasma gondii* via a nitric oxide mechanism. *Clin Immunol Immunopathol.* 1993;67(2):178-183.
  108. Peterson PK, Gekker G, Hu S, Chao CC. Intracellular survival and multiplication of *Toxoplasma gondii* in astrocytes. *J Infect Dis.* 1993;168(6): 1472-1478.
  109. Creuzet C, Robert F, Roisin MP, Van Tan H, Benes C, Dupouy-Camet J, Fagard R. Neurons in primary culture are less efficiently infected by *Toxoplasma gondii* than glial cells. *Parasitol Res.* 1998;84(1):25-30.
  110. Halonen SK, Lyman WD, Chiu FC. Growth and development of *Toxoplasma gondii* in human neurons and astrocytes. *J Neuro-pathol Exp Neurosci.* 1996;55(11):1150-1156.
  111. Jones TC, Bienz KA, Erb P. In vitro cultivation of *Toxoplasma gondii* cysts in astrocytes in the presence of gamma interferon. *Infect Immun.* 1986;51(1): 147-156.
  112. Fischer HG, Nitzgen B, Reichmann G, Gross U, Hadding U. Host cells of *Toxoplasma gondii* encystation in infected primary culture from mouse brain. *Parasitol Res.* 1997;83(7):637-641.
  113. Fischer HG, Nitzgen B, Reichmann G, Hadding U. Cytokine responses induced by *Toxoplasma gondii* in astrocytes and microglial cells. *Eur J Immunol.* 1997;27(6):1539-1548.
  114. Däubener W, Remscheid C, Nockemann S, Pilz K, Seghrouchni S, Mackenzie C, Hadding U. Anti-parasitic effector mechanisms in human brain tumor cells: role of interferon-gamma and tumor necrosis-alpha. *Eur J Immunol.* 1996;26(2):487-492.
  115. Minc-Golomb D, Tsarfaty I, Schwartz JP. Expression of inducible nitric oxide synthase by neurons following exposure to endotoxin and cytokine. *Brit J Pharmacol.* 1994;112(3):720-722.
  116. Minamitani M, Tanaka J, Suzuki Y. Pathomechanism of cerebral hypoplasia in experimental toxoplasmosis in murine fetuses. *Early Human Dev* 1996;44(1):37-50.
  117. Suzuki Y, Orellana MA, Schreiber RD, Remington JS. Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science* 1988;240:516-518.
  118. Suzuki Y, Kobayashi A. Induction of tolerance to *Toxoplasma gondii* in newborn mice by maternal antibody. *Parasitol Res.* 1990;76(5):424-427.
  119. Gazzinelli R, Hakim FT, Hieny S, Shearer GM, Sher A. Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J Immunol.* 1991;146(1):286-292.
  120. Denkers EY, Gazzinelli RT. Regulation of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev.* 1998;11:565-588.
  121. Denkers EY, Gazzinelli RT, Martin D, Sher A. Emergence of NK1.1+ cells as effectors of IFN-gamma dependent immunity to *Toxoplasma gondii* in MHC class I-deficient mice. *J Exp Med.* 1993;178(5):1465-1472.
  122. Johnson LL, VanderVegt FP, Havell EA. Gamma interferon-dependent temporary resistance to acute *Toxoplasma gondii* infection independent of CD4+ or CD8+ lymphocytes. *Infect Immunol.* 1993;61(12):5174-5180.
  123. Sher A, Oswald IP, Hieny S, Gazzinelli RT. *Toxoplasma gondii* induces a T-independent IFN-gamma response in natural killer cells that requires both adherent accessory cells and tumor necrosis factor alpha. *J Immunol.* 1993;150(9):3982-3989.
  124. Hunter CA, Subauste CS, Van Cleave VH, Remington JS. Production of gamma interferon by natural killer cells from *Toxoplasma gondii*-infected SCID mice: regulation by interleukin-10, interleukin-12, and tumor necrosis factor alpha. *Infect Immun.* 1994;62(7):2818-2824.
  125. Dupont CD, Christian DA, Hunter CA. Immune response and immunopathology during toxoplasmosis. *Semin Immunopathol.* 2012;34(6): 793-813.
  126. Adams LB, Hibbs JB Jr, Taintor RR, Krahenbuhl JL. Microbiostatic effect of murine-activated macrophage for *Toxoplasma gondii*. Role for synthesis of inorganic nitrogen oxide synthase gene in cultured vascular smooth muscle cells. *J Immunol.* 1990;144(7):2725-2729.
  127. Gazzinelli RT, Xu Y, Hieny S, Cheever A, Sher A. Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *J Immunol.* 1992;149(1):175-180.
  128. Taylor GA, Collazo CM, Yap GS, Nguyen K, Gregorio TA, Taylor LS, Eagleson B, Secrest L, Southon EA, Reid SW, Tessarollo L, Bray M, McVicar DW, Komschlies KL, Young HA, Biron CA, Sher A, Vande Woude GF. Pathogen-specific loss of host resistance in mice lacking the IFN-gamma-inducible gene IGTP. *Proc Natl Acad Sci USA* 2000;97(2):751-755.
  129. Howard JC, Hunn JP, Steinfeldt T. The IRG protein-based resistance mechanism in mice and its relation to virulence in *Toxoplasma gondii*. *Curr Opin Microbiol.* 2011;14(4):414-421.
  130. Murray HW, Szuro-Sudol A, Wellner D, Oca MJ, Granger AM, Libby DM, Rothermel CD, Rubin BY. Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma-interferon stimulated human macrophages. *Infect Immun.* 1989;57(3):845-849.
  131. Däubener W, Spors B, Hücke C, Adam R, Stins M, Kim KS, Schroten H. Restriction of *Toxoplasma gondii* growth in human brain microvascular endothelial cells by activation of indoleamine 2,3-dioxygenase. *Infect Immun.* 2001;69(10):6527-6531.
  132. Anderson SE, Remington JS. Effect of normal and activated human macrophages on *Toxoplasma gondii*. *J Exp Med.* 1974;139(5):1154-1174.
  133. Gazzinelli RT, Amichay D, Schar-ton-Kersten T, Grunvald E, Farber JM, Sher A. Role of macrophage-derived cytokines in the induction and regulation of cell mediated immunity to *Toxoplasma gondii*. *Curr Top Microbiol Immunol.* 1996;219:127-140.
  134. MacKenzie CR, Gonzalez RG, Kniep E, Roch S, Däubener W. Cytokine mediated regulation of interferon-gamma-induced IDO activation. *Adv Exp Med Biol.* 1999;467:533-539.
  135. Tait ED, Jordan KA, Dupont CD, Harris TH, Gregg B, Wilson EH, Pepper M, Dzierszynski F, Roos DS, Hunter CA. Virulence of *Toxoplasma gondii* is associated with distinct dendritic cell response and reduced numbers of activated CD8+ T cells. *J Immunol.* 2010;185(3):1502-1512.
  136. Zoroğlu SS, Yürekli M, Meram I, Söğüt S, Tutkun H, Yetkin O, Savas HA, Yanik M, Herken H, Akyol O. Pathophysiological role of nitric oxide and adrenomedullin in autism. *Cell Biochem Funct.* 2003;21(1):55-60.
  137. Söğüt S, Zoroğlu SS, Ozyurt H, Yılmaz HR, Ozuğurlu F, Sivasli E, Yetkin O, Yanik M, Tulkun H, Savas HA, Karakcioglu M, Akyol O. Changes in nitric oxide levels and antioxidant enzyme activities may have a role in the pathophysiological mechanisms involved in autism. *Clin Chim Acta* 2003;331(1-2):111-117.
  138. Sweeten TL, Posey DJ, Shankar S, McDougale CJ. High nitric oxide production in autistic disorder. A possible role for interferon-



- gamma. *Biol Psych.* 2004;55(4):434-437.
139. Cohen BI. Ammonia (NH<sub>3</sub>), nitric oxide (NO) and nitrous oxide (N<sub>2</sub>O) – the connection with infantile autism. *Autism* 2006;10(2):221-223.
  140. Moshage H, Kok B, Huizenge R, Jansen PL. Nitrite and nitrate determinations in plasma: A critical evaluation. *Clin Chem.* 1995;41(6 Pt 1):892-896.
  141. Palmieri L, Perlico AM. Mitochondrial dysfunction in autism spectrum disorders: Cause or effect ?. *Biochim Biophys Acta* 2010;1797(6-7):1130-1137.
  142. Kilbourn RG, Belloni P. Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. *J Natl Cancer Inst.* 1990;82(9):772-776.
  143. Nussler AK, Di Silvio M, Billiar TR, Hoffman RA, Geller DA, Selby R, Madariaga J, Simmons RL. Stimulation of the nitric oxide synthase pathway in human hepatocytes by cytokine and endotoxin. *J Exp Med.* 1992;176(1):261-264.
  144. Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cells: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science* 1973;180(4093)1332-1339.
  145. Diamond JR, Karnovsky MJ. Focal and segmental glomerulosclerosis: analogies to atherosclerosis. *Kidney Int.* 1988;33(5):917-924.
  146. Okayama N, Kevil CG, Correia L, Jourd'heuil D, Itoh M, Grisham MB, Alexander JS. Nitric oxide enhances hydrogen peroxide-mediated endothelial permeability in vitro. *Am J Physiol.* 1997;273(5 Pt 1):C1581-C1587.
  147. Grisham MB, Specian RD, Zimmerman TE. Effect of nitric oxide synthase inhibition on pathophysiology observed in a model of chronic granulomatous colitis. *J Pharmacol Exp Ther.* 1994;271(2):1114-1121.
  148. Henry Y, Lepoivre M, Drapier JC, Ducrocq C, Boucher JL, Guisani A. EPR characterization of molecular targets for NO in mammalian cells and organelles. *FASEB J.* 1993;7(12):1124-1134.
  149. Hunot S, Boissiere F, Fauchoux B, Brugg B, Monatt-Prigeut A, Agid Y, Hirsch EC. Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. *Neuroscience* 1996;72(2):355-363.
  150. Adegate E, Parvez SH. Nitric oxide and neuronal and pancreatic beta cell death. *Toxicology* 2000;153(1-3):143-156.
  151. Bal-Price A, Borutaite V, Brown GC. Mitochondria mediate nitric oxide-induced cell death. *Ann NY Acad Sci.* 1999;893:376-378.
  152. Parlog A, Schlüter D, Dunay IR. *Toxoplasma gondii* induced neuronal alterations. *Parasite Immunol.* 2015;37(3):159-170.
  153. Reif DW. Delayed production of nitric oxide contributes to NMDA-mediated neuronal damage. *Neuroreport* 1993;4(5):566-568.
  154. Weikert S, Freyer D, Weih M, Isaev N, Busch C, Schultze J, Megow D, Dimagl U. Rapid Ca<sup>2+</sup>-dependent NO-production from central nervous system cells in culture measured by NO-nitrite/zone chemoluminescence. *Brain Res.* 1997;748(1-2):1-11.
  155. Brown GC, Bolanos JP, Heales SJR, Clark JB. Nitric oxide produced by activated astrocytes rapidly and reversibly inhibits cellular respiration. *Neurosci Lett.* 1995;193(3):201-204.
  156. Brown GC, Foxwell N, Moncada S. Transcellular regulation of cell respiration by nitric oxide generated by activated macrophages. *FEBS Lett.* 1998;439(3):321-324.
  157. Pollak Y, Yirmiya R. Cytokine-induced changes in mood and behavior: Implications for depression due to a general medical condition, immunotherapy and antidepressive treatment. *Int J Neuropsychopharmacol.* 2002;5(4):389-399.
  158. Choi BM, Pae HO, Jang S II, Kim YM, Chung HT. Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. *J Biochem Mol Biol.* 2002;35(1): 116-126.
  159. Pua HH, Guo J, Komatsu M, He YW. Autophagy is essential for mitochondrial clearance in mature T lymphocytes. *J Immunol.* 2009;182(7): 4046-4055.
  160. Unno N, Menconi MJ, Smith M, Fink MP. Nitric oxide mediates interferon gamma-induced hyperpermeability in cultured human intestinal epithelial monolayers. *Crit Care Med.* 1995;23(7):1170-1176.
  161. Xu DZ, Lu Q, Deitch EA. Nitric oxide directly impairs barrier function. *Shock* 2002;17(2):139-145.
  162. D'Euphemia P, Celli M, Finocchiaro R, Pacifico L, Viozzi L, Zaccagnini M, Cardi E, Giardini O. Abnormal intestinal permeability in children with autism. *Acta Paediatr.* 1996;85(9):1076-1079.
  163. Salzman AL, Menconi MJ, Unno N, Ezzell RM, Casey DM, Gonzales PK, Fink MP. Nitric oxide dilates tight junctions and deplete ATP in cultured CaCo-2BBE intestinal epithelial monolayers. *Am J Physiol.* 1995;268(2 Pt 1):G361-G373.
  164. Coleman JW. NO is also a regulator of mast cell activation and mast cell-mediated inflammation *Clin Exp Immunol.* 2002;129(1):4-10.
  165. Danford DE, Smith CJ, Huber AM. Pica and mineral status in the mentally retarded. *Am J Clin Nutr.* 1982;35(5):958-967.
  166. Arbitr EA, Black D. Pica and iron deficiency anaemia. *Child Care Health Dev.* 1991;17(4):231-234.
  167. Matson JL, Hattier MA, Belva B, Matson ML. Pica in persons with developmental disabilities: approach to treatment. *Res Dev Disabil.* 2013;34(9):2564-2571.
  168. Bogdan C. The multiple function of nitric oxide in (auto)immunity. *J Exp Med.* 1998;187(9):1361-1365.
  169. Bogdan C. Nitric oxide synthase in innate and adaptive immunity: an update. *Trends Immunol.* 2015;36(3):161-178.
  170. McQuaid KE, Smyth EM, Keenan AK. Evidence for modulation of hydrogen peroxide-induced endothelial barrier dysfunction by nitric oxide in vitro. *Eur J Pharmacol.* 1996;307(2):233-241.
  171. Khan AU, Kasha M. Singlet molecular oxygen evolution upon simple acidification of aqueous hypochlorite: Application to studies on the deleterious health effects of chlorinated drinking water. *Proc Natl Acad Sci USA* 1994;91(26):12362-12364.
  172. Farias-Eisner R, Chaudhuri G, Aeberhard E, Fukuto JM. The chemistry and tumoricidal activity of nitric oxide/hydrogen peroxide and the implications to cell resistance/susceptibility. *J Biol Chem.* 1996;271(11):6144-6151.
  173. Unno N, Hodin RA, Fink MP. Acidic conditions exacerbate interferon-gamma-induced intestinal hyperpermeability: role of peroxynitrous acid. *Crit Care Med.* 1999;27(8):1429-1436.
  174. Okayama N, Grisham MB, Kevil CG, Eppihimer LA, Wink DA, Alexander JS. Effect of reactive oxygen metabolites on endothelial permeability: role of nitric oxide and iron. *Microcirculation* 1999;6(2):107-116.
  175. Watts RN, Richardson DR. The mechanism of nitrogen monoxide (NO)-mediated iron mobilization from cells. NO intercepts iron before incorporation into ferritin and indirectly mobilizes iron from ferritin in a glutathione-dependent manner. *Eur J Biochem.* 2002;269(14):3383-3392.
  176. Dimier IH, Bout DT. Interferon-gamma activated primary enterocytes inhibit *Toxoplasma gondii* replication: a role for intracellular iron. *Immunology* 1998;94(4):488-495.
  177. Weiss G, Werner-Felmayer G, Werner ER, Grünewald K, Wachter H, Hentze MW. Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J Exp Med.* 1994;180(3):969-976.
  178. Nairz M, Haschka D, Demetz E, Weiss G. Iron at the interface of immunity and infection. *Front Pharmacol.* 2014;5:152
  179. Amin SB, Myers G, Wang H. Association between neonatal iron overload and early human brain development in premature infants. *Early Human Dev.* 2012;88(8):583-587.
  180. Gazzinelli RT, Hieny S, Wynn T, Wolf S, Sher A. IL-12 is required for the T lymphocyte-independent induction of interferon-gamma by an intracellular parasite and induces resistance in T-cell-deficient hosts. *Proc Natl Acad Sci USA* 1993;90(13):6115-6119.

181. Hunter CA, Bermudez L, Beernink H, Waegell W, Remington JS. Transforming growth factor-beta inhibits interleukin 12 induced production of interferon-gamma by natural killer cells: a role for transforming growth factor-beta in the regulation of T-cell-independent resistance to *Toxoplasma gondii*. *Eur J Immunol*. 1995;25(4):994-1000.
182. Sher A, Sousa CR. Ignition of the type I response to intracellular infection by dendritic cell-derived interleukin-12. *Eur Cytokine Netw*. 1998;9(3 Suppl):65-68.
183. Miller CM, Smith NC, Johnson AM. Cytokines, nitric oxide, heat shock proteins and virulence in *Toxoplasma*. *Parasitol Today* 1999;15(10):418-422.
184. Pfefferkorn ER, Eckel M, Rebhun S. Interferon-gamma suppresses the growth of *Toxoplasma gondii* in human fibroblasts through starvation for tryptophan. *Mol Biochem Parasitol*. 1986;20(3):215-224.
185. Sibley LD, Adams LB, Funkutomi Y, Krahenbuhl JL. Tumour necrosis factor-alpha triggers antitoxoplasmal activity by IFN-gamma primed macrophages. *J Immunol*. 1991;147(7):2340-2345.
186. Gazzinelli RT, Wysocka M, Hayashi S, Denkers EY, Hieny S, Caspar P, Trinchieri G, Sher A. Parasite-induced IL12 stimulates early IFN-gamma synthesis and resistance during acute infection with *Toxoplasma gondii*. *J Immunol*. 1994;153(6):2533-2543.
187. Langermans JAM, van der Hulst ME, Nibbering PH, Hiemstra PS, Fransen L, Van Furth R. IFN-gamma induced L-arginine-dependent toxoplasmastatic activity in murine peritoneal macrophages is mediated by endogenous tumour necrosis factor-alpha. *J Immunol*. 1992;148(2):568-574.
188. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem*. 1994;269(7):4705-4708.
189. Gomez-Marin JE. No NO production during human *Toxoplasma* infection. *Parasitol Today (Letters)* 2000;16(3):131.
190. Ghigo D, Todde R, Ginsburg H, Costamagna C, Gautret P, Busso-lino F, Ulliers D, Giribaldi G, Deharo E, Gabrielli G, Pescarmona G, Bosia A. Erythrocyte stages of *Plasmodium falciparum* exhibit a high nitric oxide synthase (NOS) activity and release an NOS inducing soluble factor. *J Exp Med*. 1995;182(3):677-688.
191. Paveto C, Pereira C, Espinosa J, Montagna AE, Farber M, Esteve M, Flawia MM, Torres HN. The nitric oxide transduction pathway in *Trypanosoma cruzi*. *J Biol Chem*. 1995;270(28):16576-16579.
192. Minamiyama Y, Takemura S, Imaoka S, Funae Y, Tanimoto Y, Inoue M. Irreversible inhibition of cytochrome P450 by nitric oxide. *J Pharmacol Exp Ther*. 1997;283(3):1479-1485.
193. Khatsenko O, Kikkawa Y. Nitric oxide differentially affects constitutive cytochrome P450 isoforms in rat liver. *J Pharmacol Exp Ther*. 1997;280(3):1463-1470.
194. Gergel D, Misik V, Riesz P, Cederbaum AI. Inhibition of rat and human cytochrome P4502E1 catalytic activity and reactive oxygen radical formation by nitric oxide. *Arch Biochem Biophys*. 1997;337(2):239-250.
195. Catani MV, Bernassola F, Rossi A, Melino G. Inhibition of clotting factor XIII activity by nitric oxide. *Biochem Biophys Res Commun*. 1998;249(1):275-278.
196. Bernassola F, Rossi A, Melino G. Regulation of transaminases by nitric oxide. *Ann NY Acad Sci*. 1999;887:83-91.
197. Sigfrid LA, Cunningham JM, Beeharry N, Lortz S, Tiedge M, Lenzen S, Carlsson C, Green IC. Cytokines and nitric oxide inhibit the enzyme activity of catalase but not its protein or mRNA expression in insulin-producing cells. *J Mol Endocrinol*. 2003;31(3):509-518.
198. Morgan ET. Regulation of cytochrome P450 during inflammation and infection. *Drug Metab Rev*. 1997;29(4):1129-1188.
199. Khatsenko OG, Gross SS, Rifkind AB, Vane JR. Nitric oxide is a mediator of the decrease in cytochrome P45-dependent metabolism caused by immunostimulants. *Proc Natl Acad Sci USA* 1997;90(23):11147-11151.
200. Li Z-Y, Manthey CL, Perera PY, Sher A, Vogel SN. *Toxoplasma gondii* soluble antigen induces a subset of lipopolysaccharide-inducible genes and tyrosine phosphoproteins in peritoneal macrophages. *Infect Immun*. 1994;62(8):3434-3440.
201. Morgan ET. Regulation of cytochrome P450 by inflammatory mediators: why and how?. *Drug Metab Dispos*. 2001;29(3):207-212.
202. Tindberg N, Baldwin HA, Cross AJ, Ingelman-Sundberg M. Induction of cytochrome P450 2E1 expression in rat and gerbil astrocytes by inflammatory factors and ischemic injury. *Mol Pharmacol*. 1996;50(5):1065-1072.
203. Shimamoto Y, Kitamura H, Hoshi H, Kazusaka A, Funae Y, Imaoka S, Saito M, Fujita S. Differential alterations in levels of hepatic microsomal cytochrome P450 isoenzymes following intracerebroventricular injection of bacterial lipopolysaccharide in rats. *Arch Toxicol*. 1998;72(8):492-498.
204. Renton KW, Nicholson TE. Hepatic and central nervous system cytochrome P450 are down-regulated during lipopolysaccharide-evoked localized inflammation in brain. *J Pharmacol Exp Ther*. 2000;294(2):524-530.
205. Dobashi K, Pahan K, Chahal A, Singh I. Modulation of endogenous antioxidant enzymes by nitric oxide in rat C6 glial cells. *J Neurochem*. 1997;68(5):1896-1903.
206. Mutane-Relat J, Ourlin JC, Domergue J, Maurel P. Differential effects of cytokines on the inducible expression of CYP1A1, CYP1A2, and CYP3A4 in human hepatocytes in primary culture. *Hepatology* 1995;22(4 Pt 1):1143-1153.
207. Renaud J-P, Boucher J-L, Vadon S, Delaforge M, Mansuy D. Particular ability of liver P450s3A to catalyze the oxidation of Nw-hydroxyarginine to citrulline and nitrogen oxides and occurrence of NO synthases of a sequence very similar to the heme-binding sequences in P450s. *Biochem Biophys Res Commun*. 1993;192(1):53-60.
208. Kuo PC, Abe KY, Dafoe DC. Cytochrome P450IIIa activity and cytokine-mediated synthesis of nitric oxide. *Surgery* 1995;118(2):310-317.
209. Fantuzzi G, Galli G, Zinetti M, Fratelli M, Ghezzi P. The upregulating effect dexamethasone on tumor necrosis factor alpha production is mediated by a nitric oxide-producing cytochrome P450. *Cell Immunol*. 1995;160(2):305-308.
210. Fantuzzi G, Cantoni L, Sironi M, Ghezzi P. Inhibitors of cytochrome P450 suppress tumor necrosis factor production. *Cell Immunol*. 1993;150(2):417-424.
211. Chauhan A, Chauhan V, Brown WT, Cohen I. Oxidative stress in autism: Increased lipid peroxidation and reduced serum levels of ceruloplasmin and transferrin – the antioxidant proteins. *Life Sci*. 2004;75(21):2539-2549.
212. McGinnis WR. Oxidative stress in autism. *Alternative Ther*. 2004;10(6):22-36.
213. Weissman JR, Kelley R I, Bauman ML, Cohen BH, Murray KF, Mitchell RL, Kern RL, Natowicz MR. Mitochondrial disease in autism spectrum disorder patients: a cohort analysis. *PLoS ONE* 2008;3(11):e3815.
214. Pastural E, Ritchie S, Lu Y, Jin W, Kavianpur A, Su-Myat K, Heath D, Wood PL, Fisk M, Goodenowe DB. Novel plasma phospholipid biomarkers of autism: mitochondrial dysfunction as a putative causative mechanism. *Prostagl Leukotr Essent Fatty Acids* 2009;81(4):253-254.
215. Giulivi C, Zhang YF, Omanska-Klusek A, Ross-Inta C, Wong S, Hertz-Picciotto I, Tassone F, Pessah IN. Mitochondrial dysfunction in autism. *JAMA* 2010;304(21): 2389-2396.
216. Rossignol DA, Frye RE. Mitochondrial dysfunction in autism spectrum disorders: a systematic review and meta-analysis. *Mol Psychiatry* 2012; 17(3):290-314.
217. Zhang B, Angelidou A, Alysandratos K-D, Vasiadi M, Francis K, Asadi S, Theoharides A, Sideri K, Lykouras L, Kalogeromitos D, Theoharides TC. Mitochondrial DNA and anti-mitochondrial antibodies in serum of autistic children. *J Neuroinflamm*. 2010;7:80.

218. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ.* 2009;16(11):1438-1444.
219. Gui X, Carraway RE. Involvement of mast cells in basal and neurotensin-induced intestinal absorption of taurocholate in rats. *Am J Physiol Gastrointest Liver Physiol.* 2004;287(2):G408-G416.
220. Arnoult D, Carneiro L, Tattoli I, Girardin SE. The role of mitochondria in cellular defense against microbial infection. *Semin Immunol.* 2009;21(4):223-232.
221. Ming X, Stein TP, Brimacombe M, Johnson WG, Lambert GH, Wagner GC. Increased excretion of lipid peroxidation biomarker in autism. *Prostaglandins Leukot Essent Fatty Acids* 2005;73(5):379-384.
222. James SJ, Cutler P, Melnyk S, Jernigan S, Janak L, Gaylor DW, Neubrandner JA. Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. *Am J Clin Nutr.* 2004;80(6):1611-1617.
223. Geier DA, Geier MR. A clinical and laboratory evaluation of methionine cycle-transsulfuration and androgen pathway markers in children with autistic disorders. *Horm Res.* 2006;66(4):182-188.
224. Geier DA, Kern JK, Garver CR, Adams JB, Audhya T, Nataf R, Geier MR. Biomarkers of environmental toxicity and susceptibility in autism. *J Neurol Sci.* 2009;280(1-2): 101-108.
225. Torreilles F, Salman-Tabcheh S, Guerin M, Torreilles J. Neurodegenerative disorders: the role of peroxynitrite. *Brain Res Brain Res Rev.* 1999;30(2):153-163.
226. De la Monte SM, Bloch KD. Aberrant expression of the constitutive endothelial nitric oxide synthase gene in Alzheimer disease. *Mol Chem Neuropathol.* 1997;30(1-2):139-159.
227. Beckman JS, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. *Methods Enzymol.* 1994;233:229-240.
228. Brown GC. Regulation of mitochondrial respiration by nitric oxide inhibition of cytochrome c oxidase. *Biochim Biophys Acta* 2001;1504(1):46-57.
229. Akyol O, Zoroglu SS, Armutcu F, Sahin S, Gurel A. Nitric oxide as a physiopathological factor in neuropsychiatric disorders. *In vivo* 2004;18(3): 377-390.
230. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl-radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990;87(4):1620-1624.
231. Frye RE. Central tetrahydrobiopterin concentrations in neurodevelopmental disorders. *Front Neurosci.* 2010;4:52
232. Moreno H, Borjas L, Arrieta A, Saez L, Prasad A, Estevez J, Bonilla E. Clinical heterogeneity of the autistic syndrome: a study of 60 families. *Invest Clin.* 1992;33(1):13-31.
233. Oliveira G, Diogo L, Grazina M, Garcia P, Ataide A, Margues C, Miguel T, Borges L, Vicente AM, Oliveira CR. Mitochondrial dysfunction in autism spectrum disorders: a population based study. *Dev Med Child Neurol.* 2005;47(3):185-189.
234. Filipek PA, Juranek J, Nguyen MT, Cummings C, Gargus JJ. Relative carnitine deficiency in autism. *J Autism Dev Disord.* 2004;34(6):615-623.
235. Aldred S, Moore KM, Fitzgerald M, Waring RH. Plasma amino acid levels in children with autism and their families. *J Autism Dev Disord.* 2003;33(1):93-97.
236. Page T, Coleman M. Purine metabolism abnormalities in a hyperuricosuric subclass of autism. *Biochim Biophys Acta* 2000;1500(3):291-296.
237. Cubala-Kucharska M. The review of most frequently occurring medical disorders related to aetiology of autism and the methods of treatment. *Acta Neurobiol Exp.* 2010;70(2):141-146.
238. Karaman U, Celik T, Kiran TR, Colak C, Daldal NU. Malondialdehyde, glutathione, and nitric oxide levels in *Toxoplasma gondii* seropositive patients. *Korean J Parasitol.* 2008;46(4):293-295.
239. Elsheikha HM, Azab MS, Abousamra NK, Rahbar MH, Elghanam DM, Raafat D. Seroprevalence of and risk factors for *Toxoplasma gondii* antibodies among asymptomatic blood donors in Egypt. *Parasitol Res.* 2009;104(6):1471-1476.
240. Elsheikha HM, El-Motayam MH, Abouel-Nour MF, Morsy AT. Oxidative stress and immune-suppression in *Toxoplasma gondii* positive blood donors: implications for safe blood transfusion. *J Egypt Soc Parasitol.* 2009;39(2):421-428.
241. Mihara M, Uchiyama M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem.* 1976;86(1):271-278.
242. Clancy RM, Levartovsky D, Leszczynska-Piziak J, Yegudin J, Abramson SB. Nitric oxide reacts with intracellular glutathione and activates the hexose monophosphate shunt in human neutrophils: evidence for S-nitrosoglutathione as a bioactive intermediary. *Proc Natl Acad Sci USA* 1994;91(9):3680-3684.
243. Gazzinelli RT, Eltoun I, Wynn TA, Sher A. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-alpha and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. *J Immunol.* 1993;151(7):3672-3681.
244. Oswald IP, Wynn TA, Sher A, James SL. NO as an effector molecule of parasite killing: modulation of its synthesis by cytokines. *Comp Biochem Physiol Pharmacol Toxicol Endocrinol.* 1994;108(1):11-18.
245. Engin AB, Dogruman-Al, F, Ercin U, Celebi B, Babur C, Bukan N. Oxidative stress and tryptophan degradation pattern of acute *Toxoplasma* infection in mice. *Parasitol Res.* 2012;111(4):1725-1730.
246. Koivisto A, Matthias A, Bronnikov G, Nedergaard J. Kinetics of inhibition of respiration by NO. *FEBS Lett* 1997;417(1):75-80.
247. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah I, Van de Water J. Associations of impaired behaviors with elevated plasma chemokines in autism spectrum disorders. *J Neuroimmunol.* 2011;232(1-2): 196-199.
248. Shirakawa I, Deichmann KA, Izuhara I, Mao I, Adra C, Hopkin JM. Atopy and asthma: genetic variants of IL-4 and IL-13 signaling. *Immunol Today* 2000;21(2):60-64.
249. Mosmann TR, Sad S. The expanding universe of T cell subsets: Th1, Th2 and more. *Immunol Today* 1996;17(3):138-146.
250. O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998;8(3):275-283.
251. Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, Labit C, Leplatois P, Liauzun P, Miloux B, et al. Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature* 1993;362(6417):248-250.
252. De Waal MR, Figdor CG, Huljbens R, Mohan-Peterson S, Bennett B, Culpepper J, Dang W, Zurawski G, de Vries JE. Effect of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes. Comparison with IL-4 and modulation by IFN-gamma or IL-10. *J Immunol.* 1993;151(11):6370-6381.
253. Marodi L, Kaposzta R, Nemes E. Survival of group B *Streptococcus* type III in mononuclear phagocytes: differential regulation of bacterial killing in cord macrophages by human recombinant gamma interferon and granulocyte-macrophage colony-stimulating factor. *Infect Immun.* 2000;68(4): 2167-2170.
254. Goriely S, Vincart B, Stordeur P, Vekemans J, Willems F, Goldman M, De Wit D. Deficient IL-12(p35) gene expression by dendritic cells derived from neonatal monocytes. *J Immunol.* 2001;166(3):2141-2146.
255. Hitziger N, Dellacasa I, Albiger B, Barragan A. Dissemination of *Toxoplasma gondii* to immunoprivileged organs and role of Toll/interleukin-1 receptor signaling for host resistance assessed by in vivo bioluminescence imaging. *Cell Microbiol.* 2005;7(6):837-848.
256. Mun HS, Aosai F, Norose K, Chen M, Piao LX, Takeuchi O, Akira S, Ishikura H, Yano A. TLR2 as an essential molecule for protective immunity against *Toxoplasma gondii* infection. *Int Immunol.* 2003;15(9):1081-1087.



257. Authier H, Cassaing S, Bans V, Batigne P, Bessieres M-H, Pipy B. IL-13 pre-treatment of murine peritoneal macrophages increases their anti-Toxoplasma gondii activity induced by lipopolysaccharides. *Int J Parasitol.* 2008;38(3-4):341-352.
258. Authier H, Cassaing S, Coste A, Balard P, Gales A, Berry A, Bessieres MH, Pipy B. Interleukin-13 primes iNO synthase expression induced by LSP in mouse peritoneal macrophages. *Mol Immunol.* 2008;45(1):235-243.
259. Chaves AC, Ceravolo IP, Gomes JA, Zani CL, Romanha AJ, Gazzinelli RT. IL-4 and IL-13 regulate the induction of indoleamine 2,3-dioxygenase activity and the control of Toxoplasma gondii replication in human fibroblasts activated with IFN-gamma. *Eur J Immunol.* 2001;31(2):333-344.
260. Schultz C, Reiss I, Bucksy P, Gopel W, Gembruch U, Ziesenits S, Gortner L. Maturation changes of lymphocyte surface antigens in human blood: Comparison between fetuses neonates and adults. *Biol Neonate* 2000;78(2): 77-82.
261. Luebke RW, Parks C, Luster MI. Suppression of immune function and susceptibility to infections in humans: Association of immune function with clinical disease. *J Immunotoxicol.* 2004;1(1):15-24.
262. Upham JW, Lee PT, Holt BJ, Heaton T, Prescott SL, Sharp MJ, Sly PD, Holt PG. Development of interleukin-12-producing capacity throughout childhood. *Infect Immun.* 2002;70(12):6583-6588.
263. Bartlett JA, Schleifer SJ, Demetrikopoulos MK, Delaney BR, Shifflett SC, Keller SE. Immune function in healthy adolescents. *Clin Diagn Lab Immunol.* 1998;5(1):105-113.
264. Kang DH, Kim CJ, Suh Y. Sex differences in immune responses and immune reactivity to stress in adolescents. *Biol Res Nurse* 2004;5(4):243-254.
265. Sayles PC, Gibson GW, Johnson LL. B cells are essential for vaccination-induced resistance to virulent Toxoplasma gondii. *Infect Immun.* 2000;68(3): 1026-1033.
266. Aosai F, Chen M, Kang H-K, Mun H-S, Norose K, Piao LX, Kobayashi M, Takeuchi O, Akira S, Yano A. Toxoplasma gondii-derived heat shock protein HSP70 functions as a B cell mitogen. *Cell Stress Chaperones* 2002;7(4):357-364.
267. Wilson CB, Kollmann TR. Induction of antigen-specific immunity in human neonates and infants. *Nestle Nutr Workshop Ser Pediatr Program, Basel, Nestec Ltd., Vevey. S. Karger, A.G.* 2008;61:183-195.
268. Corbett NP, Blimkie D, Ho KC, Cai B, Sutherland DP, Kallos A, Crabtree J, Rein-Weston A, Lavoie PM, Turvey SE, Hawkins NR, Self SG, Wilson CB, Hajjar AM, Fortunato ES 3rd, Kollmann TR. Ontogeny of Toll-like receptor mediated cytokine responses of human blood mononuclear cells. *PLoS ONE* 2010;5(11):e15041.
269. Ogra PL. Ageing and its possible impact on mucosal immune responses. *Ageing Res Rev.* 2010;9(2):101-106.
270. Bryson S, Smith I. Epidemiology of autism: Prevalence, associated characteristics, and implications for research and service delivery. *Mental Retard Dev Disabil Res Rev.* 1998;4:97-103.
271. Gillberg C, Wing L. Autism: not an extremely rare disorder. *Acta Psychiatr Scand.* 1999;99(6):399-406.
272. Dalton P, Deacon R, Blamire A, Pike M, McKinlay I, Stein J, Styles P, Vincent A. Maternal neuronal antibodies associated with autism and a language disorder. *Ann Neurol.* 2003;53(4):533-537.
273. Vincent A, Dalton P, Clover Palace J, Lang B. Antibodies to neuronal targets in neurological and psychiatric diseases. *Ann NY Acad Sci.* 2003;992:48-55.
274. Martin LA, Ashwood P, Braunschweig D, Cabanlit M, Van de Water J, Amaral DG. Stereotypies and hyperactivity in rhesus monkeys exposed to IgG from mothers of children with autism. *Brain Behav Immun.* 2008;22(6): 804-816.
275. Singer HS, Morris C, Gause C, Pollard M, Zimmerman AW, Pletnikov M. Prenatal exposure to antibodies from mothers of children with autism produces neurobehavioral alterations: a pregnant dam mouse model. *J Neuroimmunol.* 2009;211(1-2):39-48.
276. Roberts CW, Cruickshank SM, Alexander J. Sex-determined resistance to Toxoplasma gondii is associated with temporal differences in cytokine production. *Infect Immun.* 1995;63(7):2549-2555.
277. Araujo FG, Slifer T. Different strains of Toxoplasma gondii induce different cytokine responses in CBA/Ca mice. *Infect Immun.* 2003;71(7):4171-4174.
278. Kasper LH. Identification of stage-specific antigens of Toxoplasma gondii. *Infect Immun.* 1989;57(3):668-672.
279. Hester J, Mullins J, Sa Q, Payne L, Mercier C, Cesbron-Delauw MF, Suzuki Y. Toxoplasma gondii antigens recognized by IgG antibodies differ between mice with and without active proliferation of tachyzoites in the brain during the chronic stage of infection. *Infect Immun.* 2012;80(10):3611-3620.
280. Prandota J. Increased generation of antibodies and autoantibodies directed against brain proteins in patients with autism and their families may be caused by T. gondii infection. Maternal and fetal microchimerisms probably play an important role in these processes acting as a "Trojan horse" in dissemination of the parasite. In: Gemma C (ed.), *Neuroinflammation. Pathogenesis, Mechanisms, and Management.* New York: Nova Science Publishers 2012;447-638.
281. Holec-Gasior L. Toxoplasma gondii recombinant antigens as tools for serodiagnosis of human toxoplasmosis. *Current status of studies.* *Clin Vac Immunol.* 2013;20(9):1343-1351.
282. Prandota J. Idiopathic intracranial hypertension may be caused by reactivation of latent cerebral toxoplasmosis. Effect of drugs and biologic substances. In: Gemma C (ed.), *Neuroinflammation. Pathogenesis, Mechanisms and Management,* Nova Science Publishers, New York 2012;273-336.
283. Prandota J. Recurrent headache as the main symptom of acquired cerebral toxoplasmosis in non-human immunodeficiency virus-infected subjects with no lymphadenopathy. The parasite may be responsible for the neurogenic inflammation postulated as a cause of different types of headaches. *Am J Ther.* 2007;14(1):63-105.
284. Prandota J. The importance of Toxoplasma gondii infection in diseases presenting with headaches. Headaches and aseptic meningitis may be manifestations of the Jarisch-Herxheimer reaction. *Int J Neurosci.* 2009;119(12):2144-2182.
285. Alvarado-Esquivel C, Niewiadomski A, Schweickert B, Lisenfeld O. Antiparasitic treatment suppresses production and avidity of Toxoplasma gondii-specific antibodies in a murine model of acute infection. *Eur J Microbiol Immunol.* 2011;1(3):249-255.
286. Krause I, Anaya JM, Fraser A, Barzilai O, Ram M, Abad V, Arango A, Garcia J, Shoenfeld Y. Anti-infectious antibodies and autoimmune-associated autoantibodies in patients with type 1 diabetes mellitus and their close family members. *Ann NY Acad Sci.* 2009;1173:633-639.
287. Prandota J. T. gondii infection acquired during pregnancy and/or after birth may be responsible for development of both type 1 and 2 diabetes mellitus. *J Diabetes Metab.* 2013;4:241.
288. Guk SM, Kook J, Jeon YH, Choi JH, Han ET, et al. Suppressed cytokine and immunoglobulin secretions by murine splenic lymphocytes infected in vitro with Toxoplasma gondii tachyzoites. *J Parasitol.* 2005;91:467-470.
289. Prandota J. Gastrointestinal tract abnormalities in autism, inflammatory bowel disease and many other clinical entities may be due to T. gondii infection. *Scientific Reports* 2012;1(4).
290. Bhadra R, Khan IA. Redefining chronic toxoplasmosis – A T cell exhaustion perspective. *PLoS Pathog* 2012;8(10): e1002903.
291. Goldszmid RS, Coppens I, Lev A, Caspar P, Mellman I, Sher A. Host ER-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in Toxoplasma gondii-infected dendritic cells. *J Exp Med.* 2009;206(2):399-410.
292. Lefevre-Pettazzoni M, Le Cam S, Wallon M, Peyron F. Delayed maturation of immunoglobulin G avidity: implication for the diagnosis of toxoplasmosis in pregnant women. *Eur J Clin Microbiol*



- Infect Dis. 2006;25(11):687-693.
293. Prandota J, Gryglas A, Fuglewicz A, Źesławska-Faleńczyk A, Ujma-Czapska B, Szenborn L, Mierzwa J. Recurrent headaches may be caused by *T. gondii* infection. *World J Clin Pediatr.* 2014;3(3):59-68.
294. Victorio M. EHMTI-0290. Headaches in patients with autism spectrum disorders (Abstract). *J Headache Pain* 2014;15(Suppl 1):B37.
295. Sullivan JC, Miller LJ, Nielsen DM, Schoen SA. The presence of migraines and its association with sensory hyperreactivity and anxiety symptomatology in children with autism spectrum disorders. *Autism* 2014;18(6):743-747.
296. McAlonan GM, Cheung V, Cheung C, Suckling J, Lam GY, Tai KS, Yip L, Murphy DG, Chua SE. Mapping the brain in autism. A voxel-based MRI study of volumetric differences and intercorrelations in autism. *Brain* 2005;128(Pt 2):268-276.
297. Hallahan B, Daily EM, McAlonan G, Loth E, Toal F, O'Brien F, Robertson D, Hales S, Murphy C, Murphy KC, Murphy DG. Brain morphometry volume in autistic spectrum disorder: a magnetic resonance imaging study of adults. *Psychol Med.* 2009;39(2):337-346.
298. Shen MD, Nordahl CW, Young GS, Wootton-Gorges SL, Lee A, Liston SE, Harrington KR, Ozonoff S, Amaral DG. Early brain enlargement and elevated extra-axial fluid in infants who develop autism spectrum disorder. *Brain* 2013;136(Pt 9):2825-2835.
299. Prandota J. Idiopathic intracranial hypertension may be caused by reactivation of latent cerebral toxoplasmosis. Effect of various diseases and clinical states. In: Gemma C (ed.), *Neuroinflammation. Pathogenesis, Mechanisms and Management*, Nova Science Publishers, New York 2012; 337-343.
300. Prandota J. Transient beneficial effects of fewer on behavior of children with autistic spectrum disorders may be caused by *T. gondii* stage differentiation during latent cerebral toxoplasmosis, associated with the changes in immune responses of the host. In: Gemma C (ed.), *Neuroinflammation. Pathogenesis, Mechanisms, and Management*. Nova Science Publishers, New York 2012;399-421.
301. Evers M, Cunningham-Rundles C, Hollander E. Heat shock protein 90 antibodies in autism. *Mol Psychiatry* 2002;7 Suppl 2:S26-S28.
302. Ohtsuka K, Suzuki T. Roles of molecular chaperones in the nervous system. *Brain Res Bull.* 2000;53(2):141-146.
303. Echeverria PC, Figueras MJ, Vogler M, Kriehuber T, de Miguel N, Deng B, Dalmaso MC, Matthews DE, Matrajt M, Haslbeck M, Buchner J, Angel SO. The Hsp90 co-chaperone p23 of *Toxoplasma gondii*: identification, functional analysis and dynamic interactome determination. *Mol Biochem Parasitol.* 2010;172(2):129-140.
304. Angel SO, Figueras MJ, Alomar ML, Echeverria PC, Deng B. *Toxoplasma gondii* Hsp90: potential roles in essential cellular processes of the parasite. *Parasitology* 2014;141(9):1138-1147.
305. Sternberger LA. *Immunocytochemistry*, 2nd ed. John Wiley & Sons, New York 1979.
306. Sabin AB, Feldman HA. Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoan parasite (*Toxoplasma*). *Science* 1948;108(2815):660-663.
307. Chapey E, Wallon M, Debize G, Rabilloud M, Peyron F. Diagnosis of congenital toxoplasmosis using whole-blood interferon-gamma release assay. *J Clin Microbiol.* 2009;48(1):41-45.

**Peer reviewers:** Mehrak Javadi Paydar, Laboratory Program in Behavioral Neuroscience, Department of Psychology, University of South Carolina, 915 Greene Street (Discovery I) Columbia, South Carolina, 29208, USA; Amir Abdoli, PhD, Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.