Increased Seroprevalence of Chronic Toxoplasmosis in Autistic Children: Special Reference to the Pathophysiology of IFN-γ and NO Overproduction

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

ABSTRACT

Autism spectrum disorders (ASD) are a heterogenous 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-γ 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 ± 2.2 years; 41 boys, 5 girls) were studied for anti-*T. gondii* IgG antibody seropositivity (ELISA kit, DRG Int), and their serum IFN-γ (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-γ 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-γ 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.

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


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INTRODUCTION

ASD

This clinical entity is a complex multifactorial neurodevelopmental disorder with genetic, immunological, and environmental contributions[4-6]. It is characterized by reduced sociability, abnormalities in verbal and nonverbal communication, restricted stereotyped interests and repetitive behaviors[7]. Manifestations onset starts in the first 3 yrs of life[8]. 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)[9]. 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[10]. Recently, Blumberg et al[11] 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)[12]. 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)[13]. 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)[14].

Despite decades of intense 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[15-16]. A number of environmental factors and associated clinical abnormalities, as well as immune irregularities have been linked to pathophysiology of ASD and neurological activation/neuroinflammation in the brain of patients with autism[17-19]. Markedly low subpopulation of CD4+ and CD8+ lymphocytes together with imbalance between Th1 and Th2 type cytokines skewing more towards Th2 arm have been demonstrated in children with ASD[20-21]. 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[22]. 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[23-24].

Accumulating evidence indicates that in schizophrenia and autism, inflammatory cytokines including IL-1β and IL-6 have a role in onset and progress of neuropsychiatric symptoms probably via perinatal inflammation[25-27]. These findings may be in line with the enhancement of intracellular replication of T. gondii by IL-6 and reversal of IFN-γ-mediated toxoplasmacidal activity, as well as with the markedly higher prevalence of anti-Toxoplasma antibodies in patients with autoimmune disease[28-30]. 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-γ had an opposite effect[31]. In turn, Shapira et al[32] 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[33], and hypothalamic-pituitary-adrenal axis function, both known to be affected during chronic T. gondii infection[34].

T. gondii

This intracellular pathogen believed to be a global threat[25-30] chronically infects approximately 30-50% of the human population, and ophthalmomimonomulogists[35] 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[36]. Recent study by Flegr et al[37] 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[38], and it was demonstrated that the protozoan ingested and digested host cystoic proteins using cathepsin L and other proteases within its endosytoplasmic system[39].

Evans et al[39] 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[40-41]. 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[42-44]. 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[40-47].

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[48]. Wang et al[49] 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[49,50].

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\(^{12-14}\). 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-γ and NO levels, and the Childhood Autism Rating Scale (CARS) values.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Two months after inoculation</th>
<th>Six months after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>34</td>
<td>22</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Thalamus</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Amygdala</td>
<td>25</td>
<td>9</td>
</tr>
<tr>
<td>Caudate putamen</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

Each group of mice was infected with 5 cysts of *T. gondii* and generated by using one-way ANOVA: expressed as the mean number of cysts collected from each group ± SD, inoculation on the 5\(^{th}\) day after birth. Results are as follows: each mean number of cysts collected from each group ± SD, generated by using one-way ANOVA: \(p < 0.05\) vs late stage infection and \(p > 0.05\) 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 2

<table>
<thead>
<tr>
<th>Group of mice</th>
<th>Number of cysts in brain</th>
<th>Congenital <em>T. gondii</em> transmission rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early stage infection</td>
<td>224 ± 59 (^{+}) ((n = 18))</td>
<td>94.74</td>
</tr>
<tr>
<td>Intermediate stage infection</td>
<td>202 ± 44 (^{+}) ((n = 19))</td>
<td>90.48</td>
</tr>
<tr>
<td>Late stage infection</td>
<td>134 ± 31 ((n = 22))</td>
<td>91.67</td>
</tr>
</tbody>
</table>

Each group of mice was infected with 5 cysts of *T. gondii* by oral inoculation on the 5\(^{th}\), 10, and 15\(^{th}\) day after gestation. Results are as follows: each mean number of cysts collected from each group ± SD, generated by using one-way ANOVA: \(p < 0.01\) vs late stage infection and \(p > 0.05\) 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\(^{38}\), with own modification).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>mAb for host cell identification</th>
<th>Frequency of cell type</th>
<th>Rate of <em>T. gondii</em> infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons</td>
<td>Anti-NF 200 kDa</td>
<td>88.0 ± 1.3(%)</td>
<td>9.5 ± 1.1(%)</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Anti-GFAP</td>
<td>7.9 ± 2.1(%)</td>
<td>9.7 ± 3.3(%)</td>
</tr>
<tr>
<td>Microglia</td>
<td>Anti-CD11</td>
<td>4.1 ± 0.9(%)</td>
<td>31.5 ± 5.9(%)</td>
</tr>
</tbody>
</table>

\(^{1}\) Determined 48 hrs post infection by double immunofluorescence (at least 100 parasitophorous vacuoles were examined for each determination). Data represent mean ± SD from three independent experiments. mAb, monoclonal antibody. GFAP, glial filament acidic protein.

### Table 4

Replication and morphology of different cell types in embryonal rat cortices (E15) (acc. to Lüder et al\(^{38}\), with own modification).

<table>
<thead>
<tr>
<th>Intracellular replication (% PV)</th>
<th>Neurons</th>
<th>Astrocytes</th>
<th>Microglia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 parasites/PV</td>
<td>66 ± 2.6</td>
<td>67.2 ± 5.0</td>
<td>93.1 ± 4.5</td>
</tr>
<tr>
<td>4-8 parasites/PV</td>
<td>30 ± 2</td>
<td>26.2 ± 1.2</td>
<td>8.9 ± 4.5</td>
</tr>
<tr>
<td>&gt; 32 parasites/PV</td>
<td>0</td>
<td>1.3 ± 1.2</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{1}\) Determined 48 hrs post infection by double immunofluorescence (at least 100 PV were examined for each determination). Data represent mean ± 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 IFN-γ 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 μl 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 μl) was then added to react with the bound antibody. Substrate (100 μl) for peroxidase was added and the reaction was stopped by stop solution (100 μl). 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 (< 32 IU/mL), a positive reaction to *T. gondii* index of 1.0 or greater (> 32 IU/mL), and an equivocal result corresponded to *T. gondii* index between 0.91-0.99.

**Detection of serum INF-γ levels**

All serum samples were tested for serum INF-γ 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 μl 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 μl) was then added to each well incubated at room temperature for 1 h. Streptavidin solution (100 μl) was next added to each well and incubated at room temperature for 45 min. 100 μl of TMB one-step substrate reagent was then added to each well incubated for 30 min, and 50 μl 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 Borics & Borics\(^{39}\). 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 μM. The method was linear from 5 to 200 μM nitrate in serum. The median (range) concentration

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in serum of 20 healthy individuals was 16 (0-42) μM[75].

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[56].

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 INF-γ and NO levels in the studied participants 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 ± 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 (χ^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 ± 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-γ 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-γ 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[52-54] 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-γ levels in autistic children with toxoplasmosis
T. gondii- positive autistic children have significantly increased serum IFN-γ concentrations as compared with Toxoplasma-free patients (Table 5). IFN-γ, a classic T cell cytokine produced also in the brain, influences more than 200 genes[55a-d], and its overproduction play an important role in development of neurodegeneration[19,20].

ASD. Children with autism have peripheral and central inflammation[2]. Neuronal activation and neuroinflammation processes[22] with significantly increased plasma proinflammatory and anti-inflammatory cytokines (IFN-γ, TNF-α, IL-1β, 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 lymphocytes) were found among 50 control children (χ^2 = 8.1143; p < 0.004392). This finding strongly support recent suggestions of Prandota[52-54] 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.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Serum INF-γ levels (pg/mL)</th>
<th>Mann-Whitney test</th>
<th>Asymptomatic significance (2-tailed)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxo-(+)</td>
<td>Median Percentiles</td>
<td>2202.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2135.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>2375.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toxo-negative</td>
<td>Median Percentiles</td>
<td>194.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>158.44</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>201.86</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

αCL, Confidence Interval. *HS, highly significant difference between the groups. L and U, lower and upper limits of significant values.

Table 7 Serum INF-γ concentrations in autistic children with and without toxoplasmosis.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Serum NO levels (μM)</th>
<th>Mann-Whitney test</th>
<th>Asymptomatic significance (2-tailed)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxo-(+)</td>
<td>Median Percentiles</td>
<td>41.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>33.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>57.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toxo-negative</td>
<td>Median Percentiles</td>
<td>8.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>10.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>10.08</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

αCL, Confidence Interval. *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.
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 (Figures 2 and 3). Recently, Schwartz [75] 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 [76,77]. In this context, decreased tryptophan metabolism found in patients with ASD [72] may reflect a defense reaction of the host since IFN-γ blocked growth of the parasite by inducing the host cells to degrade tryptophan [78]. This is in line with the finding that tryptophan loading induced oxidative stress because its metabolites generated free radicals [79], 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 [19,80,81].

Figure 1 Relationship between serum IFN-γ (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 kynureninines). 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 [74]; with own modification)
T. gondii. The parasite infects a variety of host brain and other tissue cells, and IFN-γ-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

biomechanisms affecting T. gondii survival in the host cells include: (1) mechanisms mediated by an IFN-γ responsive gene family. Several of these proteins, including IGTP (IFN-γ-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-γ; (2) production of NO by inducible NO synthase (iNOS); (3) production of various cytokines (TNF-α, IFN-γ, IL-1β, 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) [82-84]. Several molecular, biochemical and immune host defense mechanisms associated with T. gondii infection were presented in Tables 10 and 11 [85,86].

The IFN-γ 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 [87-89]. Cell sources of IFN-γ include: NK cells [90-92] (promotes iNOS expression [92]), CD4+ T cells [93,94] (promotes p47 GTPase-mediated killing of T. gondii [87,88]), and CD8+ T cells [95] (promotes tryptophan degradation [96,97]). Virulence of the parasite was associated with distinct dendritic cell (DC) responses and reduced numbers of activated CD8+ T cells [98]. Although IFN-γ may play multiple roles in resistance to the pathogen, macrophage activation is generally believed to be the critical effector function [99,100]. Because activation of these cells results in iNOS gene induction and synthesis of moderate levels of RNI generated during degradation of arginine into citrulline by iNOS [83,84]. Synthesis of RNI was also highly enhanced by certain microbial products, as well as by triggering macrophage TNF-α synthesis [89,101]. 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 [102,103], and cytokines mediated regulation of IFN-γ-induced L-tryptophan biodegrading enzyme, IDO [104] (Figure 2).

![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-γ, interferon-γ; IL, interleukin; Kyn, kynurenine; KynA, kynurenic acid; LPS, lipopolysaccharide; mRNA, messenger RNA; iNOS, inducible nitric oxide synthase; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; Trp, tryptophan; xA, xanthurenic acid. The broken lines represent possible reactions (acc. to Stone & Darlington [105], with own modification).](Image)

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 [106], with own modification).

<table>
<thead>
<tr>
<th>Inhibition of:</th>
<th>Through induction of (and/or use/interaction with):</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-L-related IFN-γ production</td>
<td>NO (negative effects on antigen presentation and cell proliferation, PGE2 (decreases IL-2 and IFN-γ production))</td>
<td>[86,88]</td>
</tr>
<tr>
<td>IFN-γ-mediated NO generation</td>
<td>IL-4, IL-10, NO (inactivation of NOS and IDO), 3-HAA, 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-kynurenine-5-hydroxymethyl-kynurenine metabolic pathway). Iron down-regulates macrophage NO production stimulated by IFN-γ plus LPS, although, on the other hand, iron deprivation is one of the mechanisms mediating anti-Toxoplasma activity. These abnormalities are consistent with the impaired iron homeostasis in the periaequatorial gray matter reported in the patients with episodic migraine and chronic daily headache. PGE2 and LTβR inhibit NOS type 2 expression, iNOS synthesis and expression, and NO production in hepatocytes and macrophages. And vice versa, NO mediates IL-1-induced PGE2 production by vascular smooth muscle cells.</td>
<td>[74, 86, 89-96]</td>
</tr>
<tr>
<td>NO antiparasitic effects</td>
<td>NO may serve as a molecular trigger of the parasite stage conversion, either by selecting for parasites that</td>
<td></td>
</tr>
</tbody>
</table>

PGE2 produced by human monocytes during T. gondii infection suppresses T-L cytokine production and induce IL2 cytokines, such as IL-10 [107-109], and decreases NO production by activated microglia [107,108]. PGE2 (as well as PGE2 and PGA2) 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 [107,108]. IDO, indoleamine 2,3-dioxygenase, the first enzyme of the kynurenine pathway, the main pathway for tryptophan metabolism, is powerfully induced by IFN-γ; inhibition of NOS consequently results in an exaggerated output of kynurenines.

The anti-inflammatory cytokines TGF-β and IL-4 suppress IDO activity in human monocytes and fibroblasts [110], which is consistent with IDO metabolic pathway being a significant contributor to the pro-inflammatory system [111]. Enhanced IDO activity was found in vivo in diseases that were associated with chronic stimulation of T1-mediated immunity, such as the CNS T. gondii infection [112,113]. Metabolites of tryptophan, L-kynurenine, picolinic acid and quinolinic acid, were found to inhibit proliferation of CD4+CD8+ T lymphocytes and NK cells, thus affecting immune response of the host to the parasite [114]. NO can directly inactivate NOS [115].

1 IL-13 exerted dual effects, i.e. a decreased and an enhanced NO production by LPS-stimulated GM-CSF-stimulated factor-derived bone marrow macrophages. 2 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-κB [116]. 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 anti-toxoplasmatic effect [117].
Prandota J et al. Seroprevalence of chronic toxoplasmosis in autistic children

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. DCs were required for optimal CD4 and CD8 T cell responses, and the phagocytosis of heat-killed or invasion-blocked parasites was not sufficient to induce T cell responses.

In summary, several cytokines such as IFN-γ, TNF-α, 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-γ 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. Higher plasma nitrite (NO$_2$)/nitrate (NO$_3$) levels (total NO, recovery from plasma approximately 87%, with 17.6% of the total level as nonstable nitrite) were also found in children with autism compared with controls. 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, were associated with mitochondrial dysfunction. The induction of a high-output inducible enzyme NOS (iNOS) is triggered primarily by IFN-γ, in combination with TNF-α and IL-1β, or endotoxin. In the central nervous system, NO at physiological concentrations acts as an intracellular messenger, but at higher levels acts as an intracellular messenger, but at higher levels it may inhibit cellular respiration and that of coincubated cells via the NO inhibition of cytochrome oxidase. Moreover, NO caused glutamate release from synaptosomes via inhibition of synaptosomal respiration, and killed cerebellar granule neurons by excitotoxic mechanisms.

Prolonged exposure of cells to NO might cause cell death by opening a mitochondrial permeability transition pore and subsequent activation of caspases, and the mode of cell death as proapoptotic or proerotic (Table 12) seemed to be determined by cellular level of ATP. (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). Excess of NO caused increased intestinal permeability (“leaky gut”), prevalent in autism, since this biomolecule mediated IFN-γ-induced hyperpermeability in cultured human intestinal epithelial cells of autistic patients compared to age- and sex-matched normal controls, along with enzymatic evidence of NO-related oxidative stress, were associated with mitochondrial dysfunction.

**Figure 4** Possible model for NO-mediated regulation of IDO in IFN-γ-primed mononuclear phagocytes. NO, nitric-oxide synthase; IDO, indoleamine 2,3-dioxygenase; L-Arg, L-arginine; L-Trp, L-tryptophan; IFN-γ, interferon-γ; 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, diethylenoaminodinitric oxide. SNP, DEANO, and SNAP release NO extracellularly, while GTN is thought to release NO intracellularly (acc. to Thomas, Mohr, & Stocker).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Astrocites</th>
<th>Microglial cells</th>
<th>Neurons</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative efficiency of infection</td>
<td>10$^3$</td>
<td>50$^4$</td>
<td>5-15$^5$</td>
<td>107-110</td>
</tr>
<tr>
<td>Cytokine release</td>
<td>IFN-γ, IL-1, IL-6, GM-CSF, NO</td>
<td>IFN-γ, IL-10, IL-6, TNF-α, NO</td>
<td>TNF-α, NO, glutamate</td>
<td>118-119</td>
</tr>
<tr>
<td>Cyst formation</td>
<td>Limited</td>
<td>Yes</td>
<td>Yes</td>
<td>110, 113</td>
</tr>
<tr>
<td>Size of cysts</td>
<td>Large (~50 μm)</td>
<td>Small (&lt;10 μm)</td>
<td>Small (~10 μm)</td>
<td>111</td>
</tr>
<tr>
<td>Size of brain cells</td>
<td>100 μm (harbor several dozen tachyzoites)</td>
<td>5-10 μm</td>
<td>10-15 μm$^6$ (contain only few tachyzoites)</td>
<td>110</td>
</tr>
<tr>
<td>Effect of IFN-γ and TNF-α</td>
<td>Encystation</td>
<td>Parasite killing</td>
<td>?</td>
<td>107, 112, 114</td>
</tr>
<tr>
<td>Inducible NOS</td>
<td>Low</td>
<td>High</td>
<td>Yes$^5$</td>
<td>115, 116</td>
</tr>
</tbody>
</table>

1. Loder et al.$^{34}$ 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. 2. 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 μm. 3. Not documented for *T. gondii* infection. GM-CSF, granulocyte-macrophage colony-stimulating factor.

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Table 12 Possible consequences on neurons of cytokines and biomolecules secreted upon T. gondii infection (acc. to: Fagard et al.[43], with own modification)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Secreted biomolecules</th>
<th>Neurotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocyte</td>
<td>IL-6, CM-CSF, TNF-α, IL-1β, arachidonic acid</td>
<td>±</td>
</tr>
<tr>
<td>Macrophage</td>
<td>NO, IL-12</td>
<td>±</td>
</tr>
<tr>
<td>Microglial cells</td>
<td>RNI, NO, H2O2, IFN-γ, Glutamate</td>
<td>+</td>
</tr>
<tr>
<td>Neuron</td>
<td>NO, TNF-α, Glutamate</td>
<td>±</td>
</tr>
<tr>
<td>Natural killer cell</td>
<td>IFN-γ, PAF, II-4, IFN-γ, IL-10</td>
<td>-</td>
</tr>
<tr>
<td>T cell</td>
<td>PAF, Glutamate</td>
<td>±</td>
</tr>
</tbody>
</table>

RNI, reactive oxygen intermediates; PAF, platelet-activating factor; CM-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[43]. Atopic concentrations were 14% higher in the brain of mice with chronic infections than in controls[46], 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[43]. Moreover, dopamine stimulated tachyzoite proliferation in human fibroblast and primary neonatal rat astrocyte cell cultures[43], 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[43,46]. Nitric oxide caused glutamate release from brain synaptosomes[43]. Interestingly, lithium chloride (a drug used in psychiatric diseases) potentiated astroglial nitric oxide synthase type-2 mRNA expression, and therefore treatment with this medication could exacerbate inflammatory responses in the brain[46].

Table 13 The induction of necrosis or apoptosis by NO donors or inhibitors of mitochondrial respiration in PC12 cells (acc. to: Bal-Pierce et al.[114], with own modification)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Necrotic cells (%)</th>
<th>Apoptotic cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.00 ± 1.40</td>
<td>0.10 ± 0.10</td>
</tr>
<tr>
<td>SNAP</td>
<td>83.80 ± 4.30</td>
<td>0.56 ± 0.10</td>
</tr>
<tr>
<td>SNAP + glucose</td>
<td>19.05 ± 1.80</td>
<td>19.45 ± 1.40</td>
</tr>
<tr>
<td>SNAP + glucose + VAD</td>
<td>14.80 ± 2.30</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>NOC-18</td>
<td>97.80 ± 0.80</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>NOC-18 + glucose</td>
<td>17.30 ± 1.80</td>
<td>0.5 ± 0.24</td>
</tr>
<tr>
<td>NOC-18 + glucose + VAD</td>
<td>8.30 ± 1.10</td>
<td>0.50 ± 0.10</td>
</tr>
<tr>
<td>Myxothiazol</td>
<td>92.50 ± 3.40</td>
<td>0.00</td>
</tr>
<tr>
<td>Myxothiazol + glucose</td>
<td>11.20 ± 3.60</td>
<td>10.38 ± 2.20</td>
</tr>
<tr>
<td>Azide</td>
<td>97.23 ± 1.50</td>
<td>0.00</td>
</tr>
<tr>
<td>Azide + glucose</td>
<td>15.20 ± 3.10</td>
<td>8.23 ± 2.80</td>
</tr>
</tbody>
</table>

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 μM myxothiazole or 2 mM azide) for 24 h. After this time the cell death was analyzed by trypan blue or propidium iodide staining (necrotic cells) or by using chromatin dye Hoechst 33342 (apoptotic cells).

monolayer[185,187]. NO as a regulator of mast cell activation and mast cell-mediated inflammation, may participate in development of this pathology[184]. Because NO directly impairs the intestinal barrier function[185], this abnormality may be responsible for the iron, calcium and zinc deficiency reported in individuals with Pica behavior[186,188]. 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[186,189,190]. 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 (H2O2)-mediated microvascular endothelial permeability, and this NO effect was concentration dependent[184]. McQuaid et al[184] demonstrated that the permeability produced by H2O2 could be exacerbated when relatively high levels of NO (100 μM sodium nitroprusside) were present, and this endothelial dysfunction may be associated with generation of singlet oxygen from H2O2[177]. NO did not affect H2O2 metabolism by endothelial cells but markedly depleted intracellular glutathione[184].

The mechanism in which NO and H2O2 cooperated to promote increased microvascular permeability probably involved the cell-surface bound iron (Fe3+)-catalyzed formation of a potent oxidant such as hydroxyl radical (·OH) generated from decomposition of H2O2 in the following reactions[146,172-174]:

\[
\text{Fe}^{3+} + \text{NO} \rightarrow \text{Fe}^{2+} + \text{NO}^{2-}
\]

\[
\text{H}_2\text{O}_2 + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{OH}^{-} + \text{OH}^{\cdot} (\text{highly reactive free radicals})
\]

and by NO-mediated depletion of intracellular glutathione[179]. 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[177]. Okayama et al[174] proposed that physiologic conditions that can substantially elevate locally H2O2 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[184]. 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[170]. This finding may be supported by the fact that iron down-regulated NO synthesis and release by macrophages[177,178]. Interestingly, it was demonstrated that macrophages loaded with iron lose their ability to kill intracellular pathogens via NO formation[179], 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)[179].

Tachyzoites of T. gondii stimulate production of IL-1β[180-183] and this proinflammatory cytokine activates NK cells and T cells to produce IFN-γ and it is this early-produced IFN-γ that is crucial for host resistance[114,117,186]. IFN-γ and TNF-α act synergistically to mediate killing of tachyzoites by macrophages[123,183,184]. 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[123,185,187] (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 NFκB[183,188]. It must be noted that Gomez-Marín[190] obtained evidence of NO generation not only in the host cells, but also in T. gondii (similarly like in some other protozoans[199,191], which has its own constitutive calcium-dependent NOS producing 2-6 μM of nitrates that could be essential in intracellular signaling. The NO defensive mechanism,
where levels of nitrates can reach 120 μM or more, is probably toxic for human and mice tissues[189].

In summary, while NO at physiological levels is beneficial for the host, especially that it induces antitoxic 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 reactivates with various molecules, including superoxide, iron, thiol compounds and various homoproteins, such as CYP450 isoforms at nearly diffusion-limited rates, and these proteins might be the primary targets[192,193]. 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 2C11 > 2B1/2 > 2E1 = 3A2 > 1A1/2. NO may regulate enzymatic activity of glutathiones, Ca-dependent enzymes, and their biologic effects, via S-nitrosylation of their crucial thiol groups (e.g. coagulation factor XⅢ) [194-197]. 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[198]. For example, administration of Nω-L-arginine methyl ester to rats treated with phenobarbital and LPS blocked the down-regulation of CYP2B1/2 activity, mRNA and protein[199]. It must be noted that not only bacterial LPS but also T. gondii soluble antigen induced a subset of LPS-inducible genes in macrophages[200].

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[192,198,201]. 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[198,201]. Inflammatory mediators derived from different CYP450s could either enhance (NO, CYP3A) or inhibit (CYP2C, CYP2J) inflammatory responses[201]. For example, CYP2E1 was induced in astrocytes during brain tissue inflammation and CYP1A1 was down-regulated[202]. Injection of LPS in the brain had profound effect on CYP1A1, -2B, -2E1, and -3A activities in the liver[203,204]. LPS caused also release of many different cytokines, including TNF-α, IL-1, IL-6, IFN-γ[198]. LPS and IFN-γ induced excessive generation of NO in rat C6 glial cells, which differentially control several endogenous antioxidant enzymes, including catalase, glutathione peroxidase, CuZn- and Mn-superoxide dismutases[209].

Cytokines administered in vivo or in vitro also have enzyme-selective effects on CYP450 expression[201]. 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[207], 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[208]. LPS-induced NO formation in animals was potentiated by dexamethasone induction of CYP3A, and the potentiation was inhibited by treloamidocin, an antibiotic inhibitor of CYP3A enzymes[209]. NO stimulates TNF-α production, and inhibition of NO generation blocked TNF-α release[200]. 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**

Oxidative stress is an important mechanism in autism and direct markers for enhanced lipoxidation were reported by several authors[71,136,211,212]. 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[213-216]. 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[217]. Zhang et al.[217] 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[81]. It should be added that mtDNA components may be released also from viable neutrophils[218], and from activated tissue mast cells, a rich source of neutropectin[219-221]. Finally, mitochondria has been long recognized for their important role in cellular defense against microbial infection[220] 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[156,212,214]. It was also demonstrated that serum lipid peroxides and urinary isoprostanes were markedly higher in children with ASD[211,221]. Patients with autism also have decreased plasma total GSH and increased oxidized GSH levels[71,213,222,224].

Inflammatrory 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[225]. Enhanced NO production play an important role in these diseases, including Down syndrome[226]. Excess of NO leads to increased formation of its metabolites and reactive nitrogen species (RNI) which have antimicrobial activity. High levels of NO (> 1 μMol) and/or peroxynitrite (ONOO−), nitrogen dioxide (NO2) and dinitrogen trioxide (N2O3) formed by reaction of NO with superoxide (O2−), 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[227,228]. Apparent hydroxyl-radical production by ONOO− resulted in endothelial injury from NO and superoxide[229]. 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[230,231]. Mitochondrial disease and dysfunction (abnormal lactate, pyruvate, ubiquinone, AspAT, low carnitine levels) have been increasingly recognized in autism[232,233,234,235].
that oxidative stress in autistic patients may be at least in part due to the parasite infection restricted to intracellular area.

In mice, the response to cytokine stimulation by IL-10, IL-4, TGF-β, and IFN-γ, IFN-α, IL-2, and negatively controlled mainly by IL-10, IL-4, TGF-β, and IL-6, IL-8, and IL-12p40, in the sera of 37 IgG-seropositive patients with T. gondii infection, Karaman et al.[239] 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.[239,240] These significant alterations in redox status between the two groups of blood donors indicate that chronic T. gondii infection is associated with oxidative stress as 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.[239,240] Clancy et al.[241] 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-α, IFN-γ, IL-2, and negatively controlled mainly by IL-10, IL-4, TGF-β, and IL-6. Other cell types, such as endothelial cells and hepatocytes, display a similar capacity for NO generation in response to cytokine stimulation.[244]

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).[239] 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 infection often is regarded as a global threat. 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 any 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.[239,240]

ASD. Studies of Ashwood et al.[243] 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-α, 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.[240]. Following stimulation, the frequency of CD3+, CD4+ and CD8+ T cells expressing activation markers CD14 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-1β, IL-6, IL-8, and IL-12p40 compared with controls (P < 0.04).[62] Moreover, plasma chemokine levels MCP-1, RANTES, and eotaxin were markedly higher in children with ASD compared to controls (P < 0.03).[63] It was noted that all these biomolecular perturbations were associated with more impaired communication and aberrant behaviors.[62,63,247]. In addition, significantly higher IL-13/IL-10 and IFN-γ/IL-10 ratios were found in children with ASD than in matched controls.[65] IL-13 is a pleiotropic cytokine that shares a receptor component and signaling pathways with IL-4.[247]. IL-13 may affect T cell functions and type 1 cell differentiation indirectly.[248] through its down-regulatory effects on the production of proinflammatory cytokines[251], particularly IL-12.[251] Neonatal antigen-presenting cells showed a limited capacity to produce IL-12, due to a defect in the expression of IL-12p35 gene.[214] In view of the importance of IL-13 in type 2 inflammatory responses and clinical allergy,[225] including eotaxin production,[66], 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.[247]. 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.[257] 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 T2 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.[257]. 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.[257]. On the other hand, Chaves et al.[259] reported that IL-13 and IL-4 negatively regulated the induction of

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**Table 14** Serum glutathione, malondialdehyde, and NO concentrations in T. gondii-seropositive patients and healthy controls (acc. to Karaman et al.[239]; with own modification).

<table>
<thead>
<tr>
<th>Bioparameter</th>
<th>Group</th>
<th>No of participants</th>
<th>Serum levels (mean ± SD)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td>Patients</td>
<td>37</td>
<td>3.96 ± 0.10</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>40</td>
<td>10.37 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>Patients</td>
<td>37</td>
<td>41.32 ± 2.05</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>40</td>
<td>9.18 ± 1.21</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Patients</td>
<td>37</td>
<td>47.47 ± 1.00</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>40</td>
<td>39.18 ± 1.29</td>
<td></td>
</tr>
</tbody>
</table>

Serum glutathione and NO levels are expressed as μM, and malondialdehyde concentrations represent nM. Results statistically significant at P < 0.05.
IDO mRNA expression, tryptophan catabolism, and the control of T. gondii replication in human fibroblasts activated with IFN-γ. 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[263,264].

**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)[260,261]. The balance of T1 and T12 cytokine production differs between neonates and adults. At birth, the response is skewed in favor of T12 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[260]. The predominance of T12 responses, even in children up to 12 years of age, decreased the efficiency of host protective responses, particularly to intracellular pathogens[261] such as T. gondii. Age contributed to the percentage of CD29+ 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[260].

Bartlett et al[260] 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+ cells in the male adolescents than in the females (F = 2.24, p < 0.03), and a lower percentage of CD4+ cells was demonstrated among males than among females (F = 5.85, p < 0.0001)[261]. Moreover, the percentage but not the number of CD29+ 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[264] also reported sex differences in immune responses and demonstrated enhanced immune reactivity in 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[279,290], are at least in part, responsible for the delayed diagnosis of autism despite evidence of prenatal changes in the brain[278,279]. 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[269] (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[269,272,275].

The important immune responses and immune reactivity findings related to age and gender in the adolescent children[263,264] 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[274]. In male animals, a rapid response to infection with high levels of TNF-α and IFN-γ 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-γ generation compared with their male counterparts may account for their higher mortality rates and cyst burdens[270]. It should be also added that different strains of T. gondii induce different cytokine responses[277], as well as stage of parasite development and generated stage-specific antigens following pathogen interconversion (tachyzoites, bradyzoites, oocyst-sporozoites) (Table 17)[278,279] 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[261].

<table>
<thead>
<tr>
<th>Table 15 Distribution of lymphocyte subtypes in the fetus, newborn and adult (acc. to Schultz et al[265]; Luebke et al[266]; with own modification).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fetus</strong></td>
</tr>
<tr>
<td><strong>Marker</strong></td>
</tr>
<tr>
<td>WBC</td>
</tr>
<tr>
<td>Lymphocytes</td>
</tr>
<tr>
<td>CD4</td>
</tr>
<tr>
<td>CD8</td>
</tr>
<tr>
<td>CD4</td>
</tr>
<tr>
<td>CD8</td>
</tr>
<tr>
<td>CD4:CD8</td>
</tr>
<tr>
<td>CD8 (B cells)</td>
</tr>
</tbody>
</table>

1 Per min. 2 Significantly different from adults. 3 Significantly different from neonates.
Table 17 | Identification of stage-specific antigens of *T. gondii*. Value of ELISA titer of pooled immune mouse antiserum to Toxoplasma tachyzoites, bradyzoites, and oocyst-sporozoites (230).

<table>
<thead>
<tr>
<th>Antiserum fraction</th>
<th>Oocyst-sporozoites</th>
<th>Antibody titer for: Tachyzoites</th>
<th>Bradyzoites</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>450</td>
<td>1350</td>
<td>12150</td>
</tr>
<tr>
<td>Bradyzoites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oocyst-sporozoites</td>
<td>36450</td>
<td>450</td>
<td>1350</td>
</tr>
<tr>
<td>Tachyzoites</td>
<td>450</td>
<td>12150</td>
<td>1350</td>
</tr>
<tr>
<td>IgM</td>
<td>1350</td>
<td>450</td>
<td>1350</td>
</tr>
<tr>
<td>Bradyzoites</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oocyst-sporozoites</td>
<td>1350</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Tachyzoites</td>
<td>1350</td>
<td>450</td>
<td>450</td>
</tr>
</tbody>
</table>

1 Parasites were used at a concentration of 10⁶ per well. Mouse antiserum 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, 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 (283).

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 (280) 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 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 Cubula-Kucharska, personal information, November 2012) (278). 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 lowered levels of IgG, IgA, and IgM due to *T. gondii* infection (284). 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) (280,281), may be associated with their exhausted secondary innate and adaptive immune responses directed against foreign *T. gondii* antigens (280), in which host-endoplasmic reticulum-parasitophorous vacuole interaction provides a route of entry for antigen cross-presentation in *T. gondii*-infected dendritic cells (280,281). 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 (280), and in the autistic children (280). 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 (287).

Lefevre-Pettazzoni et al. (291) 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. (292) 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 > 9 IU/mL). It appeared that all Toxoplasma-positive children had high avidity index indicating chronic infection. 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 (284,290). It seems therefore that the enhanced cerebrospinal fluid production and elevated extra-axial fluid found in infants, older children and adults with ASD (290-293), as well as the recurrent headaches reported in individuals with idiopathic intracranial hypertension (282,283,290), were due to congenital or acquired latent *T. gondii* infection, especially that plexus choroidaeus 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 (279) 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 (290). Thus, the increased generation of antibodies and autoantibodies against brain proteins in autistic individuals and their families should not be surprising (290), especially that for example autistic subjects had significantly elevated levels of antibodies to heat shock protein 90 (HSP90) (291), which is critical for the maintenance of proper protein synthesis and function (290). Nb. *T. gondii* HSP90 could be linked with many essential processes of the parasite such as host cell invasion, replication and tachyzoite-bradyzoite interconversion (290,294). 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 Stermerberger et al. (290) and modified and applied successfully by Conley & Jenkins (20) especially that all lots used at that time were negative for Toxoplasma antibody analyzed by the Sabin-Feldman dye test (280).

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. (297) proposed a simple test for diagnosis of congenital toxoplasmosis based on whole-blood IFN-γ 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-γ 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 effect 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.

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Prandota J et al. Seroprevalence of chronic toxoplasmosis in autistic children


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