Lack of circulating *Toxoplasma gondii* DNA in seropositive patients with bipolar or schizophrenia spectrum disorders.

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Abstract

Toxoplasmosis has been previously associated with an increased risk of having Schizophrenia or Bipolar disorder in several epidemiological studies. The aim of this observational, cross-sectional study was to examine the seroprevalence of *Toxoplasma* infection in a cohort of Italian psychiatric inpatients and to verify the presence of circulating *Toxoplasma gondii* DNA in the seropositive subjects. Sixty-three patients affected by bipolar or schizoaffective disorders according to DSM 5 criteria were enrolled. The presence of *Toxoplasma* infection was firstly examined using an indirect serological method (ELFA), and three different direct PCR-based methods were performed to detect circulating DNA in the seropositive patients. The seroprevalence of infection was 28.6%, with a significant association between higher age and the infection status. PCR, nested-PCR and Real-Time PCR revealed no positive samples for *Toxoplasma gondii.* This result is in contrast with recent data from case-control studies that detected parasite genome in patients with different neuropsychiatric diagnosis without clinical evidence of acute toxoplasmosis. Our findings are to be interpreted with caution, because of the small sample size, the heterogeneity of enrolled patients and the observational nature of the study. Further studies are needed to better define the clinical features correlated to the seropositive status in neuropsychiatric patients.

Keywords:

Toxoplasma gondii, seroprevalence, circulating parasite DNA, PCR, nested-PCR, RT-PCR, bipolar disorder, schizoaffective disorder

Highlights

- *T. gondii* was associated with increased risk of Schizophrenia or Bipolar Disorder
- We analyzed circulating *T. gondii* DNA in a cohort of Italian psychiatric inpatients
- Seropositive status for IgG anti-*T. gondii* was found in 28,6% of patients
- PCR, nested-PCR and Real-Time PCR revealed no positive samples for *T. gondii*
- No significant association between the seropositive status and diagnostic category

1. Introduction

Toxoplasmosis is the most common neurotropic protozoan infection in humans caused by the parasite *Toxoplasma gondii* (*T.gondii*). It can induce highly variable disease manifestations, depending mainly on the immunological status of the host but also on the genotype of the parasite, ranging from asymptomatic infection in immunocompetent individuals to severe forms associated with encephalitis, chorioretinitis, uveitis, or multi-organ involvement in the cases of immunocompromised individuals, congenital infection or infection with more virulent type of strains (Boothroyd and Grigg, 2002; Montoya and Liesenfeld, 2004; Remington et al., 2001).

T. gondii infects around one-third of humans worldwide but the prevalence rates vary widely among different Countries (Flegr et al., 2014); in Central Italy, a geographical area at intermediate prevalence of toxoplasmosis, the percentage is estimated to be about 25-30% (Mosti et al., 2013; Pinto et al., 2012, 2017), whereas in Ethiopia, among pregnant women attending routine antenatal care, or in other countries of South America and Africa at high-risk of toxoplasmosis, the seroprevalence can reach 80-90% (Gelaye et al., 2015; Montoya and Liesenfeld, 2004; Robert-Gangneux and Dardé, 2012).

In humans, that are intermediate hosts, the infection is commonly acquired by the oral ingestion of tissue cysts containing bradyzoites through the consumption of raw or undercooked meat infected with *T. gondii*; however, it can also be acquired via ingestion of the parasite's oocysts, which are contained in the feces of infected cats, or by contaminated water (Bahia-Oliveira et al., 2003; Jones et al., 2009; Weiss and Dubey, 2009). This parasite has a particular tropism for muscle and brain tissue, where it remains localized in the form of cysts throughout life and establishes a chronic infection stimulating the production of a variety of cytokines by microglia, astrocytes and neurons (Carruthers and Suzuki, 2007; Prandovszky et al., 2011).

In last decades, an increasing number of epidemiological studies has highlighted the potential involvement of *T. gondii* infection in the etiopathogenesis of several neuropsychiatric disorders. The role of this infectious agent in the emergence of major psychosis, such as Schizophrenia (SCH), has been widely documented (Amminger et al., 2007; Esshili et al., 2016; Hinze-Selch et al., 2007; Torrey et al., 2007, 2012; Wang et al., 2006; Yolken et al., 2017) but, more recently, the association of toxoplasmosis with Bipolar disorder (BD) has gained growing attention as well (Dickerson et al., 2014a, 2014b; Hamdani et al., 2013, 2015; Pearce et al., 2012; Sutterland et al., 2015; Tedla et al., 2011). A positive correlation has also been detected between a diagnosis of Obsessive-Compulsive

Disorder, Tourette's syndrome or tic disorder and anti-*T. gondii* antibodies (Akaltun et al., 2018a, 2018b; Flegr and Horáček, 2017; Taboas et al., 2012).

Several mechanisms by which *Toxoplasma* infection might induce neuronal function alterations underpinning the development or the progression of psychiatric disorders have been hypothesized: increased dopamine production, apoptosis inhibition, protein injection on neuronal surface membrane, increased endocannabinoid levels, neurodegeneration, neurotransmitters balance alteration, impact on neuroplasticity, induction of neuroinflamamtion and systemic phlogosis (Fabiani et al., 2013, 2015; Parlog et al., 2015; Del Grande et al., 2017a).

Almost all previous studies that have investigated the association of *T. gondii* infection with neuropsychiatric disorders were based on serological assessments, and the results were often controversial, so no conclusive cause-effect causality could be established (Alvarado-Esquivel et al., 2011; Arias et al., 2012; Cetinkaya et al., 2007; Dickerson et al., 2014b; Freedman et al., 2016; Hamdani et al., 2013; Pearce et al., 2012; Sutterland et al., 2015; Tedla et al., 2011; Torrey et al., 2007, 2012).

Noteworthy, recent evidence demonstrated the presence of circulating *T. gondii* DNA in the blood of psychiatric patients. Del Grande et al. (2017b) described the case of a patient affected by recurrent ocular toxoplasmosis and BD with psychotic features. The peculiarity of this case was that in this patient, parasite DNA was detected at the time of the occurrence of psychiatric symptoms through molecular analyses (nested-PCR) proving a reactivation of the infection. Moreover, in three case-control studies a high positivity rate for circulating *T. gondii* DNA was found in patients with SCH, Parkinson's disease (PD) and Alzheimer's disease (AD) without clinical evidence of toxoplasmosis (Fallahi et al., 2017; Omar et al., 2015; Rashno et al., 2017).

On the basis of this literature evidence, we aimed to investigate the seroprevalence of *T. gondii* infection in a sample of Italian psychiatric inpatients affected by BD or SCH spectrum disorders and to verify the presence of circulating *T. gondii* DNA in the seropositive subjects, in order to identify possible differences in clinical or demographic characteristics that can be associated with chronic toxoplasmosis. The findings would further help in understanding the possible etiopathogenetic mechanisms underpinning the association between *T. gondii* and neuropsychiatric disorders.

Material and Methods

2.1 Sample

In this observational, cross-sectional study we enrolled sixtythree inpatients, all born in Italy, with a diagnosis of Schizoaffective disorder (SZA) (*n=20*) or BD with or without psychotic features

(*n=43*), recruited among patients consecutively admitted at the hospital or day-hospital unit of the Psychiatric Clinic of the Department of Clinical and Experimental Medicine, University of Pisa, Italy. The diagnosis of SZA or BD according to DSM 5 (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) criteria was made by psychiatrists using the SCID 5 (Structured Clinical Interview for DSM-5) (First et al., 2015). The study was approved by local institutional ethical committee (No. 333, approved on May 14th 2015) and all participants provided written informed consent after the explanation of the study procedures.

An anamnestic questionnaire was administered to the enrolled patients in order to collect socio-demographic characteristics (age, birthplace, place of residence, education level, marital status, working status, family environment), risk factors of exposure to the parasite (travel in regions at high-risk of toxoplasmosis, rural dwelling, usual consumption of raw or undercooked meat, habitual contact with cats during life), the presence of pathological conditions well known to be associated with toxoplasmic infection (lifetime episodes of lymphadenopathy, abnormal psychomotor development, epilepsy, eye diseases) and family history of allergic or autoimmune diseases.

At the time of hospitalization, peripheral blood of each patient was collected in a 10 ml Vacutainer tube without anticoagulant for serological analysis and in a 5 mL Vacutainer tube with EDTA (Vacutest, Kima) for molecular analysis. Samples for serological investigations were centrifuged at 1,500 x g for 10 minutes before storage at -20°C, while those allocated to molecular analysis were immediately stored at -20 ° C.

2.2 *T. gondii* serological analysis

The serum samples were examined by the Enzyme Linked Fluorescent Assay (ELFA, Biomerieux, France) using the Vidas Toxo IgG II kit for IgG and Vidas Toxo IgM kit for IgM. The serological tests were all conducted in accordance with the manufacturer's instructions. The level of IgG was expressed in International Units/mL and samples were considered positive for IgG antibodies when the value was >8 IU/mL, indeterminate from ≥4 to ≤8 IU/mL and negative when <4 IU/mL. The IgM level was expressed as sample index (value of relative fluorescence of the sample/relative fluorescence value of the standard); ELFA results were positive when the reagent index was ≥0.65 IU/mL, indeterminate from <0.65 to ≥0.55 IU/mL and negative < 0.55 IU/mL.

2.3 *T. gondii* molecular analysis

Samples collected in EDTA of patients with serologically positive results were undergone to molecular analysis using three different PCR-based methods.

Toxoplasma DNA was extracted from 5 mL of peripheral blood collected in EDTA using Qiamp Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. During every session of extraction, along with the samples, extraction of tachyzoites DNA (kindly provided by Dr. Spano the ISS of Rome) was performed, as positive extraction control. The extracted DNA was stored in a freezer at -20°C until polymerase chain reaction (PCR).
Three different methods were carried out in order to detect protozoan DNA: PCR, nested-PCR and RT-PCR.

2.3.1 PCR

PCR was performed selecting a 529bp fragment that is repeated 200- to 300-fold in the genome of *T. gondii*, as previously described by Homan et al. (2000). Polymerase chain reaction with the 529bp fragment is more sensitive than with the 35-copy B1 gene. PCR was conducted in 0.5 mL microcentrifuge tubes containing a final volume of 50 µL composed by 60 mM Tris-HCl (ph 9.0), 2 mM MgCl2, 15 MM of (NH4) 2 SO4, 0.1% Triton X-100, 0.5 µM of each primer, 100 µM of Deoxyribonucleoside triphosphate, 0.5 U of Taq polymerase (Thermo Scientific) and 10 ng of DNA extracted.
The TOX4 primers (CGCTGCAGGGAGGAAGACGAAAGTTG) and TOX5 (CGCTGCAGACACAGTGCATCTGGATT) were selected respectively from the 5 'and 3' ends of the fragment of 529 pb.
The PCR cycling conditions consisted of an initial denaturation step at 94°C for 7 minutes, 35 amplification cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C with a final extension of 10 minutes at 72°C in a thermocycler.
The amplification product ran by horizontal electrophoresis on agarose at 1.5%. The electrophoretic running occurred in TBE 1X buffer (Tris 89 mM, Boric acid 89 mM, EDTA 0.5 M, pH 8.8) for one hour to 100 V. Ethyl bromide (0.5 mg / mL) was incorporated into the agarose gel to allow for DNA highlighting by ultraviolet exposure. The molecular weight of the obtained bands was determined by comparison with markers of known molecular size (100 bp).

2.3.2 Nested-PCR

Nested-PCR was performed employing different primer pairs which amplify the MAG1 gene that encodes a 65-kDa matrix antigen strongly expressed by the bradyzoides. Although MAG1 protein was originally described as being expressed specifically during bradyzoite development, a study showed that this protein is expressed during both tachyzoite and bradyzoite development (Ferguson and Parmley. 2002). This method was previously tested with success by Contini et al. (2002) to

improve the detection rate of toxoplasmosis reactivation in suspected HIV or HIV-susceptible patients.

Nested-PCR was initially performed in 0.5 mL microcentrifuge tubes in the final volume of 50 µL containing 1.5 mM MgCl2, 0.2 mM dNTP, 10 µm of each primer and 2 U of Taq and 10 ng of extracted DNA. The second amplification was performed using 2 µL of amplificate from the first reaction.

External primers M1OP1 (sense primer) and M1OP2 (antisense primer), and internal M1IP1 (Sense primer) and M1IP2 (antisense primer) had the following sequences, respectively: TGAGAACTCAGAGGACGTTGC (M1OP1), TCTGACTCAAGCTCGTCTGCT (M1OP2), GCATCAGCATGAGACAGAAGA (M1IP1) and CCAACTTCGAAACTGATGTCG (M1IP2).

The amplification reaction performed by a thermocycler consisted of an incubation of 10 minutes at 95°C followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 48° C, one minute at 72° C, and a final incubation of ten minutes at 72° C.

The amplification product ran by horizontal electrophoresis on agarose at 2%. The electrophoretic running occurred in TBE 1X buffer (Tris 89 mM, Boric acid 89 mM, EDTA 0.5 M, pH 8.8) for one hour to 100 V. Ethyl bromide (0.5 mg / mL) was incorporated into the agarose gel to allow for the DNA highlighting by ultraviolet exposure. The molecular weight of the obtained bands was determined by comparison with markers of known molecular size (100 bp).

2.3.3 RT-PCR

DNA extractions from IgG anti-*Toxoplasma* positive blood samples were tested for the presence of *T. gondii* through a Real-Time PCR protocol, using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). The target was a 200- to 300-fold repetitive 529 bp region of the parasite genome, detected using the primers TOX4 and TOX5, as described by Homan et al. (2000). The concentration for each primer was 0.25 µM in a final volume of 20 µl. Amplification protocol was characterised by a denaturation step of 7 minutes at 94 °C followed by 45 repeated cycles at 94°C (30 sec), 55°C (30 sec) and 72°C (30 sec). Fluorescence signals were collected at the end of every cycle and the presence of unspecific products was avoided through the analysis of the melting curve. Each sample was tested with at least four replicates.

The presence of the parasite DNA was quantified through a standard curve. The DNA deriving from *T. gondii* tachyzoites was used as template for the amplification of the 529 bp fragment. The PCR product was run on a 2% agarose gel, extracted using a commercial kit (JETQUICK Gel Extraction

Spin Kit, Genomed), and quantified through spectrophotometry (Eppendorf). The standard curve was fitted within 4 points and ranging from 1.6 µg/µl to 1.6 pg/µl.

To confirm the presence or the absence of specific products, random amplifications were fractionated on a 2% agarose gel, stained with SYBR Safe DNA Gel Stain (Thermo Fischer Scientific), and visualized by UV transillumination.

2.4 Statistical analysis

The analysis of demographic, clinical and biological characteristics of seropositive patients, versus seronegative patients, was performed using Fisher's exact test for categorical variables. The results were analyzed using GraphPad Stat Software.

Results

Anti-*T. gondii* IgG were present in eighteen patients (28.6%) in our sample; the seropositive status was found in 14 (32.5%) of the patients with BD and 4 (20%) of the patients with SZA. None of the patients was in an acute phase of infection as IgM antibodies detection was negative for the whole sample.

Comparing the socio-demographic and epidemiological characteristics of *T. gondii* seropositive and seronegative patients, differences between the two groups were detected for mean age (45.7±9.4 years vs 38.2±12.2 years), rural dwelling (44.4% vs 24.4%), raw meat consumption (88.9% vs 66.7%), ocular diseases (5.5% vs 6.7%) and family history of allergic diseases (11.1% vs 20%). However, among these differences only the age distribution was statistically significant, probably due to the small size of the sample. No differences were found in the education level, habitual contact with a cat during life, abnormal psychomotor development, incidence of epilepsy, family history of autoimmune diseases and psychiatric diagnosis as regards the seropositive status (Table 1).

The mean titre of IgG anti-*T. gondii* was 69.5 IU/mL (range 9-300 IU/mL) and the median was 46.5 IU/ml. Because of the great difference between the size of the two diagnostic categories of patients, it is difficult to evaluate the relevance of different serum intensity between the two groups (serointensity dispersion in patients is shown in Figure1).

Table 1. Socio-demographic and clinical characteristics of the sample: comparison between *Toxoplasma*-seropositive and *Toxoplasma*-seronegative patients

	Total (*n=63*)	***Toxolasma*-Seropositive (*n=18*)**	***Toxoplasma*-Seronegative (*n=45*)**	***p-value***
Age (years)	40.4±11.8	45.7±9.4	38.2±12.2	**0.029**
Gender (Female %)	46%	50%	44.4%	0.78
Education level (lower secondary school and above)	76.2%	77.8%	75.6%	1
Rural dwelling	30.2%	44.4%	24.4%	0.13
Usual consumption of raw or undercooked meat	73%	88.9%	66.7%	0.12
Habitual contact with cats during life	63.5%	66.6%	62.2%	0.78
Ocular diseases	6.3%	5.5%	6.7%	1
Epilepsy	11.1%	11.1%	11.1%	1
Family history of allergic diseases	17.5%	11.1%	20%	0.49
Family history of autoimmune diseases	4.7%	5.5%	4.4%	1
SZA *versus* BD				0.38
SZA	31,7% (n=20)	20% (n=4)	80% (n=16)	
BD	68,3% (n=43)	32,5% (n=14)	67,5% (n=29)	

PCR, nested-PCR and Real-Time PCR revealed no positive samples for *T. gondii*. Agarose gel runs with amplification products obtained with Real-time PCR are shown in Figure 2.

Discussion

This study aimed to evaluate the possible association between chronic toxoplasmosis and different psychiatric diagnosis, using both serological and molecular techniques.

In a sample of BD or SZA patients, we observed a seroprevalence rate for *T. gondii* of 28%, that is similar to that described in the general population with the same age distribution in Italy, as previously reported in some epidemiological studies (Mosti et al., 2013; Pinto et al., 2012, 2017). Although there was no statistically significant difference in the seropositive status within the two different diagnostic groups, a higher percentage of seropositivity has been detected in the group of bipolar patients, strengthening the evidence of a positive association between toxoplasmosis and BD (Abdollahian et al., 2017; Dickerson et al., 2014b; Hamdani et al., 2013; Pearce et al., 2012; Tedla et al., 2011).

Our data also showed a different age distribution between seropositive and seronegative patients. The detection of a highest age within the seropositive group is in agreement with epidemiological data that shows an increase in the incidence of toxoplasmic infection associated with age increases, and a concomitant reduction in the rate of infection in youngest people in medium and high income populations in North Europe and USA (Dubey and Jones, 2008; Hofhuis et al., 2011; Jones et al., 2007, 2018; Mosti et al., 2013; Pinto et al., 2017).

Comparing the other socio-demographic and epidemiological characteristics of *T. gondii* seropositive and seronegative subjects we did not find statically significant differences, probably due to the small size of the sample.

The main finding of our study is the lack of circulating *T. gondii* genome in the group of seropositive patients despite the use of three different molecular analysis methods. This result is in contrast with data reported in three previous different case-control studies, in which parasite genome was detected in patients with SCH, PD and AD, respectively, without clinical evidence of acute toxoplasmosis (Fallahi et al 2017; Omar et al. 2015; Rashno et al. 2017).

A cross-sectional case-control study by Omar et al. (2015) examined the serofrequency and serointensity of *T. gondii* among patients with SCH. The strength of this study was the use of both indirect (ELISA) and direct (quantitative real-time PCR) methods for examining the presence of anti-*T. gondii* IgG and DNA, respectively. Besides a higher serofrequency of *T. gondii* IgG antibodies among patients with SCH than controls, Authors reported that *T. gondii* DNA was positive in 32.67% of patients and the positivity of DNA was significantly higher in patients than controls (3.64%), with an OR of 12.9. This data strongly confirmed the positive association between toxoplasmosis and the risk of having SCH. Furthermore, 7.7% of schizophrenic patients, but none of the control subjects, was positive for IgM, suggesting the possibility of a recent onset infection.

Recently, Del Grande et al. (2017b) reported a considerable relationship between ocular manifestations of *T. gondii* infection and the onset and recurrences of psychiatric symptoms in a Brazilian female affected by BD with psychotic features and recurrent episodes of toxoplasmic chorioretinitis and uveitis. Noteworthy, this is the first case described in the literature in which infection reactivation was directly documented through molecular analyses (nested-PCR) and indirectly, by the high level of *T. gondii*-specific IgG, while ocular and psychiatric symptoms were present.

Toxoplasmosis seroprevalence was also studied in cohorts of other neuropsychiatric diseases. In a sample of 87 AD patients and 87 healthy controls without any neuronal and mental disorders, Rashno et al. (2017) found various levels of anti-*Toxoplasma* IgG in 61.5% of subjects, whereas none of the subjects was positive to *Toxoplasma*-IgM antibody. Despite a not significant

relationship between AD and toxoplasmosis, the seroprevalence of infection was higher in AD patients compared with the control group. By using the PCR, *T. gondii* DNA was detected in 52.8% of patients and, curiously, in 40.2% of control subjects.

Fallahi et al. (2017) investigated the possible association between PD and *T. gondii* infection using both serological and molecular techniques. PCR assay was performed in duplicate using the primer pair of TOXO1 and TOXO2 targeting the B1 gene of *Toxoplasma*. In this sample, no significant difference in prevalence of *T. gondii* infection was observed in serological tests based on IgG titre, while PCR assay detected a statistically significant difference between case and control groups (19.3% vs 10.4%), suggesting that patients with PD could be at higher risk for getting *Toxoplasma* infection. The IgM seropositivity was 2.6% and 0.8% in PD patients and controls, respectively.

The absence of circulating *T. gondii* DNA in seropositive patients in our sample, in contrast with previous evidence described in the literature, could be due to several factors: all patients were immunocompetent, not treated with immunosuppressive drugs and none of them had evidence of infection reactivation or acute infection, as specific IgM were negative in the whole sample, whereas in the case-control studies mentioned above some patients, as well as controls, were found to be IgM positive. Moreover, our sample is composed mainly by patients with a chronic long-term disease with several illness episodes treated with numerous antipsychotic and mood stabilizing drugs, which can cause a reduction in the level of IgG anti-*T.gondii* and DNA by inhibiting the replication of the parasite (Jones-Brando et al., 2003; Webster et al., 2006). For this reason, the effects of antipsychotic medications, as well as timing of infection should be considered in these patients, since a temporal relationship between *Toxoplasma* exposure and disease onset has been recently suggested (Leweke et al., 2004; Omar et al., 2015; Yolken et al., 2017).

Toxoplasma-DNA detection in immunocompetent subjects, especially when IgM are negative, should be accurately investigated. Data from the literature are controversial, and some Authors (Omar et al., 2015) found circulating parasite DNA also in healthy, not schizophrenic, controls. Iranian Authors (Fallahi et al., 2017) explained their data with a high rate of *T. gondii* infection in that Country and with the high sensitivity of PCR which can detect parasite DNA also in patients with chronic toxoplasmosis, as previously described by Silveira et al. (2011). Patients in that study were from Erechim, which is known to be an area with a high prevalence of ocular toxoplasmosis and where atypical *T. gondii* genotypes circulate (Carneiro et al., 2013). Non-II genotypes are common in Malaysia (Puvanesuaran et al., 2013) and Iran (Danechin et al., 2016) as well, the two Countries where studies mentioned above were carried out, whereas in Italy and Europe type II is the most frequent strain affecting humans (Ajzenberg et al., 2002; Peyron et al., 2006). The differences in genotype distribution between these different geographical areas might explain the

discrepancy between our results and those obtained by other groups of study, although this hypothesis should be further investigated.

Many pathogenetic mechanisms have been proposed to explain the disruptive role of *T. gondii* infection in the brain and its involvement in the development or progression of psychiatric disorders. Several data suggest that the parasite may directly affect neuronal functions through cyst localization in specific brain areas implicated in the onset of psychiatric symptoms, as well as through the *T. gondii*-induction of dopamine production, inhibition of host cell apoptosis and triggering type W human endogenous retroviruses (HERV-W) elements in human genome (Perron et al., 2012; Prandovszky et al., 2011). In addition to these direct effects, chronic neuronal infection causes a persistent neuroinflammation state through the production of cytokines by microglia and astrocytes which indirectly modulate several neurotransmitter pathways implicated in the pathogenesis of psychotic syndromes and aggressive behaviors, such as GABAergic and glutamatergic neurotransmission (Coccaro et al., 2013; Fabiani et al., 2013, 2015; Flegr, 2013; Yolken et al., 2009). Therefore, these mechanisms might account for the contribution of *T. gondii* to the onset and maintaining of psychiatric disorders even in absence of a reactivation of toxoplasmic infection.

The results of this study should be interpreted keeping in mind some limitations: the small sample size, that we are going to enlarge; the observational nature of the study, with the lack of a control group; and the heterogeneity of the sample, with patients in different stage of illness and under different treatments, that does not allow to draw conclusions about the etiophatogentic role of the parasite and the association with a specific diagnostic category.

However, according to our knowledge, to date this is the first study evaluating the *T. gondii* seroprevalence and the presence of circulating parasite DNA in a cohort of Italian psychiatric inpatients. A more comprehensive research in larger sample of patients is required to consolidate this findings and to better define the clinical features correlated to the seropositive status in BD.

Finally, it seems to be of importance to consider that a better understanding of the contribution of infectious agents like *T. gondii* in the pathophysiology of BD or other psychotic disorders could help in the identification of preventive measures and development of new pharmacological treatment approaches, with a significant impact on the disease course.

Disclosure statement

The authors declare that they have no conflict of interest with regard to the present work. The authors alone are responsible for the content and writing of the paper.

References

Abdollahian, E., Shafiei, R., Mokhber, N., Kalantar, K., Fata, A., 2017. Seroepidemiological study of *Toxoplasma gondii* infection among psychiatric patients in Mashhad, Northeast of Iran. Iran. J. Parasitol. 12(1), 117-122.

Ajzenberg, D., Cogné, N., Paris, L., Bessières, M., Thulliez, P., Filisetti, D., et al., 2002. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. J. Infect. Dis. 186(5), 684–689.

Akaltun, İ., Kara, S.S., Kara, T., 2018a. The relationship between *Toxoplasma gondii* IgG antibodies and generalized anxiety disorder and obsessive-compulsive disorder in children and adolescents: a new approach. Nord. J. Psychiatry. 72(1), 57-62.

Akaltun, İ., Kara, T., Sertan Kara, S., Ayaydın, H., 2018b. Seroprevalance Anti-*Toxoplasma gondii* antibodies in children and adolescents with tourette syndrome/chronic motor or vocal tic disorder: A case-control study. Psychiatry. Res. 263, 154-157.

Alvarado-Esquivel, C., Urbina-Álvarez, J.B., Estrada-Martinez, S., Toress-Castorena, A., Molotla-de-León, G., Liesenfeld, O., Dubey, J.P., 2011. *Toxoplasma gondii* infection and schizophrenia: a case control study in a low *Toxoplasma* seroprevalence Mexican population. Parasitol. Int. 60, 151-155.

Amminger, G.P., McGorry, P.D., Berger, G.E., Wade, D., Yung, A.R., Phillips, L.J., et al., 2007. Antibodies to infectious agents in individuals at ultra-high risk for psychosis. Biol. Psychiatry. 61, 1215–1217.

Arias, I., Sorlozano, A., Villegas, E., de Dios Luna, J., McKenney, K., Cervilla, J., et al., 2012. Infectious agents associated with schizophrenia: a meta-analysis. Schizophr. Res. 136, 128-136.

Bahia-Oliveira, L.M., Jones, J.L., Azevedo-Silva, J., Alves, C.C., Oréfice, F., Addiss, D.G., 2003. Highly endemic, waterborne toxoplasmosis in north Rio de Janeiro state, Brazil. Emerg. Infect. Dis. 9, 55–62.

Boothroyd, J.C., Grigg, M.E., 2002. Population biology of *Toxoplasma gondii* and its relevance to human infection: do different strains cause different disease? Curr. Opin. Microbiol. 5 (4), 438–442.

Carneiro, A.C., Andrade, G.M., Costa, J.G., Pinhero, B.V., Vascpncelos-Santos, D.V., Ferreira, A.M., et al., 2013. Genetic characterization of *Toxoplasma gondii* revealed highly diverse genotypes for isolates from newborns with congenital toxoplasmosis in southeastern Brazil. J. Clin. Microbiol. 51(3), 901-917.

Carruthers, V.B., Suzuki, Y., 2007. Effects of *Toxoplasma gondii* infection on the brain. Schizophr. Bull. 33, 745-751.

Cetinkaya, Z.S., Yazar, S., Gecici, O., Namli, M.N., 2007. Anti-*Toxoplasma gondii* antibodies in patients with schizophrenia- preliminary findings in a Turkish sample. Schizophr. Bull. 33, 789-791.

Coccaro, E.F., Lee, R., Vezina, P., 2013. Cerebrospinal fluid glutamate concentration correlates with impulsive aggression in human subjects. J. Psychiatr. Res. 47, 1247–1253.

Contini, C., Cultrera, R., Seraceni, S., Segala, D., Romani, R., Fainardi, E., et al., 2002. The role of stage-specific oligonucleotide primers in providing effective laboratory support for the molecular diagnosis of reactivated *Toxoplasma gondii* encephalitis in patients with AIDS. J. Med. Microbiol. 51, 879–890.

Danehchin, L., Razmi, G., Naghibi, A., 2016. Isolation and genotyping of Toxoplasma gondii strains in ovine aborted fetuses in Khorasan Razavi Province, Iran. Korean. J. Parasitol. 54,15-20.

Del Grande, C., Galli, L., Schiavi, E., Dell'Osso, L., Bruschi, F., 2017a. Is *Toxoplasma gondii* a Trigger of Bipolar Disorder? Pathogens. 6, 3.

Del Grande, C., Contini, C., Schiavi, E., Rutigliano, G., Maritati, M., Seraceni, S., et al., 2017b. Bipolar disorder with psychotic features and ocular toxoplasmosis. J. Nerv. Ment. Dis.. 205(3), 192-195.

Dickerson, F., Stallings, C., Origoni, A., Katsafanas, E., Schweinfurth, L., Savage, C., et al., 2014a. Antibodies to *Toxoplasma gondii* and cognitive functioning in schizophrenia, bipolar disorder, and nonpsychiatric controls. J. Nerv. Ment. Dis. 202(8), 589–593.

Dickerson, F., Stallings, C., Origoni, A., Vaughan, C., Katsafanas, E., Khushalani, S., Yolken, R., 2014b. Antibodies to *Toxoplasma gondii* in individuals with mania. Bipolar. Disord. 16(2), 129-136.

Dubey, J.P., Jones, J.L., 2008. *Toxoplasma gondii* infection in humans and animals in the United States. Int. J. Parasitol. 38(11), 1257-1278.

Esshili, A., Thabet, S., Jemli, A., Trifa, F., Mechri, A., Zaafrane, F., et al., 2016. *Toxoplasma gondii* infection in schizophrenia and associated clinical features. Psychiatry. Res. 245, 327-332.

Fabiani, S., Pinto, B., Bruschi, F., 2013. Toxoplasmosis and neuropsychiatric diseases: can serological studies establish a clear relationship? Neurol. Sci. 34, 417–425.

Fabiani, S., Pinto, B., Bonuccelli, U., Bruschi, F., 2015. Neurobiological studies on the relationship between toxoplasmosis and neuropsychiatric diseases. J. Neurol. Sci. 351, 3–8.

Fallahi, S., Rostami, A., Birjandi, M., Zebardast, N., Kheirandish, F., Spotin, A., 2017. Parkinson's disease and *Toxoplasma gondii* infection: sero-molecular assess the possible link among patients. Acta. Tropica. 173, 97-101.

Ferguson, D.J., Parmley, S.F., 2002. *Toxoplasma gondii* MAG1 protein expression. Trends. Parasitol. 18(11), 482.

First, M.B., Williams, J.B.W., Karg, R.S., Spitzer, R.L., 2015. Structured Clinical Interview for DSM-5—Research Version (SCID-5 for DSM-5, Research Version; SCID-5-RV), Version 1.0.0. American Psychiatric Association, Arlington, VA.

Flegr, J., 2013, How and why *Toxoplasma* makes us crazy. Trends. Parasitol. 29(4), 156-163.

Flegr, J., Prandota, J., Sovičková, M., Israili, Z.H., 2014. Toxoplasmosis—a global threat. Correlation of latent toxoplasmosis with specific disease burden in a set of 88 countries. PLoS. One. 9(3), e90203.

Flegr, J., Horáček, J., 2017. *Toxoplasma*-infected subjects report an Obsessive-Compulsive Disorder diagnosis more often and score higher in Obsessive-Compulsive Inventory. Eur. Psychiatry. 40, 82-87.

Freedman, D., Bao, Y., Shen, L., Schaefer, C.A., Brown, A.S., 2016. Maternal *T. gondii*, offspring bipolar disorder and neurocognition. Psychiatry. Res. 243, 382-389.

Gelaye, W., Kebede, T., Hailu, A., 2015. High prevalence of anti-toxoplasma antibodies and absence of *Toxoplasma gondii* infection risk factors among pregnant women attending routine antenatal care in two Hospitals of Addis Ababa, Ethiopia. Int. J. Infect. Dis. 34, 41-45.

Hamdani, N., Daban-Huard, C., Lajnef, M., Richard, J.R., Delavest, M., Godin, O., et al., 2013. Relationship between *Toxoplasma gondii* infection and bipolar disorder in a French sample. J. Affect. Disord. 148, 444–448.

Hamdani, N., Daban-Huard, C., Lajnef, M., Gadel, R., Le Corvoisier, P., Delavest, M., et al., 2015. Cognitive deterioration among bipolar disorder patients infected by *Toxoplasma gondii* is correlated to interleukin 6 levels. J. Affect. Disord. 179, 161–166.

Hinze-Selch, D., Däubener, W., Eggert, L., Erdag, S., Stoltenberg, R., Wilms, S., 2007. A controlled prospective study of *Toxoplasma gondii* infection in individuals with schizophrenia: Beyond seroprevalence. Schizophr. Bull. 33, 782–788.

Hofhuis, A., van Pelt, W., van Duynhoven, Y.T., Nijhuis, C.D., Mollema, L., van der Klis, F.R., et al., 2011. Decreased prevalence and age-specific risk factors for *Toxoplasma gondii* IgG antibodies in The Netherlands between 1995/1996 and 2006/2007. Epidemiol. Infect.139(4), 530-538.

Homan, W.L., Vercammenb, M.V., De Braekeleerc, J., Verschuerenc, H., 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int. J. Parasitol. 30(1), 69-75.

Jones-Brando, L., Torrey, E.F., Yolken, R.H., 2003. Drugs used in the treatment of schizophrenia and bipolar disorder inhibit the replication of *Toxoplasma gondii*. Schizophr. Res. 62, 237– 244.

Jones, J.L., Kruszon-Moran, D., Sanders-Lewis, K., Wilson, M., 2007. *Toxoplasma gondii* infection in the United States, 1999 2004, decline from the prior decade. Am. J .Trop. Med. Hyg. 77(3), 405-410.

Jones, J.L., Dargelas, V., Roberts, J., Press, C., Remington, J.S., Montoya, J.G., 2009. Risk factors for *Toxoplasma gondii* infection in the United States. Clin. Infect. Dis. 49, 878–884.

Jones, J.L., Kruszon-Moran, D., Elder, S., Rivera, H.N., Press, C., Montoya, J.G., McQuillan, G.M., 2018. *Toxoplasma gondii* Infection in the United States, 2011-2014. Am. J. Trop. Med. Hyg. 98(2), 551-557.

Leweke, F.M., Gerth, C.W., Koethe, D., Klosterkötter, J., Ruslanova, I., Krivogorsky, B., et al., 2004. Antibodies to infectious agents in individuals with recent onset schizophrenia. Eur. Arch. Psychiatry. Clin. Neurosci. 254(1), 4-8.

Montoya, J.G., Liesenfeld, O., 2004. Toxoplasmosis. Lancet. 363(9425), 1965–1976.

Mosti, M., Pinto, B., Giromella, A., Fabiani, S., Cristofani, R., Panichi, M., Bruschi, F., 2013. A 4-year evaluation of toxoplasmosis seroprevalence in the general population and in women of reproductive age in central Italy epidemiology and infection. Epidemiol. Infect. 141(10), 2192-2195.

Omar, A., Bakar, O.C., Ada, N.F., Osman, H., Osman, A., Suleiman, A.H., et al., , 2015. Seropositivity and serointensity of *Toxoplasma gondii* antibodies and DNA among patients with scizophrenia. Korean. J. Parasitol. 53(1), 29-34.

Parlog, A., Schluter, D., Dunay, I.R., 2015. *Toxoplasma gondii*-induced neuronal alterations. Parasite. Immunol. 37(3), 159–170.

Pearce, B.D., Kruszon-Moran, D., Jones, J.L., 2012. The relationship between *Toxoplasma gondii* infection and mood disorders in the third National Health and Nutrition Survey. Biol. Psychiatry. 72, 290–295.

Perron, H., Hamdani, N., Faucard, R., Lajnef, M., Jamain, S., Daban-Huard, C., et al., 2012. Molecular characteristics of Human Endogenous Retrovirus type-W in schizophrenia and bipolar disorder. Transl. Psychiatry. 2, e201.

Peyron, F., Lobry, J.R., Musset, K., Ferrandiz, J., Gomez-Marin, J.E., Petersen, E., et al., 2006. Serotyping of *Toxoplasma gondii* in chronically infected pregnant women: predominance of type II in Europe and types I and III in Colombia (South America). Microbes. Infect. 8, 2333-2340.

Pinto, B., Castagna, B., Mattei, R., Bruzzi, R., Chiumiento, L., Cristofani, R., et al., 2012. Seroprevalence for toxoplasmosis in individuals living in North West Tuscany: access to Toxo-test in central Italy. Eur. J. Clin. Microbiol. Infect. Dis. 31, 1151-1156.

Pinto, B., Mattei, R., Moscato, G.A., Cristofano, M., Giraldi, M., Scarpato, R., et al., 2017. Toxoplasma infection in individuals in central Italy: does a gender-linked risk exist? Eur. J. Clin. Microbiol. Infect. Dis. 36(4), 739-746.

Prandovszky, E., Gaskell, E., Martin, H., Dubey, J.P., Webster, J.P., McConkey, G.A., 2011. The neurotropic parasite *Toxoplasma gondii* increases dopamine metabolism. PLoS. One. 6(9), e23866.

Puvanesuaran, V.R., Noordin, R., Balakrishnan, V., 2013. Genotyping of *Toxoplasma gondii* isolates from wild boars in Peninsular Malaysia. PLoS. One. 8(4), e61730.

Rashno, M.M., Fallahi, S., Bahrami, P., 2017. Alzheimer's disease and *Toxoplasma gondii* infection; seromolecular assess the possible link among patients. Int. J. Geriatr. Psychiatry. 32, 231–234.

Remington, J.S., McLeod, R., Thulliez, P., Desmonts, G., 2001. Toxoplasmosis, in: Remington, J.S., Klein, J., Infectious Diseases of the Fetus and Newborn Infant, 5th ed. Saunders, Philadelphia, pp. 205–346.

Robert-Gangneux, F., Dardé, M.L., 2012. Epidemiology of and diagnostic strategies for toxoplasmosis. Clin. Microbiol. Rev. 25(2), 264-296.

Silveira C, Vallochi AL, Rodrigues da Silva U, Muccioli C, Holland GN, Nussenblatt RB, et al., 2011. *Toxoplasma gondii* in the peripheral blood of patients with acute and chronic toxoplasmosis. Br. J. Ophthalmol. 95(3), 396-400.

Sutterland, A.L., Fond, G., Kuin, A., Koeter, M.W., Lutter, R., van Gool, T., et al., 2015. Beyond the association. *Toxoplasma gondii* in schizophrenia, bipolar disorder, and addiction: systematic review and meta-analysis. Acta. Psychiatr. Scand. 132(3), 161-179.

Taboas, W., McKay, D., Taylor, S., 2012. Does *Toxoplasma gondii* play a role in obsessive-compulsive disorder? Psychiatry. Res. 198(1), 176-177.

Tedla, Y., Shibre, T., Ali, O., Tadele, G., Woldeamanuel, Y., Asrat, D., et al., 2011. Serum antibodies to *Toxoplasma gondii* and Herpesviridae family viruses in individuals with schizophrenia and bipolar disorder: A case-control study. Ethiop. Med. J. 49, 211–220.

Torrey, E.F, Bartko, J.J., Lun, Z., Yolken, R.H., 2007. Antibodies to *Toxoplasma gondii* in patients with schizophrenia: A meta-analysis. Schizophr. Bull. 33, 729–736.

Torrey, E.F., Bartko, J.J., Yolken, R.H., 2012. *Toxoplasma gondii* and other risk factors for schizophrenia: an update. Schizophr. Bull. 38, 642-647.

Wang, H.L., Wang, G.H., Li, Q.Y., Shu, C., Jiang, M.S., Guo, Y., 2006. Prevalence of *Toxoplasma* infection in first-episode schizophrenia and comparison between *Toxoplasma*-seropositive and *Toxoplasma*-seronegative schizophrenia. Acta. Psychiatr. Scand. 114, 40–48.

Webster, J.P., Lamberton, P.H., Donnelly, C.A., Torrey, E.F., 2006. Parasites as causative agents of human affective disorders? The impact of anti-psychotic, mood-stabilizer and anti-parasite medication on *Toxoplasma gondii*'s ability to alter host behaviour. Proc. Biol. Sci. 273, 1023-1030.

Weiss, L.M., Dubey, J.P., 2009. Toxoplasmosis: A history of clinical observations. Int. J. Parasitol. 39, 895–901.

Yolken, R.H., Dickerson, F.B., Torrey, E.F., 2009. *Toxoplasma* and schizophrenia. Parasite. Immunol. 31(11), 706-715.

Yolken, R., Torrey, E.F., Dickerson, F., 2017. Evidence of increased exposure to *Toxoplasma gondii* in individuals with recent onset psychosis but not with established schizophrenia. PLoS. Negl. Trop. Dis. 11(11), e0006040.

Figure legends:

Figure 1. Serointensity dispersion of IgG anti-*T. gondii* in seropositive patients with schizoaffective disorder (squares) and bipolar disorder (triangles).

Figure 2. Real-Time PCR analysis of samples from *T. gondii* seropositive patients with schizoaffective disorder and bipolar disorder; A: lane 1 negative control, lane 2 positive control where the arrow shows the amplification product, corresponding to a 529 bp fragment; lane 3 100 bp molecular-weight markers, lanes 4-12 samples from patients 1-9; B: lane 1 negative control, lane 2 positive control where the arrow shows the amplification product, corresponding to a 529 bp fragment; lane 3 100 bp molecular-weight markers, lanes 4-12 samples from patients 10-18.