# ANTI-DOPAMINE D2 RECEPTOR ANTIBODIES IN CHRONIC TIC DISORDERS: POTENTIAL LINK TO FLUCTUATIONS OF TIC SEVERITY

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**Abstract**

Tic severity typically fluctuates in chronic tic disorders (CTDs). Alongside psychosocial stressors, immune-mediated mechanisms were hypothesized to influence tic fluctuations. Circulating autoantibodies targeting dopamine D2 receptors (anti-D2R) have been reported across a spectrum of suspected autoimmune movement and neuropsychiatric disorders, including a subgroup of youth with CTDs.

To study the association of anti-D2R antibodies with tic exacerbations, we investigated a cohort of 137 children with CTD, recruited from 16 child psychiatry and neurology outpatient clinics participating in a prospective study. Patients were assessed at baseline, at tic exacerbations, defined as an increase of $\geq$6 points on the total tic severity sub-score of the Yale Global Tic Severity Scale, and 2 months post-exacerbation. The presence of anti-D2R antibodies in serum was evaluated using a cell-based assay in CHO-K1 D2R-transfected cells and revealed by immunofluorescence. The association within patients between visit type and presence of anti-D2R antibodies was measured with McNemar's test and repeated-measures logistic regression models, adjusting for potential demographic and clinical confounders. We also compared demographic and clinical data between patients with and without anti-D2R seroconversion during the study period.

At baseline, 9/137 (6.6%) study participants exhibited anti-D2R positive sera. At the time point of tic exacerbation, 20/137 subjects (14.6%) had anti-D2R positive sera, 11 of whom were anti-D2R negative at baseline (newly seroconverted, 8%). At post-exacerbation visit, 20 of 112 participants (17.8%) exhibited anti-D2R positive sera, 9 of whom were anti-D2R negative at the exacerbation time point (newly seroconverted, 8%). Presence of anti-D2R antibodies in serum was significantly associated with exacerbation visits when compared to baseline visits (McNemar's odds ratio=11, $p$=0.003), but not when compared to post-exacerbation visits. Repeated-measures logistic regression confirmed this association ($Z$=3.49, $p$<.0001) after adjustment for age, sex, time interval between baseline and exacerbation, exacerbation severity and use of psychotropic drugs. There was no significant difference between demographic and clinical data of participants with respect to presence/absence of anti-D2R antibodies.

Our results provide additional evidence of a potential association between immune mechanisms and the severity course of tics in youth with CTDs.

## Introduction

Chronic tic disorders, including Tourette syndrome (TS), are neurodevelopmental disorders, predominant in males, characterized by chronic, fluctuating, motor and/or phonic tics. Tics are sudden, rapid, recurrent, nonrhythmic, simple or complex motor movements or vocalizations, typically occurring at a mean onset age of 4–7 years, peaking in severity around puberty and remitting or improving substantially in 60-70% of patients by adulthood (Leckman, Peterson et al. 1997; Freeman, Fast et al. 2000; Leckman 2002; Bloch and Leckman 2009; Singer et al. 2011). Although the temporal fluctuations of tics may reflect the impact of physical and psychological stressors, the neurobiological mechanisms of this fluctuating course are still poorly understood. Alongside a likely polygenic type of inheritance, a role for environmental and immunological factors (e.g. differential expression of lymphocyte sub-populations, pro- and anti-inflammatory cytokine production, immunoglobulin synthesis) in mediating the natural history of tic disorders has been proposed, but not elucidated (Elamin et al., 2013; Robertson et ., 2017). Several lines of evidence support the hypothesis of an autoimmune origin of TS. In a recent study, positive oligoclonal bands were detected in cerebrospinal fluid in 20% of adult patients indicating an intrathecal antibody synthesis. However, specific autoantibodies directed against NMDA-, CASPR2-, LGI1-, AMPA- or GABAB1 / B2 were not detected (Baumgaertel C. et al., 2019). These results are related to another study in which sera from children with TS were screened in cell-based assays (CBA) for antibodies to NMDAR, CASPR2, LGI1, AMPAR and GABAAR: only two individuals had antibodies binding to the NMDAR and the binding was only weakly positive, whereas no other specific antibodies were detected (Baglioni V. et al., 2019).

A body of evidence indicates an immature development of connectivity within cortico-basal ganglia–thalamo–cortical (CBGTC) networks and intracortical connections involving frontal, parietal, limbic and insular cortical regions as an important neurobiological substrate for tic disorders and other related disorders such as obsessive-compulsive disorder, attention deficit hyperactivity disorder and autism spectrum disorder (Leckman, et al. 1997; Mink 2001; Ghanizadeh and Mosallaei 2009; Hariz and Robertson 2010; Robertson e al. 2015; Hirschtritt, et al. 2015). Pharmacological agents targeting monoaminergic neurotransmission, primarily D2 receptor antagonists (first and second generation antipsychotics) and partial agonists (aripiprazole), and alpha-2 agonists, have demonstrated efficacy in the treatment of tics in TS (Pringsheim, et al. 2019). Finally, different animal models showing some face validity for human tics have suggested the involvement of

different neurotransmitter systems, including GABA, acetylcholine, dopamine and histamine, within the CBGTC networks in the generation of tic-like behaviors (Mink 2001; Albin and Mink 2006; Hughes, et al. 2010; Cho, et al. 2012; Buse, et al. 2013).

D1 (D1R) and D2 (D2R) dopamine receptors are highly expressed in the cortex, hippocampus, basal ganglia (striatum and substantia nigra), with different ability to modulate cyclic adenosine monophosphate production (Beaulieu, et al. 2011; Cho, et al. 2012). A balance in the modulation of the direct and indirect pathways in the striatum is based on the different role of D1R and D2R. Dopamine-mediated signalling at the level of D1R leads to depolarization of striatal neurones attributed to the direct pathway of the CBGTC network (promoting the release of goal-directed behaviour), whereas D2R-mediated signalling hyperpolarizes the striatal neurones attributed to the indirect pathway (promoting the inhibition of unwanted behaviours (Mishra, et al. 2018).

Recent evidence showed that a subgroup of paediatric patients affected by a spectrum of movement and psychiatric disorders of suspected autoimmune aetiology may exhibit autoantibodies targeting D2R. They were detected in youth with basal ganglia encephalitis (Ramanathan et al., 2014), in acute onset psychosis (Pathmanandavel et al. 2015), and in a small subgroup of patients with TS (Dale et al., 2012). A ΔMFI (mean fluorescent intensity) value greater than mean + 3 standard deviation of the healthy control values was considered positive in cytofluorimetric cell based assay (CBA); none of control pediatric sera was positive. In Sydenham's chorea (SC) and PANDAS the anti-D2R antibodies were titled in ELISA (Cox, et al. 2013). Cox and collegues showed an anti-D2R mean titer of 6000 in normal serum control, while the mean titer was ~3 times the normal mean in SC patients and greater than twice the normal mean in patients with PANDAS.

Dale and colleagues (2012), using CBA on human embryonic kidney (HEK) cell lines transfected with the D2R cDNA, demonstrated the presence of autoantibodies directed against the dopamine D2R in 4 of 44 Tourette sera. Their experimental approach allowed the detection of antibodies to the target antigen presented in native form on surface of transfected HEK cells. However, the frequency of anti-D2R antibodies has neither been re-analyzed in a larger cohort of children withTS, nor correlated with the clinical fluctuation of tics.

The aim of this study was to investigate, using a CBA method applied to Chinese Hamster Ovary(CHO)-K1 cell lines transfected with human D2R, the frequency of anti-D2R antibodies in the serum of children or adolescents with TS or another chronic tic disorder,

aged 3-16 years, across at least two different time points (baseline and during and after a clinically relevant exacerbation of tics). In line with a potential association between immune-based mechanisms and tic exacerbations, we hypothesized that the presence of anti-D2R antibodies is over-represented at the exacerbation time point compared to the other time points, i.e. baseline and 2 months post-exacerbation, in youth with TS.

**Patients and Methods**

A total of 715 3-6 year-old children and adolescents were enrolled in the *COURSE* cohort of the European Multicentre Tics in Children Study (EMTICS), a large prospective European longitudinal observational study involving 16 centres from ten European countries and Israel (Schrag, et al. 2019). All participants had TS or another chronic tic disorder according to DSM IV-TR criteria (American Psychiatric Association Task Force on DSM-IV, 2000), and were followed up over a maximum period of 18 months, with regular bi-monthly clinical assessments (consisting of interchanging 4-monthly hospital visits and 4-monthly telephone interviews) by well-trained study clinicians to capture clinically relevant tic exacerbations; also families were asked to keep a weekly diary and contact the study team in case of a possible tic exacerbation, and if confirmed, prompt expedited hospital visit. Tic exacerbations were defined as an increase of at least six points on the total tic severity sub-score (i.e. sum of motor and vocal tic severity) of the Yale Global Tic Severity Scale (YGTSS) compared to the score during the previous assessment (Schrag, et al. 2019). Approximately two months after the confirmed expedited visit, a post-exacerbation hospital visit was scheduled aimed at capturing a subsequent decrease of tic severity. Blood serum and throat swab specimens were taken during all hospital visits, i.e., baseline, exacerbation and post-exacerbation. Group A Streptococcus (GAS) exposure at baseline was defined by the presence of a positive throat swab specimen. GAS exposure at tic exacerbation was defined by a newly or persistently positive throat swab specimen and/or significant elevation of anti-streptococcal antibody titres, i.e. anti-streptolysin O (ASOT) and anti-DNAse B titres, with the latter defined by an ASOT > 200 AND [log10 (ASO*current visit*) – log10(ASO*prior visit*)] $\geq$ 0.2 (variation between log10 for two consecutive measurements higher than or equal to 0.2), and an anti-DNAseB titre > 300 AND [log10 (anti-DNAseB*current visit*) – log10(anti-DNAseB*prior visit*)] $\geq$ 0.2.

Our study population included a subset of this prospective cohort that had developed tic exacerbations during the course of the study and had donated blood samples, which comprised 137 participants at baseline (mean ± SD age 9.98 ± 2.6 years, age range 4-16 years; 108 boys, M/F ratio = 3.7/1) and 112 participants post-exacerbation. **Table 1** presents descriptive data for the severity of tics and obsessive-compulsive symptoms, as well as for psychiatric comorbidity rates, and exposure to psychotropic drugs across the baseline, exacerbation, and post-exacerbation time points for all participants of this sub-study. The mean ± SD difference in total tic severity sub-score of the YGTSS between the exacerbation visit and the visit immediately prior to the exacerbation was 10.12 ± 4.64. The mean ± SD time interval elapsing between baseline and exacerbation was 0.56 ± 0.33 years (i.e. 204 ± 120 days, range 11-493 days).

***Expression of humanD2R on transfected CHO-K1 cell surface***

CHO-K1 cells (ATCC® CCL-61 ™) were culturedat 37 °C and 5% CO2 in DMEM high glucose with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 5% L-glutamine (Aurogene®, Italy). A day before transfection, the cells were seeded at $25x10^4$ cells/$cm^2$ on 100 µg/ml poly-L-lysine ≥ 300,000 (Sigma) treated coverslips.

N-terminal human haemagglutinin-tagged(3XHA) D2RcDNA (UMR cDNA Resource Centre University of Missouri-Rolla) (D2R/3XHA), was used for CHO-K1 lipofectamine® 2000 transfection, according to the manufacturer's instructions (Invitrogen®, US). Subsets of cells were transfected with the empty vectorpcDNA-3.1 (Invitrogen®, US) as transfection negative control (mock).

Rabbit anti-HA antibody (Invitrogen, Italy) staining quantified transfection efficiency counting 3XHA positive cells influorescent fieldcompared with the number of cells in the bright field, while the surface expression of D2R was verified by cell staining with mouse IgG2a monoclonal antibody raised against amino acids 1-50 of human D2R (Santa Cruz Biothecnology.Inc). Briefly, two days after transfection, transfected live cells were washed in DMEM supplemented with 10% FCS and incubated with 1:500 rabbit anti-HA antibody in 10% FCS DMEM for 30 min at RT, washed three times with DMEM-10% FCS and below incubated with anti-D2R 1:50 in DMEM 10% FCS for 45 min at RT. After three DMEM 10% FCS washes, the cells were incubated with both secondary antibodies conjugated with fluorophores, Alexa Fluor™ 488 donkey anti-rabbit IgG (H+L) (Invitrogen®) and Texas Red goat anti mouse IgG2a-R (Santa Cruz Biothecnology.Inc) both diluted at 1:1000 for 30 minutes at RT. Mock cells were submitted to same staining procedure. Transfected and

mock cells were washed three times with PBS and fixed in 4% PFA in PBS for 20 min a RT. Coverslipweremounted in mowiol(Sigma Aldrich).Immunofluorescence was evaluated using an Upright Microscope Eclipse Ni (Nikon) with CFI Apochromat40x.

***Cell-based assay for detection of D2R antibodies to cell surface***

Transfected and mock CHO-K1 live cells were incubated with rabbit anti-HA antibody as indicated above, washed three times in DMEM 10%FCS, and below incubated for 30 min at RT with patients' sera diluted 1:100 in 10% FCS DMEM. After three wash in DMEM 10% FCS, both 1:1000 Alexa Fluor™ 568 goat anti-human IgG (H+L) (Invitrogen®) and 1:1000 Alexa Fluor™ 488 donkey anti-rabbit IgG (H+L) (Invitrogen®), were incubated for 30 min at RT. Cells were washed three times with PBS and fixed in 4% PFA in PBS for 30 min a RT. Coverslip were mounted in mowiol (Sigma Aldrich).

Serum specimens were always tested in triplicate in three independent experiments on transfected DRD2/3XHA positive cells and on mock cells; immunofluorescence results were evaluated using an Upright Microscope Eclipse Ni (Nikon) with CFI Apochromat40x. Pictures were overlaid using NIS-Elements BR software. The CBA was considered valid if the positive cells for the 3xHA tag revealedspecificmerging signal when incubated with human sera.The presence of anti-D2R antibodies in serum specimens wasevaluated independently by two different operators, who were blinded to the type of visit. Only serum specimens who were rated positive by both independent operators were entered as positive in the analysis.

***Statistical Analysis***

Our statistical analysis was divided in three parts. Firstly, the association between visit type (baseline/exacerbation, exacerbation/post-exacerbation) and presence/absence of anti-D2R antibodies in serum was measured using the McNemar test. This test evaluated whether different visit types yield similar proportions of anti-D2R positive results within the same subjects.

Secondly, we used repeated-measures logistic regression analysis to measure the strength and statistical significance of the associations that were observed on the McNemar test after adjustment for a number of potential confounding variables. These included age at baseline, sex, time interval between baseline and exacerbation visits, YGTSS total tic severity score change between exacerbation visit and the visit immediately prior to exacerbation (i.e. a measure of exacerbation severity), and use of psychotropic medication

in the previous two weeks. Because in a proportion of patients tic exacerbations had been captured after a time interval from the previous visit/telephone interview longer than 10 weeks, we conducted sensitivity analyses after exclusion of these patients.

In the third part of our analysis, we compared demographic (age at study entry, sex) and clinical (YGTSS total tic severity sub-score at baseline, presence of comorbid diagnosis of OCD and ADHD at baseline, exposure to any psychotropic drug or to antipsychotics during the two weeks prior to exacerbation, and exposure to GAS) variables across different patient categories based on the temporal pattern of anti-D2R antibody seroconversion, i.e. *non-seroconverters*, *early peri-exacerbation seroconverters* (who became antibody-positive at the exacerbation time point), and *late peri-exacerbation seroconverters* (who became antibody-positive at the post-exacerbation time point). These comparisons were performed using t tests or Mann-Whitney U test, as appropriate, and chi-square or Fisher's exact test statistics. Statistical significance was set at the levels of $P<0.05$ for tests of proportions, and, to adjust for multiple comparisons in repeated-measures logistic regression and between-group comparisons, at $P<0.05/6 = 0.008$. All analyses were performed using STATA SE14 software.

## Results

*Frequency of anti-D2R antibodies across study visits*

All DR2 IgG antibody assessments were carried out on DR2/3xHA expressing CHO-K1 cells. Transfection efficiency was settled at 30% of the total cells. In **Figure 1**, co-expression of D2R and 3xHA at cell surface of transfected cells and D2R-negative staining on mock cells are shown in a representative experiment; anti-D2R positive and negative sera are also shown in **Figure 1**.The two independent raters did not agree on only 8 of the 394 (2%) analyzed sera, and these samples were therefore not included in the analysis due to uncertain classification. The binding of all anti-D2R positive sera resulted in almost homogeneous intensity. No background was present following the incubation of the transfected cells with only secondary antibodies (data not shown).

**Table 2** shows the number and percentage of positive sera for the presence of anti-D2R antibodies at each visit type. At baseline, 9 of 137 (6.6%) study participants exhibited anti-D2R positive sera. At the time point of tic exacerbation, 20 of the 137 subjects (14.6%) had anti-D2R positive sera, 11 of whom (8%) were anti-D2R negative at baseline (newly seroconverted). At post-exacerbation visit, 20 of 112 participants (17.9%) exhibited anti-D2R positive sera, 9 of whom were anti-D2R negative at the exacerbation time point (newly

seroconverted, 8%). All 9 participants who were anti-D2R positive at baseline remained positive at exacerbation, and 4 of these 9 remained positive at the post exacerbation visit. Twenty participants who were anti-D2R negative at baseline exhibited seroconversion to anti-D2R positivity: 11 seroconverted at the exacerbation time point (*early peri-exacerbation seroconverters*) and 9 seroconverted at the post-exacerbation time point (*late peri-exacerbation seroconverters*). The remaining 108 participants remained anti-D2R negative throughout the whole study duration.

*Association between visit type and presence of anti-D2R antibodies*

McNemar's test revealed a positive association between the presence of anti-D2R antibodies in serum and visit type. The presence of anti-D2R antibodies was significantly more frequent at exacerbation visits compared to baseline (McNemar's $\chi^2$=8.33; exact McNemar significance probability = 0.0063; odds ratio 11, 95% confidence interval 1.60-473.47). Conversely, there was no difference in frequency of anti-D2R antibody status between exacerbation and post-exacerbation visits (McNemar's $\chi^2$=0.00; exact McNemar significance probability = 1.00; odds ratio 1, 95% confidence interval 0.39-2.54).

Repeated-measures logistic regression univariate analysis confirmed the presence of a significant association between presence of anti-D2R antibodies and exacerbation visit, when contrasted to baseline visit (Z = 2.93; regression coefficient 0.78, 95% confidence interval 0.26-1.30, $p$ = 0.003). After adjustment for age at study entry, sex, time interval (years) between baseline and exacerbation, YGTSS total tic severity score change between exacerbation visit and the visit immediately prior to exacerbation and use of psychotropic medication in the previous 2 weeks, this association survived (Z = 3.49; regression coefficient 0.95, 95% confidence interval 0.42-1.49, $p$ < 0.0001).

In 18 of the 137 patients enrolled, tic exacerbation had been captured after a time interval from the previous visit/telephone interview longer than 10 weeks. We therefore performed the same repeated-measures logistic regression after excluding these 18 patients, who did not differ from the remaining 119 for age at study entry and sex ($p$ > 0.05) whereas those whose tic exacerbation had been captured more than 10 weeks after the previous encounter manifested greater YGTSS total tic severity score change between exacerbation visit and the visit immediately prior to exacerbation (12.61 ± 7.12) compared to those whose tic exacerbation had been captured 10 weeks or less after the previous encounter (9.74 ± 3.98; $p$ = 0.01). This sensitivity analysis confirmed the significant association between presence of anti-D2R antibodies and exacerbation visit (univariate

analysis: Z = 2.71; regression coefficient 0.91, 95% confidence interval 0.25-1.57, $p = 0.007$; multivariate analysis: Z = 3.36; regression coefficient 1.17, 95% confidence interval 0.49-1.85, $p = 0.001$).

*Relationship between clinical features and anti-D2R antibody status*

Participants who never seroconverted (n = 108) did not differ significantly from the whole group of participants who seroconverted, either before baseline or during the peri-exacerbation periods (n = 31, Table 2), for sex distribution, age at study entry, frequency of recent exposure to any psychotropic medication or dopamine receptor blocking agents in particular, group A streptococcal infection exposure at baseline or at exacerbation, total tic severity on YGTSS at baseline, and frequency of comorbid diagnosis of OCD and ADHD at baseline (**Table 3**). Likewise, early peri-exacerbation seroconverters (N=11) did not significantly differ from late peri-exacerbation seroconverters (N = 9) with respect to time interval between the exacerbation visit and the previous visit, as well as to YGTSS total tic severity sub-score between the exacerbation visit and the one preceding the exacerbation visit (i.e. 'severity' of the exacerbation – **Table 4**).

## Discussion

The present study explored the frequency of circulating serum autoantibodies targeting the human D2 dopamine receptor in a relatively large prospective cohort of youth with TS, and evaluated the temporal association between these autoantibodies and the occurrence of clinically relevant exacerbations of tic severity in the same population. Circulating anti-D2R antibodies were identified in more than 7% of individuals with TS at their study entry (baseline) visit. This visit represents a cross-sectional snapshot of the general population of children and adolescents with TS attending specialist clinics, thereby indicating that anti-D2R antibodies can be found in a small, but not negligible, subgroup of patients with this neurodevelopmental disorder. Given the fluctuating nature of chronic tic disorders, it is likely that, at baseline, our clinical population included a mixed representation of stable, recently increased and recently decreased tic severity states. We have then assessed the presence of anti-D2R antibodies in the same individuals at a subsequent time point corresponding to an exacerbation of tic severity defined based on a change in YGTSS total tic severity score, and, for a subgroup of study participants, at a third post-exacerbation time point falling approximately two months after exacerbation. We observed an increased frequency and positive association of anti-D2R antibodies with the exacerbation time point compared to

baseline, indicating a potential temporal co-occurrence of clinical fluctuations and peri-exacerbation seroconversion in a subgroup of TS patients. We also detected a return to a negative anti-D2R antibody status during the post-exacerbation period in a subgroup of participants, whereas others showed persistently positive anti-D2R antibody status. Finally, we did not observe any association between demographic and clinical features of TS and the presence or seroconversion of anti-D2R antibody status. Particularly, we could not identify any association between these autoantibodies and a recent exposure or related immunological response to group A Streptococcal pharyngeal infection, an environmental factor proposed to play a role in pediatric acute neuropsychiatric syndromes presenting, amongst other symptoms, with tics.

The cell-based assay that we implemented to measure serum antibodies to the human D2R was methodologically consistent with the protocol reported by Dale and colleagues (Dale, et al. 2012), who detected this autoantibody specificity in about 10% of a smaller clinical sample of individuals with TS. This group of authors also demonstrated the presence of these autoantibodies in a subgroup of paediatric patients affected by autoimmune neuropsychiatric disorders like basal ganglia encephalitis and Sydenham's chorea, as well as in a proportion of patients at their first acute psychotic episode. Other authors have expanded the spectrum of patients bearing circulating anti-D2R autoantibodies to patients fulfilling the diagnostic criteria for Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infection (PANDAS) (Cunningham & Cox, 2016).

In our experimental procedure, the choice of the CHO-K1 D2R-transfected cells is supported by literature data demonstrating the membrane expression of human D2R (Baragli et al., 2007; Kasai et al., 2018). In our study, the expression of the human D2R on the CHO-K1 transfected cell surface was evaluated by immunofluorescence using an anti-human D2R antibody developed against residues 1-50 within the extracellular N-terminus of the receptor. Our choice was motivated by studies demonstrating that the N-terminal region of the D2R, rich in glycosylation sites, may play an important role in receptor surface trafficking and integration into the plasma membrane (Cho, et al. 2012).

More specifically, other authors showed that aminoacids 23 to 37 of D2R N-terminus promote the expression of the D2R on the cell surface (Sinmaz, et al. 2016), and the region between amino acids 20 and 29 bind the majority of antibody-positive sera from movement and psychiatric disorder patients. This particular epitope has conformational value for the receptor and the related antigenic site, given that changes in amino acid residues within the epitope may strongly decrease autoantibody binding.

It is noteworthy that, similar to what we observed in our clinical population, the binding patterns of the majority of patient sera in Sinmaz et al's (2016) paper was not associated with any particular feature of the clinical phenotype. At the same time, the association of anti-D2R antibodies with tic exacerbation in our cohort suggests a potential contribution of these autoantibodies to the severity of the core feature of this disorder. However, approximately half of the study participants who seroconverted around the time of tic exacerbation exhibited newly anti-D2R positive sera only during the two months after exacerbation, which suggests that the time course of this immunological response is heterogeneous across patients and does not always precede the behavioural fluctuation. It is possible that screening for anti-D2R antibodies in the cerebrospinal fluid (CSF) at the same time points might have provided additional information on their potential central effect in association with tic exacerbations, notwithstanding the obvious logistic difficulties of collecting CSF specimens from a sufficiently large clinical sample of patients with TS. Importantly, we took into account the possible confounding effect of D2R blocking pharmacological agents, and concluded that it is highly unlikely that exposure to these drugs might have influenced our results.

What could be the significance of anti-D2R autoantibodies in the context of TS? Immune abnormalities have been postulated to be involved in the pathogenesis of behavioural changes in different neurodevelopmental conditions, including autism, TS and obsessive-compulsive disorder (Edmiston et al., 2017; Frick &Pittenger, 2016; Trifiletti& Packard, 1999; Martino et al., 2015). Both a hyperactive immune activity and over-expression of genes controlling immune-inflammatory responses in the striatum involving the microglia have been documented in patients with TS (Martino et al., 2015; Lennington et al., 2016). However, there is no evidence of a TS-specific immunopathogenic molecular pathway, as well as of antibody-mediated mechanisms similar to those postulated for Sydenham's chorea. It is therefore possible that changes in the levels or receptor affinity of anti-D2R autoantibodies could simply be epiphenomenal to a general activation of $T_H2$-mediated immune responses at a systemic level. Alternatively, a direct effect of these autoantibodies on D2R surface expression might be a contributing factor that promotes or enhances a change in tic severity. In this respect, autoantibodies have been hypothesized to down-regulate the membrane surface expression of D2R in a way that resembles the dynamic regulation and resensitization of D2R exerted in vivo by dopamine (Beaulieu, et al. 2011). A similar receptor internalization effect was shown for anti-NMDAR and anti-AMPAR antibodies in the context of related encephalitides (Dalmau, et al. 2008; Lai, et al. 2009;

Hughes, et al. 2010), representing a relevant general mechanism of pathogenic neuroimmune autoantibodies.

Some limitations of our study should be acknowledged. Our indirect immunofluorescence methodology to detect autoantibodies is not a quantitative assay. Previous studies that measured D2R-specific IgG in human subjects either quantified titres by standard enzyme-linked immunosorbent assay (ELISA [Cox et al., 2015]), or used a flow cytometry live cell-based assay to compare patients to healthy volunteers (Pathmanandavel et al., 2015). In the latter study, specific serum antibody binding to D2R was measured as the difference between mean fluorescence intensity (ΔMFI) of specific IgG binding to HEK293-D2R transfected cells and mock cells in both patients and healthy volunteers; a ΔMFI value greater than mean + 3 SDs of values of the health control subjects was considered positive. Our experimental paradigm evaluated the temporal association between D2R autoantibodies and the occurrence of clinically relevant exacerbations of tic severity in the same population of patients by indirect immunofluorescence. Our indirect immunofluorescence system does not enable to grade anti-D2R positivity for each independent positive serum in a reliable fashion. However, staining of cultured cells as antigenic source allowed the specific recognition of autoantibodies to native antigens. The presence of multiple antigenic targets (3xHA and D2R) on live cell surface is likely to have provided good accuracy even without requiring more sophisticated analyses. Furthermore, due to the controversial debate on the possible involvement of anti D2R autoantibodies in Tourette sera, we have used mock cells as negative reference control: a serum was considered positive only if on the mock cells it did not yield a fluorescence signal while showing surface fluorescent binding on D2R-transfected cells regardless of the immunofluorescence intensity (**Figure 1**). Finally, another important limitation is the lack of prospective anti-D2R antibody determination for study participants who did not experience clinically relevant exacerbations during the follow-up period, as this would have provided further information on the temporal dynamics of anti-D2R antibody status in this clinical population regardless of exacerbations.

In conclusion, to the best of our knowledge this is the first study exploring the association between antibodies targeting the human D2 dopamine receptor and the clinical course of TS. The observed association between the presence of these antibodies and clinically relevant tic exacerbations needs to be replicated by different laboratories, but our observation replicates that of a previous study (Dale et al., 2012) in that it highlights the presence of this antibody species in a subgroup of youth with TS. Future studies focusing

on immunophenotyping and cytokine profiles of patients in our prospective cohort will expand on the relationship between anti-D2R antibodies and systemic immune inflammatory responses. Similarly, the association between infectious triggers other than group A Streptococcus or psychosocial stress levels and the seroconversion of anti-D2R antibody status will be the objective of future analyses from our cohort.

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**Fig. 1**. **A.** Immunofluorescence on live DRD2/3XHA transfected cells and mock cells. Upward, the anti-HA in green and anti-DR2 in red co-labeling showed co-expression at cell surface of 3XHA and DR2 in transfected cells. Down, no signal was present in the co-labeling of the mock cells transfected with the empty vector. **B.** Sera immunoreactivity to DRD2 on transfected cells. Upward, a D2R IgG positive serum (in red) binds to the anti-HA positive cells (in green); down, a D2R IgG negative serum. Scale bar 50 μm.