

Detection of auto-antibodies to DAT in the serum: Interactions with DAT genotype and psycho-stimulant therapy for ADHD



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ABSTRACT

Interest is rising for auto-immune contribution in neuro-psychiatry. We evaluated the auto-antibodies against dopamine transporter (DAT aAbs) in 61 children (46 ADHD who met DSM-IV-TR criteria, 15 healthy controls).

Methods: ADHD patients were assigned, according to severity, either to a non-pharmacological therapy (NPT, N = 32) or to a pharmacological treatment (PT, N = 14) with methylphenidate (MPH). In ADHD children, blood samples were withdrawn twice, at recruitment (T0 basal) and after 6 weeks (T1); following 16 excluded subjects, DAT genotype was characterized (9-repeat or 10-repeat alleles; N = 15 each). After 18 months of NPT or PT, some patients (carrying at least one 9-repeat allele) were blood sampled again (T2), for comparison with healthy controls (final n = 8).

Results: Compared to NPT, basal DAT aAbs titers were higher within most severe patients (then assigned to PT), specifically if carrying a DAT 10/10 genotype. DAT aAbs levels of NPT group resulted highly correlated with distinct subscales of Conners' Parent/Teacher Scales ($R_s > 0.34$), especially within DAT 10/10 genotype ($R_s > 0.53$). While T1 titers were elevated over T0 baseline for NPT children, such an increase was not observed in PT patients carrying at least one 9-repeat allele, who also showed behavioral response to subchronic MPH. After 12–24 months of MPH exposure, DAT aAbs titers in PT subjects were comparable to those of healthy controls, while titers remained significantly elevated in NPT patients. Data warrant further research on serum DAT aAbs, which could be used to confirm ADHD diagnosis and/or to monitor therapeutic efficacy of MPH.

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1. Introduction

Attention deficit hyperactivity disorder (ADHD) has been internationally recognized as a medical neuro-developmental condition (Curatolo et al., 2009; Davis et al., 2011; Purper-Ouakil et al., 2011; Cortese, 2012); global interest in long-term consequences of ADHD and of psycho-stimulant administration for ADHD is on the rise (Hinshaw et al., 2011). According to the current criteria of Diagnostic and Statistical Manual of Mental Disorders, 4th edition – Text Revision (DSM IV-TR), ADHD prevalent symptoms include problems in maintaining attention, excessive motor activity, and impulsivity, which often lead to poor academic performance and impaired social interactions (American Psychiatric Association, 2000). These symptoms

develop early in up to 5% of children (Polanczyk et al., 2007), and can persist into adolescence and adulthood (Biderman et al., 2006). Frequently comorbid with ADHD are other impulse-control disorders, like oppositional defiant disorder, conduct disorder, substance abuse and/or dependence problems (see Hollander et al., 2000, 2005 for pathological gambling), all of which may be conceptualized as part of the addictive disorder spectrum (Fontenelle et al., 2011).

Although the multi-factorial etiology of ADHD is still unclear, evidence suggests that the disorder is linked to imbalanced levels of dopamine neurotransmitter. Some accounts present ADHD as a motivational dysfunction (Sonuga-Barke, 2005), arising from altered processes within fronto-striatal circuits (Oades, 1998; Sagvolden and Sergeant, 1998; Chambers and Potenza, 2003). For this reason, one focus of ADHD research has converged on the brain dopamine transporter (DAT) in the clinics and in preclinical models. It has been proposed that specific ADHD symptoms may arise from a modification in DAT expression and function (Jucaite et al., 2005; Bannan, 2005; Berridge et al., 2007):

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human DAT1 gene has a VNTR polymorphism of 40 bp (3–11 repeats) in the 3' untranslated region, with 9- and 10-repeat variants being the most common in Caucasians. 10-repeat VNTR polymorphism of DAT has been associated with ADHD and with obsessive-compulsive disorder (Cook et al., 1995; Gill et al., 1997; Sharp et al., 2009; Scherk et al., 2009; Yang et al., 2007; Greenwood et al., 2013). DAT1 high-risk allele (i.e. the 10-repeat allele) can explain 1%–4% of the overall variance in ADHD symptoms; its relation with hyperactive–impulsive symptoms is stronger and more reliable than that with inattentive symptoms (Waldman et al., 1998).

However, although the presence of DAT1 high-risk alleles may influence the severity of the disorder and may explain why as many as 30% of ADHD children do not respond to psycho-stimulants (Madras et al., 2002), other non-genetic factors are crucial for the onset of ADHD symptoms. To account for a possible DAT alteration, a rising interest exists for auto-immune processes and psycho-immunological interactions (Shulman, 2009; Graus et al., 2010a,b). A breach in blood–brain barrier (BBB) integrity, due to conditions of stress (Kuang et al., 2004) or owing to a traumatic injury to the brain (like e.g. as complication of a difficult delivery, Ankeny and Popovich, 2010), could implicate the draining of CNS antigens to peripheral lymphoid organs, with subsequent auto-immune responses (Diamond et al., 2009; Levin et al., 2010). According to recent literature, anti-neuronal antibodies may target a wide range of CNS proteins, including neuro-receptors (Davies et al., 2007; Graus et al., 2008; Zuliani et al., 2012). Behavioral dysfunction might possibly stem from anti-neuronal auto-antibodies (aAbs) that would presumably compromise neural function (Granstrom et al., 2006). Circulating aAbs against neuro-receptors are reliable biomarkers for Systemic Lupus Erythematosus (Cohen-Solal and Diamond, 2011), intractable seizures (Rogers et al., 1994; Twyman et al., 1995), brain ischemic stroke (Dambinova et al., 2003), Hashimoto's encephalopathy (Chong et al., 2003) and Sydenham's chorea. Other two neurological conditions with a claimed role for aAbs and related neuro-psychiatric symptoms are the Limbic and the NMDAR-Ab encephalitis. While seizures are prominent with GABA-B receptor (GABAB-R) aAbs, there may be psychiatric features with AMPA receptor (AMPA-R) aAbs (Lai et al., 2009; Graus et al., 2010a,b; Lancaster et al., 2010; Zandi et al., 2010) and with NMDA receptor (NMDA-R) aAbs (Dalmau et al., 2011). Many of these patients are children who may initially seek for psychiatric wards for acute anxiety, behavioral change or psychosis. Interestingly, a role for auto-immunity in general and for aAbs in particular has been claimed for Tourette's (Hoekstra and Minderaa, 2005; Martino et al., 2009; Rizzo et al., 2010), for obsessive–compulsive disorder (Teixeira et al., 2014), and for ADHD as well (Passarelli et al., 2013; Hegvik et al., 2014).

Circulating aAbs to CNS antigens can be also detected in animal models (Dambinova et al., 1997, 1998; Vincent et al., 1999; Kowal et al., 2006; Knight et al., 2007; Capone et al., 2008; Colasanti et al., 2009), as well as in opiate-treated mice (Granstrom et al., 2006). In this line, we recently proposed (Adriani et al., 2012) that a DAT altered turnover/degradation ratio may lead to an over-production of neuro-receptor fragments, which might then overcome the BBB and spill into the blood, where they can generate an auto-immune reaction. This auto-immune challenge could in turn lead to an enduring and possibly detectable interference with the dopamine (DA) neurotransmission as well as to DA-related behavioral changes, like ADHD symptoms (i.e. impulsivity and hyperactivity). Thus, purpose of the present study was 1) to ascertain the presence in the blood of circulating auto-antibodies (aAbs) targeting some epitopes of the DAT protein, 2) to measure levels of such DAT aAbs, by means of an ELISA assay, as a function of therapy with or without pharmacological MPH treatment, 3) to correlate these DAT aAbs titers with clinical scores of ADHD symptoms, and 4) to further evaluate the above parameters as a function of the individual DAT genotype. We hypothesized that a more clear account of observed symptoms could be served by taking into consideration the interaction between genetic and auto-immune parameters.

2. Material and methods

2.1. Recruitment of patients and healthy controls

Participants included 61 children. We recruited 48 patients with a formal diagnosis of ADHD, with a female to male ratio of 1:5, referred to Child Psychiatry Unit of Tor Vergata University from April 2010 to March 2012. Two of these recruited ADHD children were dropped out, since they turned out to be non-responders to the recruitment commitments; we also recruited 15 healthy children (handled under the same routine conditions from April 2013 to March 2014, in the same period as the patients' re-sampling, see below) to act as controls. All subjects had a full Scale IQ over 84, as assessed by the Wechsler Intelligence Scale – III edition (Wechsler, 1991). They were evaluated by child neuro-psychiatrists who determined the diagnosis of ADHD, according to DSM IV-TR criteria (American Psychiatric Association, 2000); a medical work-up excluded any other neuro-genetic disease or immune disorder, as well as any psychiatric comorbidity (conduct disorder, obsessive–compulsive disorder, Tourette's, depression, bipolar disorder, psychosis), assessed by the Schedule for Affective Disorders and Schizophrenia for School Age Children – Present and Lifetime Version (K-SADS-PL) (Kaufman et al., 1997). Post-recruitment exclusions were also made for coeliac or diabetic disease, and in case of recent fever or allergy, as resulting from standard blood screen.

The clinical sample was divided into two, based on therapeutic intervention decided at enrollment: children with milder symptoms, which did not need pharmacological treatment, underwent cognitive-behavioral therapy and/or periodic follow-up (unmedicated, NPT group; N = 32); severe children, with a significant impairment of adaptive functioning in different areas of life, were assigned to pharmacological treatment with MPH (PT group; N = 14).

The study was formally approved by ISS Ethical Committee (Prot. CE-ISS 09/270 of 15 July 2009, scientific responsible and PI: W.A.). Informed consent procedures included searching for consent from the child (using age-adequate approaches) and illustrating to parents the standard consent form; the parents gave their written informed consent for the child to participate in this study. All potential participants who decided not to participate in the study were not disadvantaged in any way by not participating. Also, we declare that collected biological materials were used solely to the purpose of this study; the responsible person for pediatric privacy is one of authors (M.C.P.). The rules set by the Code of Ethics of the World Medical Association (Declaration of Helsinki), which has been printed in the British Medical Journal (18 July 1964), were respected.

2.2. Clinical assessment

Each patient was evaluated by a staff of trained child neuro-psychiatrists at our "S. Alessandro" clinical Unit, according to the DSM-IV and ICD-10 criteria for ADHD. Information was gathered from the clinical interviews and questionnaires with the parents, teacher and from direct observations of the patients.

Parents completed SNAP-IV that elicits DSM-IV TR criteria for ADHD on a four-point scale of frequency (Swanson, 1983), also giving information about ADHD subtypes (inattentive, hyperactive–impulsive, combined type). ADHD symptoms were also determined using *Conners' Parent Rating Scale*; each item was scored according to the published measure from 0 (Not true at all) to 3 (Very much true) (Conners et al., 1998). The semi-structured *Schedule for Affective Disorders and Schizophrenia – Present and Lifetime version* (K-SADS/PL) and also the *Child Behavior Checklist/4–18* (CBCL; Achenbach, 1991) were used separately, to elicit parents' and patients' reports of signs and symptoms that might indicate possible co-morbidities.

The *Children's Global Assessment Scale* (CGAS) was used by clinicians to measure the overall severity of social and psychiatric functioning for children ages of 4–16 years (Shaffer et al., 1983). CGAS scores range between 1 and 100, with higher scores indicating better functioning.

The full clinical assessment was performed at time of recruitment, while both parents completed the Conners' Parent Rating Scale and clinicians completed the CGAS twice, at time T0 and T1. The patient's teacher completed (only once, at time of recruitment) the Conners' Teacher Rating Scale.

2.3. Biochemical and genetic assessment

After recruitment, in the 46 ADHD children we were able to collect two blood samplings. *Time T0*: basal withdrawal, at recruitment (early in the morning and/or without breakfast, i.e. when stomach was empty; also used for standard blood screen); *Time T1*: second withdrawal, taken either after 6 weeks from time T0 (for subjects who did not undergo pharmacological therapy, i.e. NPT group) or after 6 weeks from when MPH had reached the therapeutic dosage (for subjects which were assigned, at recruitment, under pharmacological treatment, i.e. PT group). Blood was withdrawn from patients using standard phlebotomy procedures: it was collected in a sterile 10-mL plain centrifuge tube and allowed to clot for 5–10 min, then centrifuged for 10 min at 1500 rpm in a standard centrifuge. The serum was stored at -80°C until use.

Of 46 patients, 16 children were excluded a posteriori, due to recent fever/allergy before blood sampling, as altered values were detected in standard blood screen at T0. Ten children had higher lymphocytes count ($41.0 \pm 0.84\%$, up to 47.0%) and seven children had higher eosinophils count ($4.0 \pm 0.78\%$, up to 11.1%) than standard norm for healthy controls (four children had both); two children were classified as non-responders to MPH and switched to atomoxetine treatment; one child was also excluded since he was the only one not belonging to Caucasian ethnicity. In the remaining children ($N = 30$), we collected buccal samples using a Catch-all sample collection Swab (Epicentre, USA), in order to verify specifically the DAT1 gene VNTR polymorphism in the 3' untranslated region (3'UTR), rs 28363170 (Vandenbergh et al., 1992; Sano et al., 1993). Of these, 15 had a DAT 10/10 genotype while 15 were carrying at least one 9-repeat allele.

We recruited 15 subjects as healthy controls. Of these, only 3 had a DAT 10/10 genotype (too few to be further assessed) while 12 were carrying at least one 9-repeat allele. After excluding 4 children with recent fever or allergy, we ended up with 8 healthy subjects. Hence, ADHD children with at least one 9-repeat allele were further contacted for re-sampling, after one-to-two years (average: 18 months) of exposure to MPH or to unmedicated conditions (PT or NPT, respectively). For the latter, we were able to involve just 4 patients (out of 11). We aimed to re-assess their DAT aAbs titers (*time T2*), to be compared with healthy controls; therefore, in addition to T0 baseline, blood samples were taken twice in these children, months apart, to improve reliability of findings. All these subjects were withdrawn blood samples in the same conditions as for time T0.

2.4. PCR methods

Genomic DNA was prepared from buccal swab samples by using the BuccalAmp™ DNA Extraction Kit, following the manufacturer's instructions (Epicentre, USA). Briefly, after collecting buccal cells, the swab end was placed into a tube containing QuickExtract DNA extraction solution and rotated a minimum of five times. The tube was vortex mixed for 10 s and incubated at 65°C for 1 min. After vortex mix for 15 s, the tube was transferred to 98°C and incubated for 2 min. After vortex mix for 15 s, the DNA was stored at -20°C until processing. The yield of DNA is usually between 2 and 14 ng/ μL .

The 3'-UTR repeated sequence of the DAT1 gene was amplified by the polymerase chain reaction (PCR). The primer sequences employed were 5'-TGT GGT GTA GGG AAC GGC CTG AG-3' (DAT1-F) and 5'-CTT CCT GGA GGT CAC GGC TCA AGG-3' (DAT1-R). The PCR amplification was carried out in a final volume of 50 μL containing 3 μL of genomic DNA prepared using the Buccal Amp DNA extraction kit, 1.5 mM of MgCl_2 , 200 μM of dNTP, 50 mM of KCl, 10 mM of Tris-HCl (pH 8.3), 0.25 μM of each primer, and 1 U of Promega Taq DNA polymerase. The

PCR amplification was performed for 35 cycles consisting of 94°C for 45 s, 57°C for 30 s, and 72°C for 30 s. The genotype was estimated from the size of the PCR product analyzed by electrophoresis on 6% acrylamide gels stained with ethidium bromide.

2.5. ELISA methods

We used a novel and patented DAT-EIA-kit (patent holder: ISS, Italy), based on the ELISA method - most commonly used for determination in the serum of anti-neuronal auto-antibodies (aAbs) - exploiting peptide fragments of the human DAT sequence as antigens (see Adriani et al., 2012). Briefly, the concentration of DAT-aAbs is determined using modified synthetic peptides, corresponding to a fragment of DAT, as antigens. These peptide fragments were designed by selecting a 19-amino acid portion of the DAT sequence based on the most immuno-reactive portion (Granstrom et al. 10-AUG-2012 provisional patent; 10-AUG-2013 International Patent Application No. PCT/EP2013/066845), and custom synthesized by one of authors (O.G.).

The DAT-fragment antigen was affinely pre-adsorbed to the microplate (0.5 $\mu\text{g}/\text{well}$). In a first incubation step (1 h, 25°C), the serum samples (diluted 1:100) are applied (80 μL per each well) on the immuno-plate (Costar, USA): antibodies react with the solid phase bound antigen. After intensive washing (PBS with 0.05% Tween 20, pH 7.4), the captured antibodies are detected using monoclonal murine antibodies (catalog number A8667; Sigma Aldrich, USA) to human IgG (diluted 5 mL/L in vehicle: albumine 0.5 g/L, PBS-Tween20 5 mL/L, thimerosal 0.02 g/L, sodium azide 0.05 g/L, distilled water; Sigma Aldrich, USA) conjugated with horseradish peroxidase (HP). This secondary antibody (diluted 1:20,000) is added (80 μL per each well) and incubated (1 h, 25°C). Immuno-complex is semi-quantitatively determined by HRP/TMB-detection reaction: the substrate solution, TMB (catalog number 09743; Fluka, USA) is added (80 μL per each well). Stopping solution (2 N HCl) is then added converting the color to yellow. The plate is scanned at 450 nm on a Microplate reader 3550 (Bio-Rad, USA). The intensity of the yellow color is directly proportional to the concentration of DAT-aAbs in the serum sample.

2.6. Statistical analysis

The analyses of behavioral scales, of titers, and of correlations were run within all recruited ADHD patients ($N = 46$); after 16 were excluded a posteriori for recent fever/allergy, the analyses involved only genotyped ADHD children ($N = 30$). Subjects segregated into two DAT genotype groups ($N = 15$ each). Patients with DAT 9/9 genotype were too few to be reliably used for statistic purposes: henceforth, DAT 9/9 and 9/10 genotypes were pooled and referred to as "subjects with at least one 9-repeat DAT allele" (collectively termed 9/x).

First, we investigated the role of factors such as "group" (NPT vs. PT patients), "genotype" (DAT 10/10 vs. 9/x), and "time" (T0 vs. T1). The following variables were evaluated: 1) scores obtained in CGAS and in subscales of Conners' and 2) semi-quantitative anti-DAT titers, through the optical density values, obtained with the DAT-EIA-kit's ELISA assay.

Second, we run a full correlation analysis between anti-DAT titers, resulting from the DAT-EIA-kit's ELISA assay, and scores obtained by patients (in CGAS values assigned by clinicians; in Conners' Scales, compiled by parents and by teacher of the recruited children).

Third, we investigated the anti-DAT titers once again (*time T2*), after an 18-month period of exposure to MPH or to unmedicated conditions (NPT vs. PT patients, respectively), and compared them to the healthy controls (only DAT 9/x genotype, final $n = 8$).

3. Results

As outlined in [Material and methods](#), we enrolled 46 ADHD subjects and 15 healthy controls; we analyzed aAbs titers and psychometric scales of all ADHD subjects; after a posteriori exclusion, we came to

DAT genotype of 30 ADHD subjects (15 subjects per genotype), and eight healthy controls of DAT 9/x genotype. In general, the titer of DAT-aAbs was detectable (optical density around 0.81 in average) in all healthy controls and ADHD children, with a consistent elevation (optical density more than 1–1.1) in specific cases. As a whole, we report slightly but consistently elevated levels of DAT aAbs in the most severe ADHD patients; thus, determination of serum anti-DAT titers can be proposed as a suitable biomarker to help ADHD diagnosis.

3.1. Symptom scales: CGAS and Conners'

The average values of symptom scales (filled in by the mothers) and of CGAS values (assigned by the clinician) show, between time T0 and T1, a globally stable profile of ADHD symptoms in the group of children under non-pharmacological therapy (NPT, 32 subjects before the exclusion made a posteriori), as illustrated in Fig. 1 upper panel. The Student t-test analysis did not yield any significant results hence suggesting no improvement in the absence of medication.

In the group of children placed under pharmacological treatment (PT, 14 subjects before the exclusion made a posteriori), the average values of symptoms (scales filled in by their mothers and CGAS values assigned by the clinician) showed clearly more severe ADHD symptoms, compared to those subjects who did not need to take a pharmacological therapy. As expected, a period of 6 weeks under MPH treatment led to an important reduction of ADHD symptoms between T0 and T1: the reduction of symptoms was significant for CGAS ($t(26) = 3.67$), for the attention deficit sub-scale ($t(26) = 3.13$), and for the ADHD index ($t(26) = 3.27$), as the Student t-test analysis demonstrated (Fig. 1 lower panel).

The Student t-test analysis that compared Conners' scales and CGAS values between the two groups (NPT vs PT subjects) revealed, indeed:

- Time T0: a significant difference in score for CGAS values ($t(44) = 3.79$), for the attention deficit sub-scale ($t(44) = 2.92$) and for the ADHD index ($t(44) = 2.10$): this finding suggests that the subjects, later assigned to the MPH treatment group, were characterized indeed by more severe ADHD symptoms compared to subjects who did not need to take a pharmacological therapy.
- Time T1: no significant differences were evidenced. This indicates that, after 6 weeks of pharmacological treatment, these subjects showed an important reduction in symptoms, so that they could no longer be distinguished from subjects who did not take any pharmacological therapy. The recovery of symptoms by MPH did lead to children who appeared similar to unmedicated ones.

3.1.1. Role of DAT genotype

The group of subjects who did not take pharmacological therapy (9 were excluded for recent fever/allergy; 23 were genotyped out of 32 total) was split into two subgroups, namely in carriers of a DAT 10/10 (12 subjects) vs 9/x (11 subjects) genotype. It could be clearly observed that:

- Genotype 10/10 ($n = 12$): ADHD symptoms were unchanged from time T0 to T1 (Fig. 2 upper panel), suggesting no improvement at all following a non-pharmacological intervention.
- Genotype 9/x ($n = 11$): these subjects showed a more severe ADHD symptomatology at time T0, when compared to those carrying a 10/10 genotype ($t(21) = 2.39$). At time T1, these subjects showed a slight reduction of all symptoms (possibly, due to parent

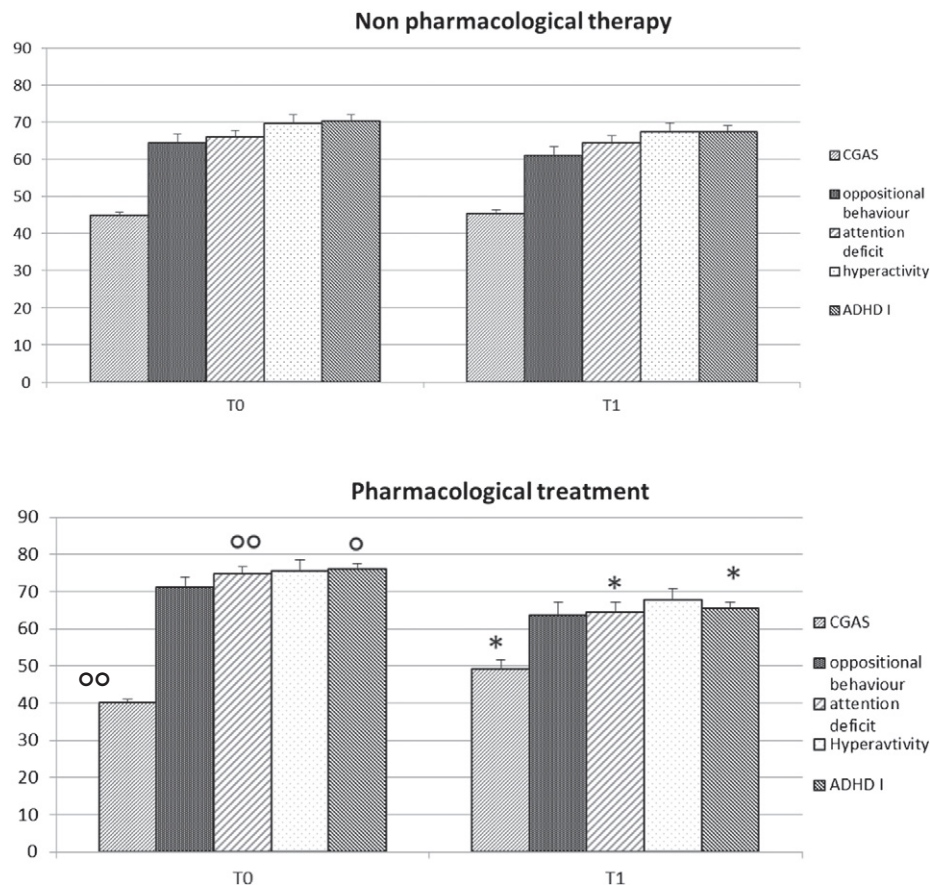


Fig. 1. (Upper panel) – Psychometric scales in recruited ADHD children not assigned to a pharmacotherapy (NPT, $N = 32$). (Lower panel) – Psychometric scales in recruited ADHD children assigned to pharmacological treatment (PT, $N = 14$). Data include also ADHD children which were then not genotyped due to recent fever/allergy. * denotes significance in comparing PT to NPT; * denotes significance in comparing T1 to T0.

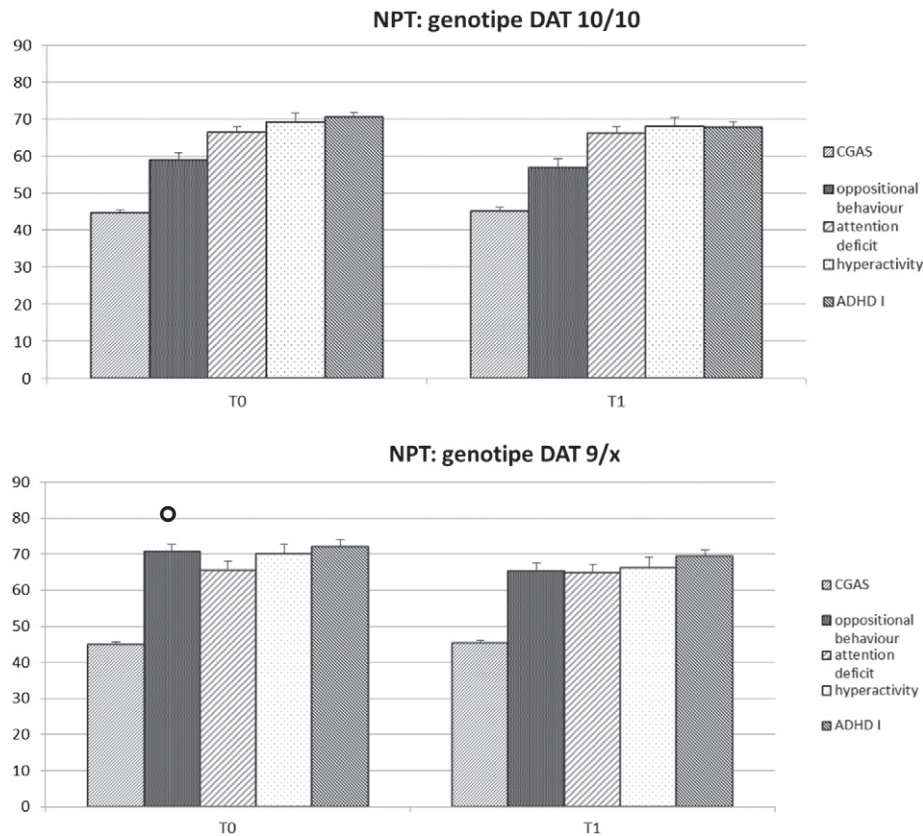


Fig. 2. (Upper panel)—Psychometric scales in genotype 10/10 children in NPT group ($n = 12$). (Lower panel)—Psychometric scales in genotype 9/x children of the NPT group ($n = 11$).

counseling and/or behavioral therapy); however, the oppositional behavior was still more severe compared to subjects carrying a 10/10 genotype (Fig. 2 lower panel). Compared to 10/10 patients, the DAT 9/x ones could be suggested to be more prone to recovery in general, even without medication.

Among subjects who needed to take pharmacological treatment, 7 were excluded and 7 were genotyped (out of 14 total). It is of note that (out of 7 excluded patients in the PT group) two were non-responder to MPH and switched to atomoxetine, while five had recent fever/allergy. Then, this group was split into two subgroups, namely, carriers of DAT 10/10 (3 subjects: Fig. 3 upper panel) vs DAT 9/x (4 subjects: Fig. 3 lower panel) genotype. We observed that:

- Genotype 10/10 ($n = 3$): subjects with both 10-repeat alleles showed just a trend towards a MPH-induced reduction of ADHD symptoms, according to CGAS and Conners' scales values, between T0 and T1; this reduction was however slight and did not reach statistical significance.
- Genotype 9/x ($n = 4$): subjects with at least a DAT 9-repeat allele showed symptoms of inattention, hyperactivity and ADHD Index which were slightly more severe than those found in subjects with a 10/10 genotype, at time T0. These subjects, however, showed a better therapeutical response to MPH: in fact, a significant reduction of CGAS ($t(6) = 2,80$) and of attention deficit Conners' sub-scale ($t(6) = 2,97$) was demonstrated by the Student t-test analysis.

3.2. Anti-DAT titer in plasma samples

The average aAbs titers in subjects who did not take pharmacological therapy showed a slight trend towards an increase in values from time T0 to T1 (Fig. 4 upper panel). This unexpected rise could represent a

“physiological” phenomenon: at time T0, the sample was taken early in the morning (along with a routine blood screen), and could be therefore representative of the basal DAT aAbs titer; conversely, the sample withdrawn at time T1 could be used to estimate the “physiological” levels, reached in the late morning (and/or after having had a breakfast).

The average aAbs titer in subjects under pharmacological treatment showed, between time T0 and T1 time, a quite stable value (Fig. 4 lower panel). In these subjects, after 6 weeks of MPH treatment, we could not find an increasing titer, as we would have expected based on the findings from the non-pharmacological therapy (NPT group). Noteworthy, at T1 time, patients were not taking the morning dose of the drug, therefore any differences between PT and NPT subjects can be considered as indicative of subchronic (and not acute) drug effects.

When comparing the two groups (PT vs NPT), at time T0, we can observe that those subjects, later to be assigned to pharmacological treatment, presented higher basal anti-DAT titers compared to those subjects later assigned to a non-pharmacological therapy. These data allow us to hypothesize elevated basal anti-DAT titers in association with a more severe type of ADHD. Indeed, those PT patients were actually in need of a pharmacological approach. On the other hand, at time T1, this difference was no longer evident, because aAbs titers appeared to be similar in the two groups. Noteworthy, the analysis of behavioral profiles also evidenced very similar scores between the two groups (Fig. 1). Thus, a close relationship between changes in behavior and in the immune marker apparently emerges. Indeed, when PT and NPT subjects were detected with similar aAbs titers (at T1, i.e. after 6 weeks of MPH or unmedicated conditions, respectively), they also showed an indistinguishable behavioral profile. Notably, in the very same subjects, DAT aAbs titers did differ quite clearly when the symptoms also differed (i.e. at time T0). Thus, serum DAT aAbs titers could represent a valid and useful tool to monitor, among others, the efficacy of MPH.

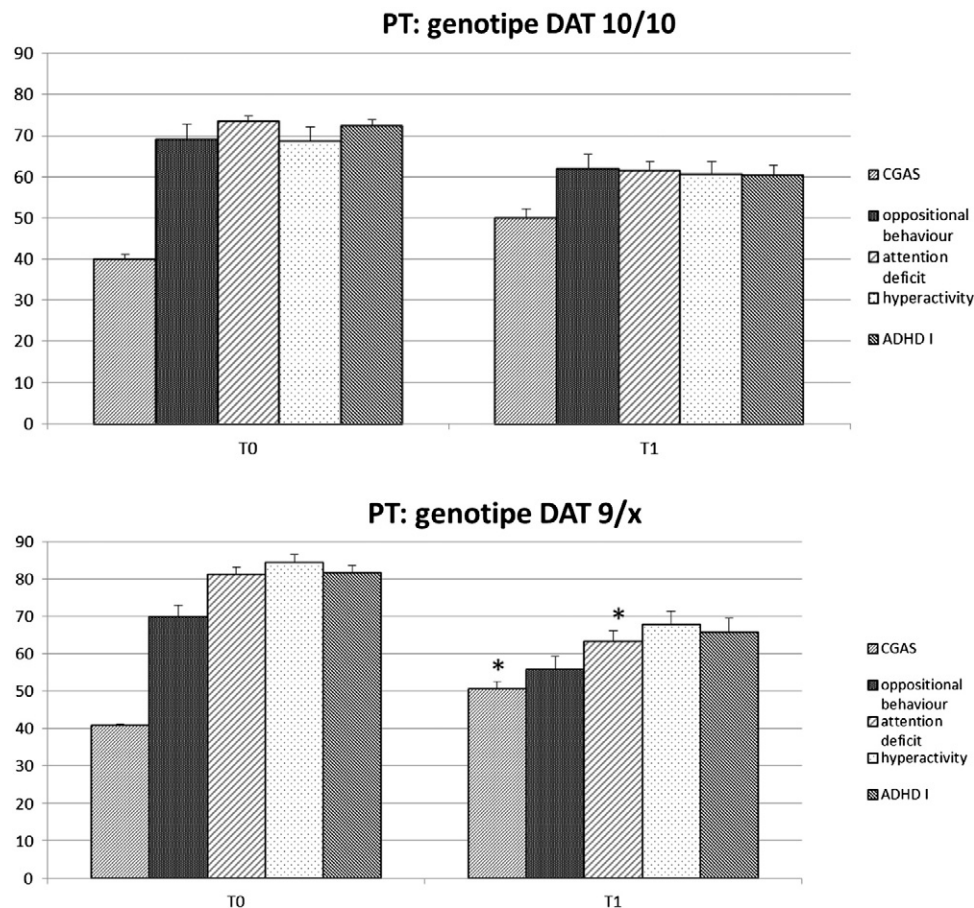


Fig. 3. (Upper panel)—Psychometric scales in genotype 10/10 children in PT group ($n = 3$). (Lower panel)—Psychometric scales in genotype 9/x children of the PT group ($n = 4$).

3.2.1. Role of DAT genotype

By splitting the group of milder ADHD subjects, who did not need to take pharmacological therapy (NPT), into the two different DAT genotypes, it could be observed that:

- Time T0: basal aAbs titers did not differ for subjects carrying a 10/10 genotype (Fig. 5a) when compared to 9/x subjects (Fig. 5b);
- Time T1: subjects carrying a 9/x genotype showed a significant elevation of anti-DAT titers from time T0 to T1 ($t(20) = 2.01$); however, at time T1, aAbs titers in subjects carrying a 9/x genotype didn't significantly differ from T1 titers in patients with a 10/10 genotype ($t(21) = 0.67$). As such, a slight trend to increase can be proposed for 10/10 patients as well.

By splitting the group of severe ADHD subjects, placed under MPH treatment (PT group), into the two different DAT genotypes, it can be observed that:

- Time T0: basal DAT aAbs titers of DAT 10/10 subjects (Fig. 5c) were almost double when compared to basal titers of 9/x subjects (Fig. 5d). It is interesting to note that all subjects, with severe symptoms and DAT 10/10 genotype, could be characterized by very high basal anti-DAT titers.
- Time T1: for the 10/10 genotype, the aAbs titer was significantly increased when compared to that of 9/x subjects ($t(5) = 2.59$). For the DAT 9/x genotype, there were no significant differences when the T1 titer was compared to the basal T0 titer. In conclusion, a high and drug-resistant titer (both basal and after treatment) may occur in DAT 10/10 people, i.e. the ones that also show a lower drug response as far as behavior is concerned (Fig. 3 upper panel). Conversely, while the titer should apparently rise from T0 to T1 (see e.g. in NPT patients), the aAbs titers are surprisingly not rising (i.e. T0 followed

by no such an increase at T1) in the group under MPH treatment, carrying a DAT 9/x genotype.

3.3. Correlation between behavioral scales and DAT-aAbs

Anti-DAT titers and the behavioral scales filled in by the parents at T0 or T1 appeared significantly correlated in subjects under non-pharmacological therapy. A significant correlation was found between titers, both at time T1 and basal, and the values of inattention indicated in questionnaires compiled by fathers; also, a significant trend was found between basal titers and hyperactivity values indicated in questionnaires compiled by teachers. These correlation values are illustrated in Table 1. This profile of data seems to indicate that, when the inattention levels (indicated in questionnaires compiled by the fathers and/or the motor behavior suggested by the teachers) do increase, also the DAT-aAbs circulating in the blood show an increase.

When considering the role of genotype in the NPT group, the observed correlation between aAbs titers and the attention disorder (i.e. the score in questionnaires compiled by the father) disappears for the 9/x subgroup. On the other hand, for the 10/10 subgroup, the correlations were confirmed and extended. A statistically significant correlation emerged between titers (both basal and at time T1) and all the behavioral scales, indicated in questionnaires compiled by fathers. Moreover, it can be found a significant relationship between anti-DAT titers (both basal and at time T1) and the oppositional behavior indicated in questionnaires compiled by the teacher (Table 2).

Correlations between DAT-aAbs and behavioral scales for subjects under pharmacological treatment (PT group) did not show any statistically significant correlation (data not shown).

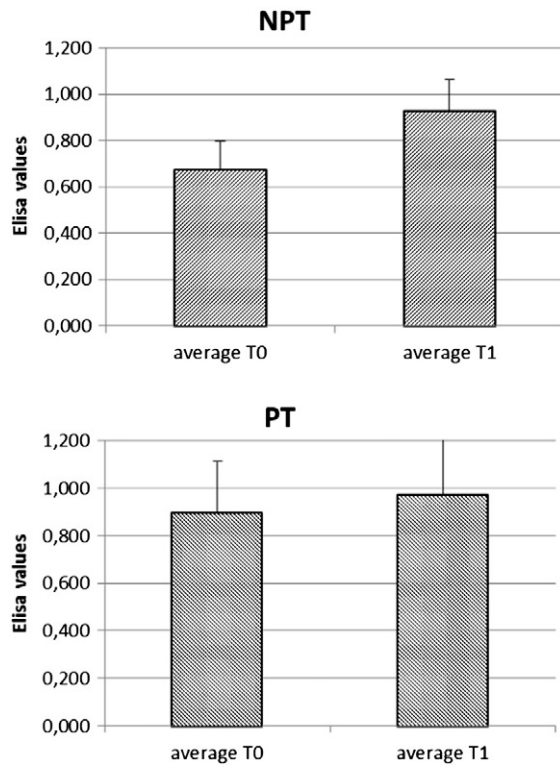


Fig. 4. (Upper panel)—hDAT aAbs titer in recruited ADHD children not assigned in a pharmacotherapy (NPT, $N = 32$). (Lower panel)—hDAT aAbs titer in recruited ADHD children assigned to pharmacological treatment (PT, $N = 14$). Data include also ADHD children which were then not genotyped due to recent fever/allergy.

3.4. Summary and comparison with control healthy subjects

Considering the role of genotype in the comparison between the two groups, we observe that:

- 1) Among 15 subjects carrying DAT 10/10 genotype, those three most severe cases, placed under a pharmacological treatment, turned out to show a doubled titer when compared to those 12 less severe cases, that were not in the need of a pharmacological therapy: this, both at time T0 (basal titer) and significantly at time T1 ($t(13) = 2.06$) after 6 weeks of therapy;
- 2) Subjects with a DAT 9/x genotype did not show any difference in basal titer (time T0) between the two PT and NPT groups; in the T1 serum sample, measure of aAbs titers revealed a significant “physiological” increase in the 11 NPT subjects (Fig. 5b). Notably, no such elevation was found in the 4 PT patients. Indeed, along with behavioral amelioration, a rise in titers was apparently prevented by a 6-week MPH treatment.

The recruitment of 15 healthy controls, carried out around 18 months after the main study (at the time of patients' re-sampling, time T2), provided reliable samples from 8 subjects, who were carrying at least one 9-repeat allele and not excluded a posteriori. These data were compared with eight 9/x patients, re-sampled (at the same time) after one-to-two years (time T2) of exposure to MPH or to the unmedicated condition (PT or NPT respectively). Interestingly, the control group presented a DAT aAbs titer of 0.838 ± 0.178 in average, a median of 0.601 and a third quartile of 1.027; the unmedicated ADHD children had a DAT aAbs titer of 1.673 ± 0.201 in average, with a median of 1.758 and a third quartile of 2.162; finally, the patients with 18-month long, daily exposure to MPH had a titer of 1.095 ± 0.188 , with a median of 1.001 and a third quartile of 1.266.

The sample of unmedicated, 9/x ADHD children showed elevated titers compared to controls ($t(14)$; $p = 0.004$); conversely, the 9/x ADHD

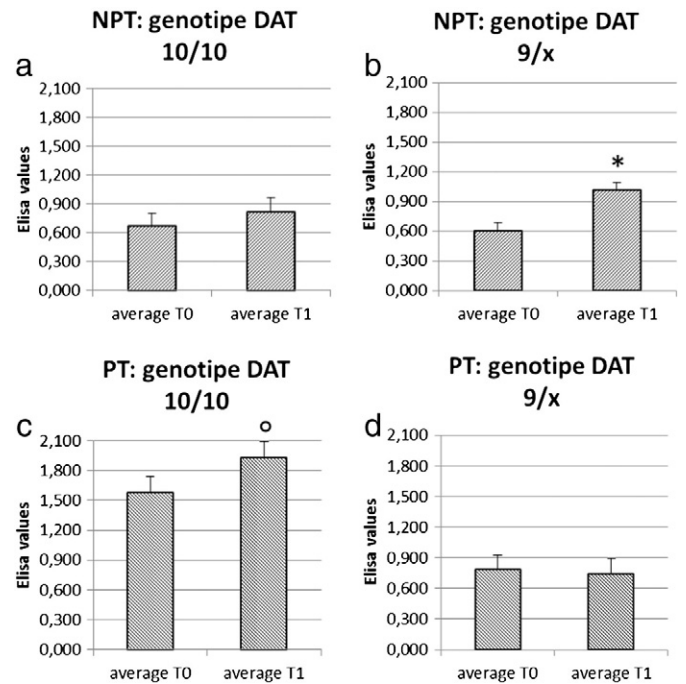


Fig. 5. (Panel a)—hDAT aAbs in children of NPT group, genotype DAT 10/10 ($n = 12$). (Panel b)—hDAT aAbs in children of NPT group, genotype DAT 9/x ($n = 11$). (Panel c)—hDAT aAbs in children in the PT group, genotype DAT 10/10 ($n = 3$). (Panel d)—hDAT aAbs in children in the PT group, genotype DAT 9/x ($n = 4$). * denotes significance in comparing PT to NPT; ^o denotes significance in comparing T1 to T0.

children under MPH treatment showed significantly reduced DAT aAbs titers ($t(14)$; $p = 0.029$), which were hardly distinguishable from those of controls. This latter finding, collected from eight re-sampled patients, can be compared with findings already collected from the very same 9/x children at T1: indeed, a 6-week MPH exposure (PT group, see Fig. 5d) was already found to prevent the rise of T1 titers, otherwise shown by the unmedicated subjects (NPT group, see Fig. 5b). As a whole, a main suggestion seems to emerge from these data: the majority of recruited ADHD children showed DAT aAbs titers (between 0.4 and 0.9) which were fully comparable to those of healthy subjects, carrying a 9/x genotype.

Interestingly, specific profiles emerged as a function of the genotype. The 10/10 ADHD children, in the three most severe cases needing drug treatment, showed an elevated titer (almost doubled, and much more than 1–1.1); also, in 12 less severe cases, the titer turned out to correlate quite well with symptoms' severity. Their DAT aAbs titer resulted, however, not sensitive to MPH exposure, and these subjects also did not show reliable relief of behavioral symptoms. The 9/x ADHD children showed – conversely – a quite interesting similarity between serum titers and behavioral symptoms, in that both appeared sensitive to MPH. Noteworthy, the 4 most severe cases showed relief of symptoms after just 6 weeks of therapy and, furthermore, the elevation of titers (expected based on all NPT-group findings) was prevented if subjects were medicated; this, both after 6 weeks (time T1) and following a longer, 1-to-2 year period (time T2). However, the aAbs titer did not correlate with individual symptoms' severity, neither before nor after medication.

4. Discussion

Nowadays, the diagnosis of ADHD is mainly based on structured interviews, questionnaires, and physicians' own judgment following clinical observation. Nevertheless, the diagnosis of ADHD is not unequivocal; indeed, the behavioral nature of these criteria renders ADHD diagnosis yet a debated question, at least in borderline conditions. There is currently

Table 1

Values of Pearson's R in correlations run between behavioral scores (obtained in CGAS and in various Conners' subscales compiled by parents and teachers, see Columns) and the DAT-aAbs titers, for children assigned to the non-pharmacology therapy (NPT) group. Data include also ADHD children which were then not genotyped due to recent fever/allergy (N = 32). First line: behavioral scores at recruitment correlated to baseline titers (at T0); Second line: behavioral scores at 6-week follow-up correlated to corresponding titers (at T1); Third line: behavioral scores at recruitment (c T0) correlated to 6-week follow-up titers (Ab at T1). The few cases where correlation is significant are marked in bold for R > 0.34 (N = 32).

	NPT												
	CGAS	Conners' mother				Conners' father				Conners' teacher			
		Oppositive behavior.	Impaired attention.	Hyperactivity	ADHD index	Oppositive behavior.	Impaired attention.	Hyperactivity	ADHD index	Oppositive behavior.	Impaired attention.	Hyperactivity	ADHD index
Mean T0	0.091	−0.056	0.158	−0.122	−0.007	0.106	0.514	0.133	0.269	0.151	0.253	0.329	0.295
Mean T1	0.057	0.102	0.047	0.013	0.048	0.078	0.010	−0.042	0.021				
c T0 vs Ab T1	0.032	0.005	0.123	−0.004	0.016	0.176	0.419	0.202	0.274	0.093	0.123	0.246	0.185

much interest in the search for new biological and measurable markers, possibly detectable via a simple biochemical tool and with non-invasive, peripheral approaches (Scassellati et al., 2012).

The clinical-assessment data from subjects in the NPT group have shown a globally stable profile of ADHD symptoms over time (i.e. between recruitment and follow-up, at 6 weeks after recruitment). This stable profile was particularly evident for patients with DAT 10/10 genotype. On the other hand, subjects later assigned to a pharmacological treatment (PT) showed, at time of recruitment, more severe ADHD symptoms compared to NPT subjects, confirming that PT patients were actually in the need to take a pharmacological therapy. Furthermore, after 6 weeks under MPH treatment, some subjects showed an important and significant reduction in ADHD symptoms. By considering DAT genotype, we found that a reduction of ADHD symptoms over time (between recruitment and follow-up, 6 weeks after MPH reached the therapeutic dosage) was marked (and statistically significant) only in the subgroup of subjects with a DAT 9/x genotype. This confirms a greater efficacy of pharmacological treatment, in reducing ADHD symptoms, for patients bearing at least one 9-repeat DAT allele.

Our primary aim was to measure the blood levels of auto-antibodies (aAbs) targeting epitopes of the DAT protein. Interestingly, all children (including healthy controls) revealed measurable levels of DAT-directed aAbs. Notably, among patients, specific subjects showed an elevated titer. Moreover, as a function of genotype, titers apparently depended on behavioral symptoms and/or on MPH exposure. A first notion is that severe ADHD subjects – which were assigned to MPH

pharmacological treatment – presented, at recruitment (time T0), higher anti-DAT titers compared to the *mild* ADHD group – which was assigned to a non-pharmacological therapy. These data suggest an association between elevated basal anti-DAT titers and a more severe profile of ADHD symptoms, therefore identifying the need of a pharmacological approach.

A second notion is that levels of these aAbs were not stable in unmedicated conditions. Subjects who entered a non-pharmacological therapy showed increasing titers from time T0 to T1, and further elevation when re-sampled 1-to-2 years later. This unexpected rise could represent, possibly, a “patho-physiological” phenomenon. It should be noted that NPT children were left in unmedicated conditions between T1 and T2, leaving room for the hypothesis that anti-DAT titers may indicate a progressively deteriorating immunological condition. We cannot however exclude the existence of a circadian physiological fluctuation in the aAbs titer, with increasing levels of circulating aAbs in the late morning and/or after food intake.

After 6 weeks from recruitment, overlapping behavioral profiles and similar aAbs titers were observed between subjects exposed to unmedicated conditions vs MPH treatment. Noteworthy, these two groups were *different* at the time of recruitment (i.e. PT patients showed elevated aAbs titer and more severe symptoms' profile). After 6 weeks from recruitment, children placed under pharmacological treatment did not show the rise in T1 aAbs titer over T0 baseline, conversely observed for NPT patients. In the PT subjects, the subchronic exposure to MPH treatment was apparently preventing the increase in anti-DAT titer.

Table 2

Same as in Table 1, but divided for genotype DAT 10/10 (Panel a, n = 12) vs. DAT 9/x (Panel b, n = 11). Teachers' evaluation was collected only at recruitment and not at 6-week follow-up. Interestingly, in DAT 10/10 patients at risk for ADHD, higher DAT-aAbs titers are related with poorer assessment by Conners' scales. Correlation is significant when marked in bold, for R > 0.53 (n = 12).

Panel a													
NPT: genotype 10/10													
CGAS	Conners' mother				Conners' father				Conners' teacher				ADHD index
	Oppositive behavior.	Impaired attention.	Hyperactivity	ADHD index	Oppositive behavior.	Impaired attention.	Hyperactivity	ADHD index	Oppositive behavior.	Impaired attention.	Hyperactivity	ADHD index	
Mean T0	−0.349	0.237	0.374	−0.150	−0.212	0.610	0.740	0.545	0.543	0.578	0.302	0.400	0.399
Mean T1	−0.002	0.184	−0.157	−0.243	−0.338	0.193	−0.157	−0.243	−0.355				
c T0 vs Ab T1	−0.263	0.112	0.103	−0.131	−0.404	0.434	0.543	0.457	0.325	0.503	0.324	0.448	0.419
Panel b													
NPT: genotype 9/x													
CGAS	Conners' mother				Conners' father				Conners' teacher				ADHD index
	Oppositive behavior.	Impaired attention.	Hyperactivity	ADHD index	Oppositive behavior.	Impaired attention.	Hyperactivity	ADHD index	Oppositive behavior.	Impaired attention.	Hyperactivity	ADHD index	
Mean T0	0.179	−0.300	0.233	−0.261	0.090	−0.428	0.267	−0.117	0.020	−0.154	0.895	0.070	0.351
Mean T1	−0.225	0.103	0.315	0.261	0.286	−0.129	0.120	0.033	0.082				
c T0 vs Ab T1	−0.155	−0.154	0.230	0.173	0.188	−0.084	0.232	0.171	0.233	−0.533	0.490	−0.270	−0.097

This information can lead us to propose, therefore, that anti-DAT titers could be used to monitor the efficacy of MPH.

4.1. Role of DAT genotype

By splitting the whole PT and NPT subjects into two subgroups, according to DAT genotype, we could observe that subjects carrying a 9/x genotype (and under non-pharmacological therapy, NPT) showed indeed the aforementioned, statistically significant increase of anti-DAT titers from T0 baseline to time T1. In other words, in the sample withdrawn at time T1, the titers revealed a significant and possibly “patho-physiological” increase over T0 baseline. This profile, however, was only evident in the group of NPT subjects, and notably no such an increase was found due to a 6-week MPH treatment. We suggest that, in DAT 9/x subjects placed under pharmacological treatment, a sub-chronic exposure to MPH prevented the increase of aAbs titers between T0 and T1, otherwise observed for subjects carrying a DAT 9/x genotype but not placed under pharmacological therapy. It is tempting to speculate that therapeutic effects of MPH might be accompanied by a specific modulation (i.e. preventing the spontaneous increase) of DAT aAbs titers.

In the group that needed to undergo MPH treatment, subjects with a DAT 10/10 genotype showed a very high levels of DAT-aAbs: the anti-DAT titers were almost double when compared both to those of DAT 9/x subjects, and to those of less severe DAT 10/10 patients, not placed under MPH. Anti-DAT levels were higher at baseline and further elevated after a 6-week pharmacological treatment, accompanied by the very slight and not significant reduction in symptoms' severity, reported by the clinician and parents. This suggests that subjects with a DAT 10/10 genotype could be characterized by a lower therapeutic response to MPH treatment (Madras et al., 2002). It is tempting to speculate that reduced drug efficacy comes along with lack of any effect over the very high (and rising) anti-DAT titer. This may suggest that, in case of ADHD subjects who are homozygous for DAT 10-repeat alleles, high and drug-resistant DAT aAbs titers can identify those patients in need of alternative, non-psychostimulant drug treatment (like e.g. atomoxetine).

4.2. Behavioral scales/anti-DAT titer correlations

The analysis of correlations was run between behavioral scales (filled in by the clinician, the parents and the teacher) and DAT aAbs titers. A profile of correlation was found in subjects under a non-pharmacological therapy. A significant correlation was found between basal aAbs titers and the values of inattention indicated by fathers; also, a significant trend was found between basal aAbs titers and hyperactivity values as indicated by patient's teacher. This means that, when inattention levels suggested by fathers and/or the motor behavior suggested by teachers is increased, the circulating DAT-aAbs may turn out to be elevated.

By splitting NPT patients into two subgroups, according to DAT genotype, the above correlations were confirmed and further extended for the DAT 10/10 subgroup. A statistically significant correlation emerged between titers (both basal and at time T1) and *all* the behavioral scales suggested by fathers. Moreover, we could find a significant relationship between titers (both basal and at time T1) and oppositional behavior indicated by the teacher. Therefore, in homozygous subjects carrying the DAT 10-repeat allele, the highest levels of DAT-aAbs came along with the more problematic behavior of children. Consistently, the most severe ADHD cases, in need of drug treatment (PT group), showed almost doubled DAT aAbs titers (see above). On the other hand, the correlation between scales and titers completely disappeared for DAT 9/x subgroup.

4.3. Patho-physiological remarks and perspectives

Abnormal dopamine levels and deregulation of dopaminergic components, expressed in immune cells, are associated with some auto-immune disorders (Pacheco et al., 2014). Dopamine can probably

activate T-cell function indirectly, by suppressing T-regulatory cell (Treg) (Kipnis et al., 2004; Cosentino et al., 2007; Levite, 2008). Treg is involved in auto-immunity, and secretes IL-10 and TGF- β : because of its inhibitory properties, reduced activity of Treg (due to abnormal dopamine) could lead to uncontrolled activation and function of effector T-cells and to auto-immunity (Pacheco et al., 2010). Furthermore, dendritic-cell derived dopamine promotes IL-23 production and, thereby, enhances Th17 responses with inflammation (Pacheco et al., 2014). These findings suggest that dopamine can mediate communication between immune cells, and the cross-talk between the immune and nervous systems (Cosentino et al., 2007; Nakano et al., 2009; Sarkar et al., 2010; Buttarelli et al., 2011; Pacheco et al., 2014).

Thus, over-expression of DAT in lymphocytes of ADHD children could imply dysregulation of immune and neuro-immune systems. A reduced level of dopamine is typical of auto-immune pathologies (Buttarelli et al., 2011). Blockade of DAT by MPH may normalize the dopamine levels, which in turn may act on the lymphocytes. It was reported, consistently, that MPH may induce (i) a hyperactivity of the immune system and a heightened tendency to respond to antigenic or immunogenic stimuli, (ii) hyper-gamma-globulinemia, and (iii) reduction of T-helper/inducer cells (Auci et al., 1997). The issue of a role for neuro-immune components in ADHD and other developmental, neuro-psychiatric conditions deserves however further investigation.

4.4. Conclusion

Replication of our findings is needed: it will be necessary to investigate the selectivity of epitopes (i.e. to ascertain that aAbs recognize epitopes of DAT over other proteins), as well as their specificity (i.e. how do these DAT aAbs vary in other mental or immunological conditions which are not related to ADHD). For validation of DAT aAbs as a tool for diagnosis and/or therapy monitoring in subjects with ADHD, we would need to clarify the etiological implications: namely, the nature of the correlation between anti-DAT titers, on one hand, and clinical features of ADHD-like behavioral symptoms, on the other hand.

Present data do indicate for the first time that detectable levels of a DAT-directed, circulating auto-antibody can be evidenced in a pediatric population, including children with ADHD diagnosis. In addition, we propose that anti-DAT titers and DAT genotyping might possibly be used together, to determine a priority for drug intervention based on severity of child's symptoms. In other words, the elevation of serum anti-DAT titers and restoration of basal levels by few weeks of MPH can be proposed as bio-markers, to support diagnosed ADHD and its drug therapy. These biological indices could turn out to be useful: 1) to provide or verify diagnostic criteria, 2) to predict the evolution of symptoms over time, and 3) to monitor the efficacy of MPH treatment.

In the end, further study is warranted to deepen the knowledge about ADHD neurobiology, by exploring the relationship between this neuro-psychiatric disorder and the immune system. The presence of elevated circulating aAbs directed against DAT in ADHD subjects may well open new avenues of (pre)clinical research, as neuro-immune modulations are increasingly worthy of being explored.

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