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Methylphenidate ameliorates the homeostatic balance between levels of kynurenines in ADHD children

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ABSTRACT

The kynurenine pathway of tryptophan metabolism has been involved in ADHD We quantified basal levels and daily fluctuations of tryptophan and several kynurenine metabolites, as well as their changes after treatment with methylphenidate (MPH).

A total of 179 children were recruited, grouped into ADHD ($n = 130$) and healthy controls (CG,$n = 49$). Blood samples were drawn at 20:00 and 09:00 h and only in the ADHD group after 4.63±2.3 months of treatment. Nocturnal urine was collected between both draws. Factorial analysis (Stata12.0) was performed with Groups, Time, Hour of Day and Depressive Symptoms (DS) as factors.

MPH significantly increased plasma Kynurenic acid (2.4 ± 1.03/2.78±1.3 ng/mL; baseline/post-treatment, morning; $z = 1.96$,$p<0.05$) and Xanthurenic acid (2.39±0.95/2.88±1.19 ng/mL; baseline/post, morning; $z = 2.7$,$p<0.007$) levels, both with higher values in the evening. In DS+ patients, MPH caused a pronounced decrease in evening Anthranilic acid [3.08±5.02/ 1.82±1.46 ng/mL, $z = 2.68$,$p = 0.0074$] until matching values to other subgroups. In urine, MPH decreased the excretion of both Nicotinamide and Quinolinic acids, but only in the DS- subgroup.

The kynurenine pathway may participate in the highly clinical favorable response to MPH. The observed changes could be considered as protective (i.e. increased plasma kynurenic acid vs. decreased quinolinic acid excretion) based on the knowledge of its physiological homeostatic functions.

1. Introduction

In addition to the strong contribution of genetic factors to the appearance of Attention Deficit and/or Hyperactivity Disorder (ADHD), social and environmental risk factors should also be considered, especially if they are present in the pre- and early postnatal periods during brain development (Sagiv et al., 2013).

The kynurenine pathway (KP) is a set of enzymatic reactions involved in the tryptophan (TRP) catabolism that mainly lead to the formation of ATP (Stone and Darlington, 2013). L-tryptophan catabolism

Abbreviations: 3HAA, 3-hydroxy-indoleacetic acid; 3HK, 3-hydroxy-kynurenine; 5HIAA, 5-hydroxy-indoleacetic acid; AA, Anthranilic acid; AAT, α-amino-adipate transaminases; AD, Attention deficit; ADHD, Attention-deficit/hyperactivity disorder; CD, Conduct disorder; CDI, Children's Depression Inventory, scale; DS, Depressive symptoms; DSM-5, Diagnostic and Statistical Manual of the American Psychiatric Association, 5th edition; EDAH, Spanish acronym of scale for Evaluation of ADHD; HI, Hyperactive-impulsive; IDO, Indoleamine 2,3-dioxygenase; KA, Kynurenic acid; KBIT, Kaufman Brief Intelligence Test; KP, kynurenine pathway; KYN, (L-)Kynurenine; LC–MS/MS, liquid-chromatography–tandem mass spectrometry; MPH, Methylphenidate.

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by the hepatic enzymes tryptophan 2,3-dioxygenase (TDO) and cerebral indoleamine 2,3-dioxygenase (IDO) represents the rate-limiting step in the synthesis of L-kynurenine (KYN), the first KP metabolite. Once synthesized, KYN is metabolized through three distinct pathways to form kynurenic acid (KA), 3-hydroxy-kynurenine (3HK) and anthranilic acid (AA) (Dostal et al., 2017).

Most KP metabolites are neuroactive. Among others, KA acts as a glutamate antagonist by inhibiting the activation of N-methyl-D-aspartate receptor (NMDAR). QA is predominantly synthesized by infiltrating macrophages and microglia, intermediates neurotoxicity by acting as a NMDAR agonist and by increasing free radical production. Xanthurenic acid (XA) is an allosteric agonist of metabotropic glutamate (mGlu) 2/3 receptors, although its main function is to inhibit vesicular glutamate transporters (Neale et al., 2014).

During brain development, stress (Michels et al., 2018) and immune activation (associated with increased levels of nitric oxide, IFN-γ and cytokines) (Badawy, 2017) induces IDO activity, which in turn controls plasma TRP availability for the KP in the CNS (Wichers et al., 2005). These inflammatory pathologies increase the production of toxic quinolinic acid (QA) (Guillemin, 2012) that predominates over the neuroprotective KA (Stone and Darlington, 2013).

These deleterious changes in the KP during early neurodevelopment might disrupt the age-related changes in discrete brain areas and connectivity. Because glutamate is the major brain excitatory neurotransmitter, there is evidence supporting NMDA dysfunction in the pathogenesis of ADHD (Chang et al., 2014). In both children (Evangelisti et al., 2017) and adults (Aarsland et al., 2015), ADHD is associated with lower serum levels of the first metabolites derived from each of the three distinct KPs (KA, 3HK, AA), with reduced concentrations of XA (a 3HK metabolite). By contrast, TRP and KYN serum levels are higher in ADHD patients in comparison with controls (Evangelisti et al., 2017). On the other hand, depressive symptoms, which is a comorbidity that reach a high incidence among ADHD population, are largely related to changes in TRP metabolism (McCusker et al., 2013; Wu et al., 2018).

Based on this knowledge, we hypothesized that the KP would be involved in the ADHD neurobiology and/or the response to methylphenidate (MPH). Consequently, our study aims to examine putative changes in the levels and daily fluctuations of TRP and several KP metabolites in ADHD children, as well as to evaluate the direction of such putative changes after chronic treatment with MPH.

2. Methods

2.1. Subjects

Children (n = 179; 135 males, 44 females) aged 5–14 years (9.61±2.55 y) were included in a prospective, quasi-experimental and open-label clinical trial. The hospital-based sample consisted of two groups, an ADHD group in which each patient was assessed at least twice and, consequently, acted as his/her own control; and a Control Group only as reference. A total of 130 children who met the DSM-IV-TR/ICD-9 ADHD criteria were included in the ADHD group. The Control Group (CG; n = 49) was mainly composed of their siblings (n = 35), who were simultaneously recruited.

2.2. Assesment

A personal medical history and physical examination were first obtained. The following questionnaires were administered as well: a) the DSM-IV-TR criteria checklist, which was completed by the patient's teacher; b) the evaluation of deficit of attention and hyperactivity scale (EDAH is the Spanish acronym) (Sánchez et al., 2010), in duplicate, one for the teacher and the other for the child's parents; c) the Children's Depression Inventory (CDI) (Kovacs, 1992), completed by patients ≥8 years of age; d) a 1-week sleep diary; and e) the d2 Test of Attention (Brickenkamp, 1997). The EDAH is based on the DSM-IV-TR criteria to aid in the identification of children with ADHD and/or conduct disorder (CD). The EDAH (Farré i Riba and Narbona-García, 1998) is a 20-item ADHD scale that uses structured observation by teachers and is divided into items for "attention deficit" (5 items), "hyperactivity" (5 items) and "behavioral problems" (10 items). Each item is in turn classified into a 4-point frequency scale. Cronbach's alpha values are 0.94 and 0.91 for "Hyperactivity" and "Attention Deficit", respectively. Based on the EDAH scores, the ADHD group was quantitatively subclassified into two clinical subgroups: a) children who had predominantly attention deficit (AD) if their scores were >9 for deficit of attention) and <10 for hyperactivity-impulsivity), with a total score <30; b) and children with predominantly hyperactive-impulsive subtype with comorbid CD (HI/CD), if they had scores <10 for AD, >9 for HI (hyperactivity); and/or a total >29. Thirty-four of the 78 children (44%) who were included into the HI/CD group met criteria for the diagnosis of HI without CD. Of the 44 children with CD symptoms, 33 (42%) showed a predominance of HI symptoms over CD symptoms, whereas the remaining patients (11/78; 14%) had a prevalence of CD symptoms over HI symptoms. Only 26 of these 78 participants (33%) did not meet further criteria for AD. Since externalizing behavioral problems and aggression seem to be related to both the hyperactive-impulsive ADHD symptom domain and the overall ADHD symptom severity (Connor and Ford, 2012), we decided to include these patients into the HI/CD group for analysis purposes

The d2-test represents an estimation of individual attention and concentration performance through the quantification of two scoring keys: errors of omission and errors of commission.

Depressive symptoms (DS) were assessed through interviews conducted with patients and parents as respondents, and quantitatively through the CDI test, which is a self-report assessment of depression for children. The CDI test is comprised of two subscales containing items that are more related to depression than anxiety. To define our subgroups, we considered the sum of both subscales, with a cut-off >17 points considered pathological.

All children were evaluated with the KBIT [Kaufman Brief Intelligence Test], an abbreviated intelligence test containing a vocabulary subtest (verbal intelligence) and a matrices subtest (non verbal intelligence).

Written informed consent was obtained from all parents and children aged ≥12 years. No control subject was treated with any drug and they were only assessed once. The study design was approved by the Hospital Ethics Committee.

Exclusion criteria included: 1) K-BIT score <85; 2) previous or current treatment with antiepileptic drugs; 3) other ADHD treatments or other pathological conditions; and 4) revocation of consent.

Somatometric characteristics of participants and mean values of their scores for the different scales and questionnaires are provided in Table 1.

2.3. Treatment

The only used drug was prolonged-release methylphenidate (OROS-MPH formulation), initially at 0.5 mg/kg/day. The dosage was adjusted based on the response and tolerance to treatment. The mean initial dose of MPH was 25.81±10.35 mg, and the final dose was 31.85±10.68 mg. At recruitment, all patients were MPH-naive and no other pharmacological or psychological treatment was administered before conclusion of the protocol.

2.4. Measurements

Fasting blood samples were taken in the absence of an acute or severe illness, at 20:00 and 09:00 h of the following day. Nocturnal urine samples were collected during this 13-hour interval, and the excretion of urinary metabolites was expressed as ng/mgCr. In the ADHD group, an identical study protocol was repeated after 4.61±2.29 months of daily

Table 1
Demographic characteristics, anthropometry, ADHD subtype, CDI and Intellectual scores for the control and the ADHD groups.

	Control (n = 49)	ADHD (n = 130)	Statistics	
			t	P value
Age (year)	10.35±2.55	9.47±2.52	2.06	0.04
Sex (Male/Female)	33/16	102/28	X^2=0.56	0.45
Height (m)	1.47±0.17	1.37±0.17	3.06	0.001
Weight (kg)	44.18±15.14	36.50±15.35	2.75	0.0033
Body Mass Index (kg/m^2)	19.81±4.09	18.76±4.17	1.39	0.16
Attention Deficit (AD)	4.12±3.22	10.49±2.78	11.45	<0.0001
Hyperactivity - impulsivity (HI)	3.95±2.61	9.10±3.31	10.16	<0.0001
Conduct Disorder (CD)	4.70±3.20	13.27 ± 6.01	11.48	<0.0001
AD+HI	8.07±4.69	19.56±4.38	13.92	<0.0001
Global Deficit	12.77±6.35	32.83±9.01	15.58	<0.0001
CDI_ Negative Mood (NM)	2.34±2.313	5.40±4.28	5.74	<0.0001
CDI_Negative Self-Esteem (NSE)	4.83±3.35	8.32±5.45	4.70	<0.0001
CDI_total score	7.17±4.87	6.63±8.71	6.01	<0.0001
KBIT total score	107.88±12.29	103.98±11.23	1.19	0.24

Data are expressed as the mean ± SD (standard deviation). M: male; F: female. BMI: body mass index; NM: value of the "negative mood" subscale of the CDI (Childhood Depression Inventory); NSE: value of the "negative self-esteem" subscale of the CDI; Z: z-value on the Wilcoxon signed-rank test. K-BIT total score: combined punctuation of the Kaufman Brief Intelligence Test.

MPH administration. All samples were stored at −80 °C until analysis.

2.5. Analytical method

Liquid-chromatography–tandem mass spectrometry (LC–MS/MS) (Fazio et al., 2015) was the method used to measure levels of tryptophan, kynurenic acid, anthranilic acid, xanthurenic acid, quinolinic acid and nicotinamide (NCTA). Previous to the analysis, only the serum samples were filtered with a nylon filter of 0.22 μm. One hundred microliters of serum were deproteinized using 100μl of internal standard (IS) working solution (50 μM in TCA 4%). Samples were vortex-mixed centrifuged at 14,000 rpm for 5 min. Fifty microliters of the clean upper layer were injected into the chromatographic system. UPLC™ analysis was performed using an Acquity UPLC class (Waters, Manchester, United Kingdom) with a mass-mass detector XEVO-TQS, which included a quaternary pump, an auto-sampler, a solvent degasser and a column oven. The mass spectrometry method was performed on a XEVO-TQS system equipped with a Turbo Ion Spray source. The detector was set in the positive and negative ion mode. The capillary and cone voltages were set at 0.6 kV and 40 V, respectively; the source temperature was 150 °C; and the desolvation temperature was 500 °C. The instrument was set in the multiple reaction monitoring (MRM) mode. Data were acquired and processed by MassLynk v4.1 software. The chromatographic conditions were as follows: Channel A, water added with 0.01% formic acid and ammonium 0.05%; Channel B, acetonitrile; flow, 0.2 mL/min; gradient, t0: 5% B; t3: 70% B; t5: 5% B; run time: 9 min. Acquity BEH C18 1.7 μm 2.1 × 50 mm was used as a chromatography column. Analytic parameters were estimated by two calibration lines, one line from 1 μg/L to 20 μg/L and the other from 20 μg/L to 500 μg/L.

2.6. Statistics

For comparisons between EDAH and CDI scores (ordinal variables), Wilcoxon signed-rank tests (paired samples) were used for inferential statistics. For comparisons between patients and each study variable, the factors in the factorial models were as follows: a) Groups with two categories: AD and HI/CD; b) Patients nested in ADHD presentation types groups and depression subgroups (CDI); c) Hour, with two categories (morning and evening), and crossed with ADHD group and depression subgroups; and d) Time, with two levels before and after treatment. Time was a crossed factor with Subgroups and Hour. Subgroups, Hour and Time were fixed-effect factors, and Patients was a randomeffects factor. Comparisons between cases and controls were performed using the same analysis repeated in two different situations because there was only one measure for controls: baseline in cases compared with controls and after treatment in cases compared with controls. Missing values (~15% in our sample) were substituted by Stata Multiple Imputation Data software. The factorial model had the following three factors: 1) Group with three categories (controls, AD and HI/CD); 2) Subjects (Controls and Patients) nested in groups, and Patients nested in CDI subgroups; and 3) Hour, with two categories, morning and evening, that was crossed with Group. Group and Hour were fixed-effect factors, and Subjects was a random-effect factor. For both types of comparisons, an analysis of variance (ANOVA) table was built, and higher interactions were determined. If these were significant, multiple pairwise comparisons were made using Bonferroníś post-hoc analysis, and if not, these corrections were applied to the principal effects in the table. The experimental quantities for these comparisons were not "t" as expected, because we used "z", the normal approximations for "t's", considering the global sample sizes. The reported analyses were crude analyses and adjusted analyses (in all comparisons) according to age and sex, which were carried out using analysis of covariace (ANCOVA) methodology. In all cases, the interactions were studied for levels <0.15, and the latest comparisons were considered significant at p<0.05 after applying the penalty provided by the correction. When analyzing the variances in different groups, homogeneous transformations were carried out on data using natural logarithms to achieve uniformity. All analyses were performed using STATA 12.0.

3. Results

Anthropometric values, ADHD, CDI and intellectual scores for both the ADHD and the CGs are shown in Table 1. The mean heights and weights were significantly higher in the CG, partly because control patients were slightly older than ADHD patients. However, there were no body mass index (BMI) differences. After treatment with MPH, the mean patient height experienced no changes whereas the weight decreased (Molina-Carballo et al., 2013). Although not significantly different [data not shown], prevalence of DS was 20.7% in patients with AD and 24.4% in the HI/CD subgroup; and more common among girls than among boys (34.8% vs. 20.2%, respectively). More than 80% of participants experienced improvements in EDAH scores after treatment with MPH as reported by parents.

3.1. Tryptophan

Serum TRP levels were really similar between the CG and the ADHD group, with only slightly higher values in the CG. Evening concentrations were significantly higher (z = 4.89,p<0.0001) in both groups. TRP levels or their daily profile were not modified by MPH or DS factors. TRP concentrations were slightly higher in the HI/CD subgroup (z = 1.64,p = 0.09) than in the CG. The lowest baseline TRP concentrations were measured in patients with AD and DS-, and the highest ones in those with DS+, with no significant differences.

3.2. Kynurenic acid

Baseline KA levels were similar between the CG Table 2) and the ADHD group, and experienced dramatic differences influenced by the Hour factor (z = 2.89,p<0.004), with predominant KA values in the evening. MPH induced an increase in the KA levels Table 2) in all data pairs (z = 1.96,p<0.05). The increase after MPH was more marked in

Table 2
Tryptophan and kynurenine metabolites in serum, in Control Group, vs ADHD group values before and after prolonged-release methylphenidate in ADHD groups as a function of the absence/presence of depressive symptomatology.

Metabolites	Baseline ADHD				ADHD, after methylphenidate (MPH)			
	Depressive symptomatology							
	No (n = 105)		Yes (n = 25)		No (n = 105)		Yes (n = 25)	
	Morning	Evening	Morning	Evening	Morning	Evening	Morning	Evening
Tryptophan	623.5 ± 149.9	743.3 ± 196.4	730.0 ± 258.5	810.6 ± 212.0	657.0 ± 253.1	744.5 ± 179.4	582.1 ± 197.4	767±239.3
Kynurenic acid	2.40±0.95	2.78±1.08	2.46±1.33	3.23±1.16	2.83±1.30	3.16±1.39	2.48±1.36	3.00±1.13
Xanthurenic acid	2.35±0.86	2.88±0.93	2.54±1.29	3.48±0.98	2.85±1.20	3.24±0.99	3.15±1.27	3.25±1.06
Anthranilic acid	1.86±1.23	1.61±0.86	1.85±1.08	2.88±4.60*	1.79±0.95	2.18± 2.04	1.73±1.04	1.78±1.40

Values are expressed as the mean (standard deviation). Comparisons between groups/subgroups: (*) $z = 2.72$, $p = 0.007$ vs baseline night, ADHD with DS-. (ϴ) $z = 1.95$, $p = 0.05$ vs baseline day DA with DS-.

DS- patients, although no statistical differences were observed for the DS factor. There were no differences in the nocturnal urinary excretion of KA for any study factor (groups/subgroups, MPH or DS).

3.3. Xanthurenic acid

MPH treatment were associated with a significant increase of XA ($z = 2.7$, $p = 0.007$) with higher evening concentrations in comparison with the morning levels, and a consequent increase of its daily fluctuations ($z = 3.85$,$p = 0.0001$), Table 3. However, the subgroup of patients with HI/CD and DS+ showed a higher XA excretion ($z = 2.59$,$p = 0.0096$) in relation to the same subgroup with DS-. After treatment, XA excretion only decreased in the HI/CD with DS+ subgroup, which contributed to erase the baseline differences, Fig. 1.

3.4. Anthranilic acid

Morning values of AA were slightly lower in the CG (1.54±0.89 ng/mL) than in ADHD patients (1.86±1.23 ng/mL, baseline without DS), without any daily fluctuation, Table 2. In the ADHD group, MPH did not modify the morning concentrations, but increased the levels in the evening. In ADHD patients with DS+, the AA values in the evening were higher at baseline (2.88±4.6 vs. 1.61±0.86 ng/mL, DS+ vs. DS-; $z = 2.72$,$p = 0.007$), Fig. 2. This difference disappeared after treatment with an inversion of the profile, showing lower concentrations ($z = 1.65$, $p<0.1$) without morning/evening differences. In the HI/CD subgroup, both the AA basal profile and the response to MPH were dependent on the DS factor: i.e., in DS- patients, MPH increased the evening values (1.6 ± 0.83 vs. 2.36±2.43 ng/mL). However, in DS+ patients, MPH caused a pronounced decrease (−59%) in the evening AA concentrations ($z = 2.68$,$p = 0.0074$], until equilibrating the AA levels with those observed in the other subgroups.

Table 3
Tryptophan and kynurenine metabolites in urine, ADHD group values before and after methylphenidate in the ADHD groups as a function of the absence/presence of depressive symptomatology.

Urine metabolites (ug/mgCr)	ADHD group, depressive symptomatology			
	No (n = 105)		Yes (n = 25)	
	Baseline	Post	Baseline	Post
Tryptophan*	54.54±38.32	46.21±28.58	43.97±23.60	41.91±16.71
Kynurenic acid	7.34±3.59	6.94±2.45	6.49±2.61	5.86±3.43
Xanthurenic cid ϴ	2.46±1.19	1.97±0.80	3.12±0.64 ¥	2.33±1.05 ¶
Quinolinic acid	25.77±16.90	17.11±8.09 §	21.47±9.81	21.46±13.39
Nicotinamide	0.74±0.31	0.63±0.38 £	0.45±0.29	0.53±0.25 ₴

Values are expressed as the mean (standard deviation). Comparisons between groups/subgroups: (*) $z = 1.83$, $p = 0.07$ baseline vs post, for the whole ADHD. (ϴ) $z = 1.76$, $p = 0.08$ ADHD baseline vs post. (¥) $z = 1.84$, $p = 0.06$ vs. baseline ADHD with DS-. (¶) $z = 2.64$, $p = 0.008$ vs baseline ADHD with DS+.(§) $z = 3.62$, $p = 0.00028$ vs baseline ADHD with DS-. (£) $z = 2.04$, $p = 0.04$ vs. baseline ADHD with DS-. (₴) $z = 2.24$, $p = 0.02$ vs baseline ADHD with DS+.

3.5. Quinolinic acid

Similar to the other metabolites, there were no differences in the urinary excretion of QA between the CG and the ADHD group at baseline, with only a slightly lower excretion in the CG, Table 3. MPH induced a decrease ($z = 3.11$,$p = 0.0018$) in the excretion of QA, Fig. 3, which was similar for both ADHD groups, until reaching even lower values than those observed in the CG. Nevertheless, this decrease was only shown by those ADHD patients with DS- ($z = 3.82$;$p = 0.00029$).

3.6. Nicotinamide

The baseline urinary concentrations were slightly lower in the CG than in the ADHD patients, Table 3. There were no differences in the excretion of NCTA between ADHD subgroups or after treatment with MPH. After considering the DS factor, the NCTA excretion was significantly higher in ADHD patients with DS- ($z = 2.04$,$p = 0.04$). The subgroup of ADHD patients with DS+ experienced an increase in the NCTA excretion after MPH, until matching the levels shown by the rest of ADHD patients and erasing the baseline difference. In the DS- patients the profile was the opposite, with a decreased excretion after MPH. Although the excretion rate after MPH showed a trend to balance out, higher excretion values in ADHD patients with DS- persisted, maintaining significant differences in comparison with the ADHD and DS+ subgroup ($z = 2.24$,$p = 0.02$), Fig. 3.

4. Discussion

This is the first study reporting in controls and ADHD children, serum concentrations and daily fluctuations of TRP and several kynurenine breakdown products, at baseline and (in ADHD) after treatment with MPH. Previous works (Aarsland et al., 2015; Evangelisti et al., 2017) did not explore the morning/evening fluctuations of KP metabolites in ADHD. KP was highly activated during the day and decreased at night in healthy neonates (Muñoz-Hoyos et al., 1998), while in children with seizures, there was an increase in the nocturnal production of several kynurenines that blunted the normal morning-evening variation (Muñoz-Hoyos et al., 1997). We've also measured several metabolites of KP in nocturnal urine.

The data were analized grouping by the two main ADHD subtypes. We found no significant differences when comparing the CG and the whole ADHD group. The composition of our CG, which included healthy siblings of ADHD patients, could prevent us from detecting differences in the concentrations of kynurenines if exists, because these children may share some genetic influences and also many environmental factors).

However, there some differences when analyzing the ADHD group separately and including the comorbid depressive symptoms in the factorial analysis. Overall, we found an increase in the concentration of some putative protective metabolites (i.e., kynurenic acid) and a lower

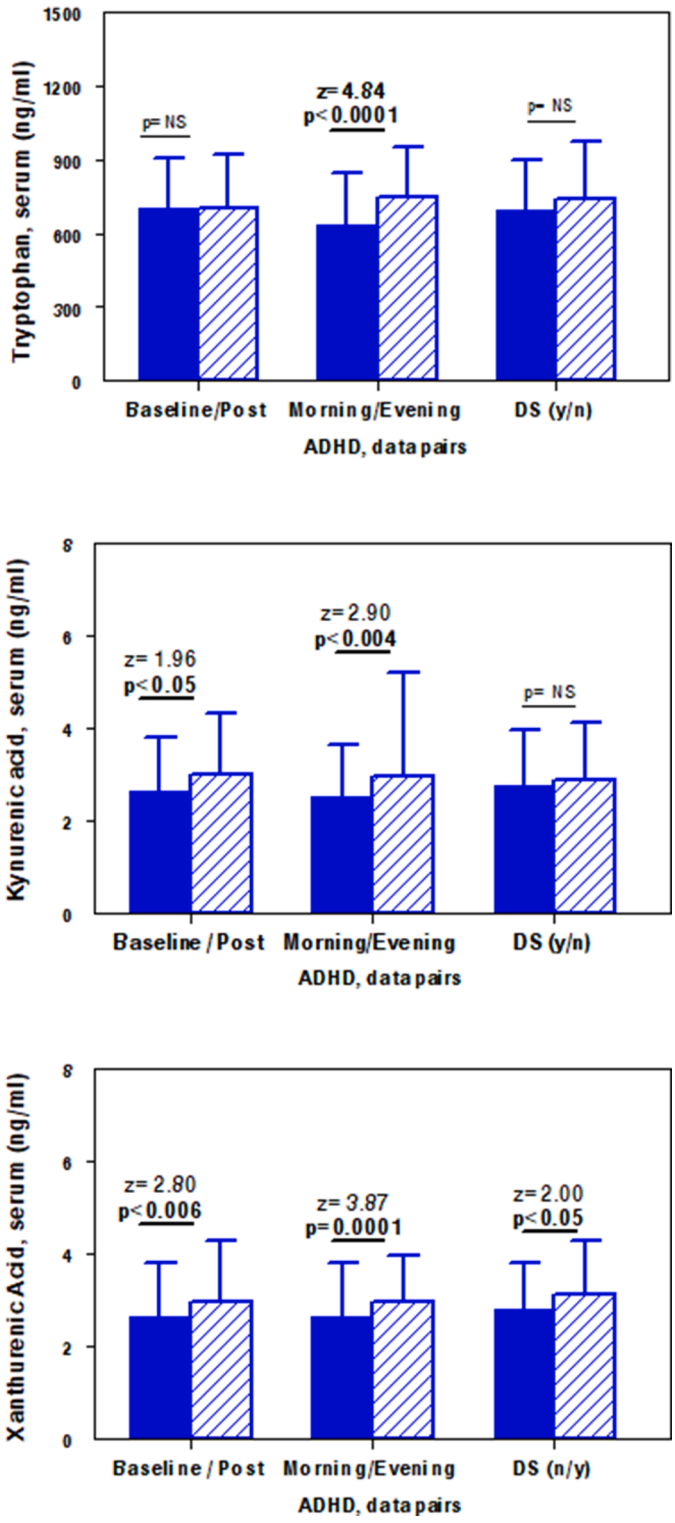

Fig. 1. Comparisons of serum concentrations of tryptophan, kynurenic acid and xanthurenic acid in the ADHD group. Data for paired values of each one of the three variables used for the factorial analysis: Time: Baseline/Post-treatment; Hour: Morning/Evening; and DS: absence (n)/presence (y) of Depressive symptoms (DS). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

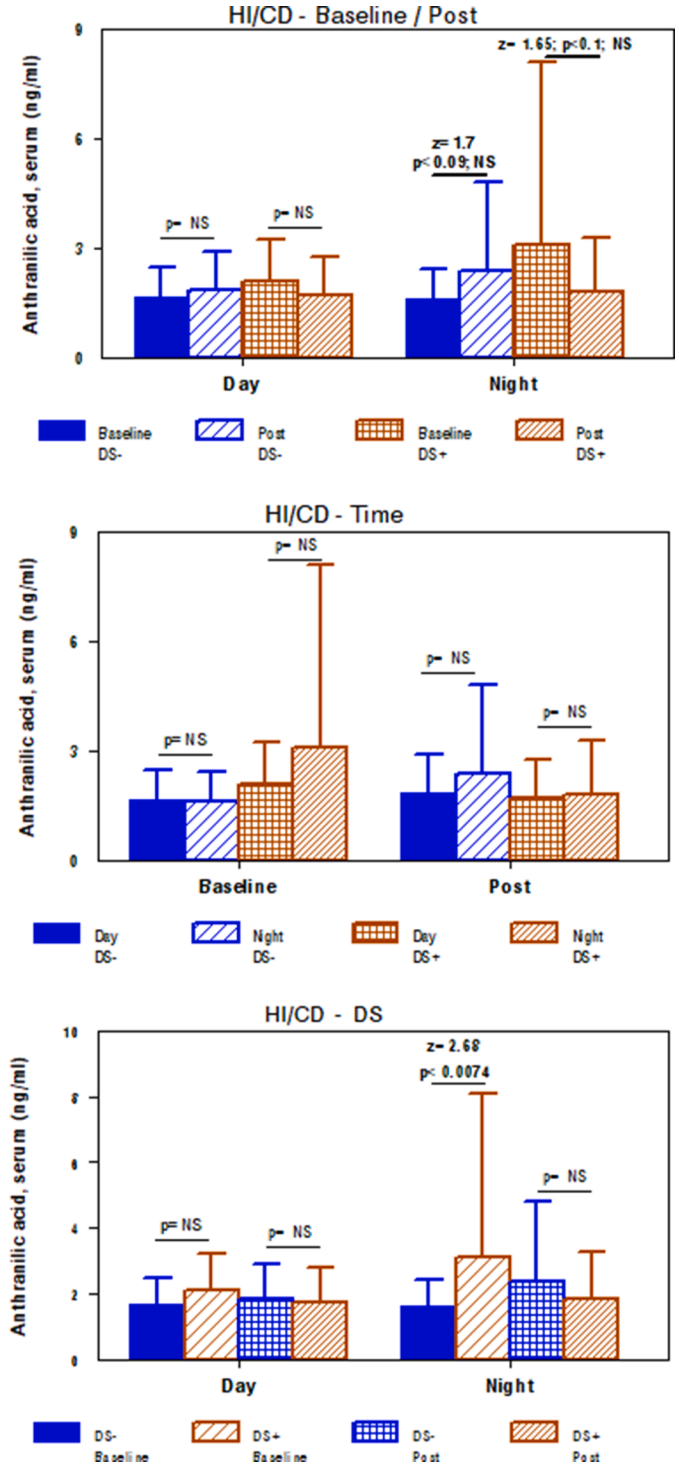

Fig. 2. Comparisons of serum anthranilic acid concentrations in the HI/CD subgroup according to the depressive symptom (DS) factor. All data paired comparisons are shown for each one of the two values of the three dichotomous variables (Time, Hour, DS) used for the factorial analysis. DS: absence (n)/presence (y) of Depressive symptoms (DS), during the morning (left figure, left) and during the evening (right figure). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

excretion of neurotoxic metabolites, possibly due to a decreased production (i.e., quinolinic acid).

During brain development environmental risk factors (eg. stress and infections) (Sagiv et al., 2013) stimulate the KP metabolism which results in an early-onset and long-lasting dysregulation of glutamate homeostasis (Notarangelo and Pocivavsek, 2017). Glutamate modulates the synaptic transmission and neuronal excitability on dopaminergic neurons in the ventral-tegmental area (VTA) (Floren et al., 2020) and in

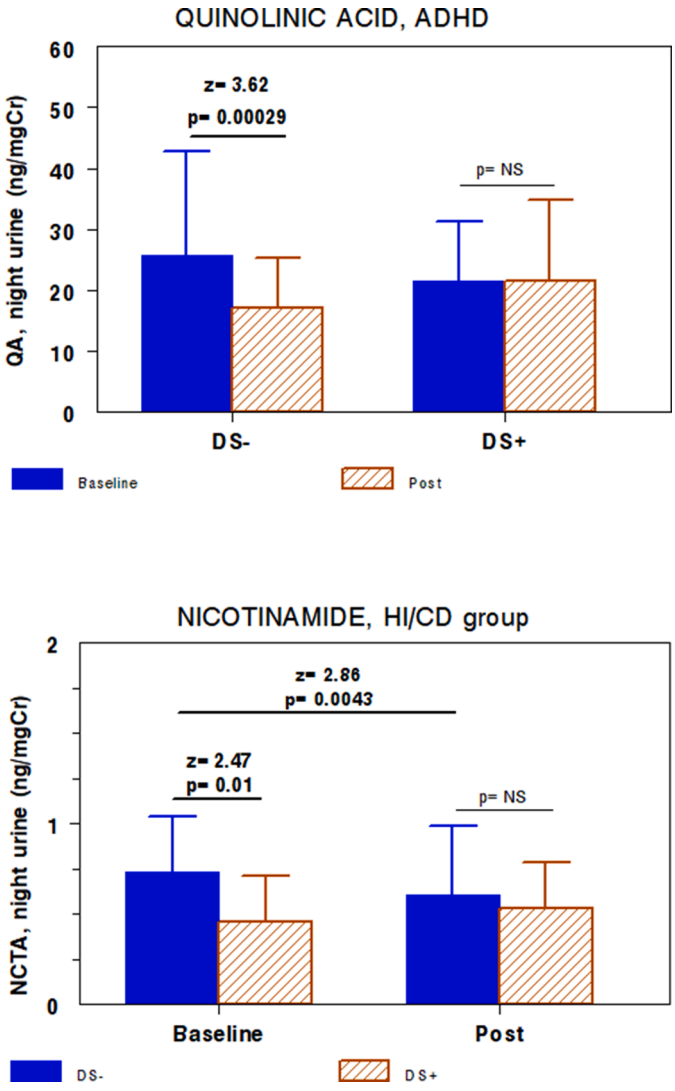

Fig. 3. Comparisons of nocturnal urinary excretion of quinolinic acid (top) and nicotinamide (bottom) in the ADHD group and in the HI/CD subgroup, respectively. Baseline=before treatment, Post=post-treatment; Depressive symptoms: absence (DS-) /presence (DS+).

prefrontal cortex (PFC), in coordination with dopamine and norepinephrine, improving attention and decreasing impulsive anger and frustration (Fuster, 2006). Modulation of HT1 receptors in VTA also may be involved (Salman et al., 2019).

In the set of subjects in the study, serum TRP levels were greater in the evening, with a trend towards lower excretion after MPH. Other studies reported a subtle increase in the TRP levels with a raised availability and a reduced breakdown in ADHD children (Oades et al., 2010b), with no differences in the concentrations of the serotonin-derived metabolite 5HIAA or kynurenines (Oades et al., 2010b). Similarly, ADHD adults showed several differences in the serum concentrations of TRP and kynurenine compared with controls (Aarsland et al., 2015),

With respect to KA levels, there were no differences in the baseline KA between our CG and the ADHD groups, similar to no differences reported between ADHD groups (Oades et al., 2010b). However, MPH induced a significant increase in KA serum levels with no alteration of its daily profile (higher evening concentrations) and no changes in its nocturnal excretion. In line with our data, other studies reported either a slightly decreased neuroprotective KA at baseline (Aarsland et al., 2015) or a tendency to increase after MPH (Oades et al., 2010a). To our knowledge, our work represents the first evidence of higher levels of KA in the evening that even increase after MPH, suggesting a neuroprotective role. Although 60% of brain kynurenine is produced systemically (Schwarcz et al., 2012) and KA hardly crosses the blood-brain barrier, changes in serum KA have been reported after treatment (Myint et al., 2011; Schwarcz and Stone, 2017).

The increase of KA after MPH is accompanied by a slight decline in the levels of brain-derived neurotrophic factor (BDNF) (Cubero-Millán et al., 2017; Pei-Chen Chang et al., 2020). These results in relation to BDNF are consistent with a previous work (Muñoz-Hoyos et al., 2011) suggesting that low basal concentrations of neuroendocrine mediators in response to chronic stress (Pei-Chen Chang et al., 2020), and inadequate responses to stimuli, may be derived from repeated biological adaptations to an increasingly stressful life. This profile may also be applicable to modifications of neurosteroids after MPH in ADHD (Molina-Carballo et al., 2014).

Postnatal administration of NMDA antagonists induces a behavior which seems similar to ADHD (Fredriksson and Archer, 2004), disrupting the attention-shifting ability (Tsukada et al., 2005). NMDAR activation plays a key role in the control of response inhibition, whereas NMDAR hypofunction may lead to increased impulsivity and perseverative responses (Bauer et al., 2016). Alterations in the dopamine and glutamate systems in ADHD relate to dysfunction in sustained and divided attention. Gene variants of NMDAR subunit GRIN2B may interact with four adversity measures [low socioeconomic status, preterm delivery, maternal smoking during pregnancy and the absence of breastfeeding] and predict children with the poorest attentional outcomes among communities with low socioeconomic status (Riva et al., 2015).

As stimulation of the extrasynaptic NMDAR mediates glutamate toxicity, the imbalance between intrasynaptic decreases and extrasynaptic increases in NMDA signaling may lead to neurotoxicity, neuronal loss and decreased regional brain volumes. NMDAR can be inhibited or activated by the KP metabolites KA and QUIN, respectively (Notarangelo and Pocivavsek, 2017). Although KA may cause cognitive dysfunction as a glutamate receptor antagonist, the same blocking action can protect the brain against the excitotoxic effects derived from an abnormally intense activation of the glutamate receptor.

Apart from KA, other KP metabolites also influence glutamatergic activity in distinct ways, (Fazio et al., 2017). XA may inhibit glutamatergic transmission by blocking L-glutamate transporters into the synaptic vesicles, therefore reducing the synaptic release (Neale et al., 2014). Small reductions in plasma XA concentrations in children (Evangelisti et al., 2017) and adults with ADHD (Aarsland et al., 2015) were reported. In our study, the XA rise after treatment could participate in the improvement of DS in children with ADHD, taking into account that serum serotonin levels did not change (Cubero-Millán et al., 2014). The evening predominance of baseline XA levels and its higher increase in the evening after treatment with MPH can support this hypothesis. The increase of serum XA levels after MPH was associated with a decrease in its nocturnal excretion in the HI/CD with DS+ subgroup, possibly due to the optimization of its metabolic route.

The third immediate downstream KP metabolite is anthranilic acid (AA), which showed marked variations associated with changes in the redox-active compound 3HAA (Dorta et al., 2017). Most inflammatory disorders, including depression, present decreased levels of 3HAA accompanied by an increase in AA. In our small subgroup of ADHD patients with DS+, there were significantly higher baseline AA concentrations in the evening. This observation reinforces the hypothesis that the activation of the KP in patients with ADHD would still be more intense in the DS+ subgroup. MPH treatment was associated with a 59% decrease of baseline AA evening concentrations only in the DS+ patients, until matching with values found in the DS- subgroup.

Differences in the nocturnal excretion of NCTA were also related to the DS factor. In the DS- patients, the reduction of its excretion after MPH could indicate a either a higher metabolic usage of NCTA or a

lower endogenous production due to reduced activation of the KP. The latter is consistent with the significant decrease in the excretion of QA as its metabolic precursor. Statistically, the reduction in the excretion of QA was more noticeable than that NCTA excretion. This might be due to the effect of MPH, which may covers the needs for $NAD^+$by enhancing its production through the rescue way, derived from dietary niacin. However, the changes in excretion of NCTA after MPH were the opposite in the subgroup of patients with ADHD and DS+. ADHD patients with DS+ showed a lower baseline excretion of NCTA that increased MPH treatment. On the other hand, the reduced excretion of QA after MPH only occurred in the ADHD with DS- subgroup, until equilibrating with the baseline excretion observed in the other subgroups. This observation reinforces the hypothesis about an activation of the KP in ADHD, which may be more intense in DS+ patients and less susceptible to being modified by MPH treatment. This activation may comes with higher levels of plasma C-reactive protein and interleukin-6 regardless of presence of CD comorbidity (Pei-Chen Chang et al., 2020). A subgroup of ADHD adults also could have abnormal cytokine levels (Corominas-Roso et al., 2017).

Apart from changes in the KP metabolites, MPH treatment it́s associated with modifications in the others ways of TRP metabolism: a) the indole TRP metabolites showed changes that, interestingly, were also related to the presence of DS (Fernández-López et al., 2020), and b) serotonin and melatonin showed subtle favourable changes in both serum concentrations and daily fluctuations (Cubero-Millán et al., 2014). In this sense, the “normalization” effect of psychostimulant medication (Oades et al., 2010b) was previously postulated in both children (Oades et al., 2010a) and adults (Aarsland et al., 2015).

Besides the characteristics of our CG, the main limitation of our study is that kynurenine and other kynurenine metabolites were not quantified due to methodological limitations.

5. Conclusion

Our data suggest that modifications of several KP metabolites, which modulate glutamatergic activity, are involved in the highly favorable clinical response to methylphenidate in children with ADHD. Since maintenance of the tryptophan-kynurenine equilibrium seems to be critical for homeostasis, the predominance of these changes in the evening will be related to the achievement of a more optimum balance. Additionally, the main factor that influences the direction and quantity of the reported KP modifications is the presence of depressive symptoms.

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Author contributions

AMC, JU and AMH designed the study and wrote the protocol. LFL, ACR, ICM, IMC, AJC, EBJ, and ASCM collected the sample, managed the literature searches and analyses. AMC and AMH undertook the statistical analysis; ICM and AMC wrote the first draft of the manuscript. All researchers contributed to and approved the final version of the manuscript.

Additional information

The authors declare that they have no conflict of interest.

Declaration of Competing Interests

All of the researchers declare not to have any conflict of interest

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