No Tryptophan, Tyrosine and Phenylalanine Abnormalities in Children with Attention-Deficit/Hyperactivity Disorder

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Abstract

Background

The aim of the current study was to explore the role of aromatic amino acids (AAAs) in blood in relation to attention-deficit/hyperactivity disorder (ADHD). Given their impact on the synthesis of serotonin and dopamine, decreased concentrations of the AAAs tryptophan, tyrosine and phenylalanine in blood may contribute to the expression of ADHD symptoms. Decreased AAA blood concentrations, in turn, may be related to lowered dietary protein intake or to abnormal AAA catabolism, as evidenced by increased urinary AAA concentrations.

Methods

Eighty-three children with ADHD (75% males) and 72 typically developing (TD) children (51% males), aged 6 to 13 years, participated in the study. AAA concentrations were assessed in blood spots and an 18-hour urinary sample. A nutritional diary was filled out by parents to calculate dietary protein intake. Parent and teacher questionnaires assessed symptoms of ADHD, oppositional defiant disorder, conduct disorder, and autism spectrum disorder.

Results

Children with ADHD showed normal AAA concentrations in blood spots and urine, as well as normal protein intake compared to controls. No associations between AAA concentrations and symptoms of ADHD or comorbid psychiatric disorders were found.

Conclusions

This study is the first to explore AAA metabolism in children with ADHD using a well-defined and relatively large sample. We found that AAA deficiencies are not related to ADHD. The results do not support treatment with AAA supplements in children with ADHD. Future
studies regarding the cause of serotonin and dopamine alterations in ADHD should focus on other explanations, such as effects of altered transport of AAAs.

**Introduction**

Attention-deficit/hyperactivity disorder (ADHD) is a childhood psychiatric disorder characterized by a persistent pattern of age-inappropriate inattention and/or hyperactivity-impulsivity [1]. Several risk factors have been proposed for the disorder, including environmental [2] and genetic [3] factors. Environmental factors include, among others, dietary abnormalities and psychosocial adversity, although odds ratios obtained for these risk factors are small or not significant [2]. Currently one of the main theories on genetic risk factors for ADHD involves aberrant dopaminergic neurotransmission [4, 5]. Dopamine receptor and transporter genes play a significant role in ADHD [6, 7], which may explain decreased dopamine levels in ADHD [8]. Other genetic studies provide evidence for an association between the serotonergic system and ADHD [9–11], in line with aberrant postsynaptic serotonin levels found in some individuals with ADHD [12]. Abnormal functioning of the dopamine and serotonin system has also been associated with neurocognitive deficits found in ADHD, such as cognitive impulsivity and poor executive attention [8]. Similarly, dopamine and serotonin abnormalities have been associated with psychiatric disorders that are highly comorbid with ADHD, including oppositional defiant disorder (ODD; [13]), conduct disorder (CD; [14]) and autism spectrum disorder (ASD; [15]).

While dopamine and serotonin hypotheses dominate current scientific work into ADHD, candidate gene study results are conflicting and effect sizes are small [3]. In addition to genetic risks of altered functioning of the neurotransmitter transporters and receptors, a potential interesting line of research focuses on the biosynthesis of dopamine and serotonin. Dopamine and serotonin are synthesized from aromatic amino acids (AAAs); the AAAs tyrosine and phenylalanine are precursors of dopamine and the AAA tryptophan is required for the synthesis of serotonin. While there are many other factors that affect the synthesis of dopamine and serotonin (including the transport of AAAs through the blood-brain barrier, and the availability of co-enzymes), normal circulating blood concentrations of AAAs are a first prerequisite for the synthesis of these neurotransmitters [16, 17]. Amino acids are a constituent of protein in foods, such as meat, bananas and milk [18, 19]. Phenylalanine and tryptophan are both essential AAAs, and therefore must be obtained by dietary means, but tyrosine can also be synthesized in the body from phenylalanine [20]. A lowered ingestion of protein or a malabsorption of AAAs may cause a decreased availability of AAAs [19]. In the current study we explore the hypothesis that decreased AAA blood concentrations contribute to ADHD symptom expression, assuming a relation between AAA blood concentrations and aberrant neurotransmission of dopamine and serotonin in ADHD.

Thus far, five case-control studies have been published on AAA blood concentrations in individuals with ADHD [21–25]. Three studies, of which two describing the same sample [21, 22], reported lower plasma concentrations of tryptophan, tyrosine and phenylalanine in ADHD [21–23]. The other two studies, however, showed increased concentrations of free tryptophan in ADHD [24] and a trend towards increased serum concentrations of tryptophan in children with ADHD [25]. All five studies are limited by non-standardized assessments of ADHD and small sample sizes (ranging from N = 12 to N = 48), and therefore further research into the availability of AAAs in ADHD is warranted. If blood concentrations of AAAs are
decreased in an ADHD sample, this may be caused by reduced protein intake, malabsorption or increased catabolism of AAAs. Although there is little evidence for dietary abnormalities in ADHD [2], thus far no studies have specifically examined protein intake in ADHD. Increased urinary concentrations of AAAs may be indicative of an abnormal catabolism [26, 27] and four studies have investigated this hypothesis in small ADHD samples [21, 22, 28, 29]. While there is no evidence of abnormal levels of urinary tyrosine and phenylalanine concentrations in ADHD [21, 22, 28], one study showed increased urinary tryptophan concentrations, suggesting abnormal AAA catabolism [29]. Taken together, the currently available studies provide some evidence for an altered AAA availability in ADHD, although more research, with greater sample sizes and standardized procedures to assess ADHD, is required to gain more insight into the potential contribution of AAAs to the expression of ADHD symptoms.

The hypothesis that AAA concentrations are related to ADHD symptoms is the basis for a number of depletion and supplementation studies. Depletion of dietary tryptophan was found to impair sustained attention in adults with ADHD [30], and to weaken behavioural inhibition in hostile children with ADHD [31]. Supplementation with tryptophan, on the other hand, resulted in a decrease of ADHD symptoms in children with ADHD [32]. Tyrosine supplementation decreased ADHD symptoms in adults with ADHD [33], but showed no effects on behavioural functioning in children with ADHD [32]. Phenylalanine supplementation in adults with ADHD caused a decrease of restlessness and an increase on the ability to concentrate at trend level [34], but in children no effects were reported for phenylalanine supplementation on ADHD symptoms [35]. However, also these depletion and supplementation studies are limited by non-standardized assessments of ADHD and small sample sizes (ranging from N = 10 to N = 20), as well as the lack of control groups, hampering conclusions regarding the relation between AAAs and ADHD. Therefore, there is a need of further research to support the hypothesis that AAA concentrations may contribute to the expression of ADHD symptoms.

Another aspect that requires further research, is the association between AAAs and symptoms of childhood psychiatric disorders that are highly comorbid with ADHD. As pointed out, dopamine and serotonin abnormalities have also been associated with ODD, CD and ASD. Indeed, tryptophan depletion have been shown to induce aggressive behaviour [36, 37], and increased tryptophan levels have been found associated with childhood ASD [38], suggesting that AAA abnormalities might contribute to the expression of symptoms of ODD, CD and ASD. Given the heterogeneous evidence of AAA abnormalities in ADHD, comorbid psychiatric conditions might act as possible confounding (mediating) or exacerbating (moderating) factors, and should therefore be taken into account when studying AAA concentrations in ADHD.

To summarize, there is inconsistent evidence that AAAs, acting as precursors of dopamine and serotonin, contribute to the expression of ADHD symptoms. The mostly outdated studies on this topic performed thus far are hampered by methodological shortcomings. Therefore, our aim was to explore concentrations of tryptophan, phenylalanine and tyrosine in a well-phenotyped sample of children with ADHD as compared to a control sample consisting of typically developing (TD) children. We firstly hypothesized that children with ADHD would show decreased blood concentrations of tryptophan, tyrosine and phenylalanine compared to controls, and that below average AAA concentrations would increase the risk of being diagnosed with ADHD. Secondly, we hypothesized that blood AAA concentrations would be related to ADHD symptoms. Thirdly, we hypothesized that abnormal blood AAA concentrations would be related to a decreased protein ingestion or by an aberrant AAA catabolism as evidenced by increased urinary AAA concentrations. Finally, we studied the possible confounding effects of symptoms of ODD, CD and ASD on our findings.
Materials and Methods

Participants

Subjects were 83 children with ADHD (75% males) and 72 TD children (51% males), aged between 6 and 13 years. Inclusion criteria for the ADHD group were: (a) a clinical diagnosis of ADHD according to DSM-IV criteria, (b) confirmation of this diagnosis by the Diagnostic Interview Schedule for Children, fourth edition, administered to parents (DISC-IV-P; [39]), (c) significant ADHD symptoms, as indicated by parent ratings >90th percentile on at least one of the ADHD scales (Inattention and Hyperactivity/Impulsivity scales) of the Disruptive Behaviour Disorder Rating Scale (DBDRS; [40]), and (d) pervasive ADHD symptoms, as indicated by teacher ratings >75th percentile on at least one of the ADHD scales of DBDRS. Having a comorbid diagnosis (for example ODD or ASD) was no exclusion criterion, neither was treatment with stimulant medication. Children on stimulant medication (N = 50, 60% of the ADHD group) discontinued drug use 24 hours before testing, in order to allow complete wash-out [41], and during participation in our study. Inclusion criteria for the TD group were: (a) absence of a clinical diagnosis of any developmental or behavioural disorder (including ADHD and ODD), and (b) scores <90th percentile on both parent- and teacher-rated ADHD scales of the DBDRS.

Materials

Behaviour. Parents of children eligible for inclusion in the ADHD group were assessed with the disruptive behaviour disorder section of the DISC-IV-P. The DISC-IV-P is a widely used standardized diagnostic interview for the assessment of DSM-IV childhood psychiatric disorders, with adequate psychometric properties [39].

Parents and teachers of children in both the ADHD and TD group completed the DBDRS to assess ADHD symptoms and symptoms of ODD and CD. The DBDRS contains four scales measuring symptoms of inattention, hyperactivity/impulsivity, ODD and CD on a 4-point Likert scale (ranging from 0 to 3), with higher scores indicating worse symptoms. Adequate psychometric properties have been reported for the DBDRS [42].

The Strengths and Weaknesses of ADHD-symptoms and Normal Behaviour rating scale (SWAN; [43, 44]) was completed by parents and teachers to assess symptoms of ADHD. This widely used questionnaire contains two subscales; the Inattention scale and the Hyperactivity/Impulsivity scale, each comprising 9 items. Items are scored on a 7-point Likert scale (ranging from -3 to +3), with higher scores indicating worse symptoms. The items are based on the DSM-IV symptoms of ADHD, but reflect both ends (strong and weak) of the behaviour described in each ADHD symptom. Mean scores on both subscales were used as dependent variables. Adequate psychometric properties have been reported for the SWAN [45].

ASD symptoms were assessed using the 65–item Social Responsiveness Scale (SRS; [46, 47]), completed by parents and teachers. The items of the SRS are based on the DSM-IV symptom domains of ASD, including impairment in social interaction, communicative deficits and restricted/stereotypic patterns of behaviours or interests. The SRS uses a 4–point Likert scale (ranging from 0 to 3), and the summed item score on the total SRS scale was used as dependent measure, with higher scores indicating worse symptoms. The SRS has adequate psychometric properties [46, 48].

Blood spots. To investigate blood concentrations of tryptophan, tyrosine and phenylalanine, a dried blood spot technique was used. Collecting blood spots is less invasive for children than taking venous blood samples and the dried blood spot technique is sufficiently robust and stable for diagnostic purposes [49–51]. Blood spot AAA concentrations are highly correlated
with serum AAA concentrations (rs ranging from .86 to .96) [52]. A blood spot of each child was collected using a disposable safety lancet. Three blood drops were spotted onto a blood stain card. A 5.5mm punch of a dried blood spot was mixed with 100μl of an internal standard solution (containing 29μM L-phenylalanine-D5, 6μM L-tyrosine-D4 and 5μM L-tryptophan-D5) and 400μl methanol in a Gas Chromatography vial (GC-vial) and shaken for 15 minutes in an ultrasonic bath. The supernatant was transferred in another GC-vial and evaporated under nitrogen at 30°C. Subsequently, the sample was butyalted with 100μl of 5.5% acetyl chloride (in n-butanol) at 60°C for 15 minutes. Afterwards, the butanol-layer was evaporated under nitrogen (at 30°C) and the residue was dissolved in 500μl acetonitrile. Blood spot concentrations of tryptophan, tyrosine and phenylalanine were determined by positive electrospray liquid chromatography–tandem mass spectrometry (LC-MS/MS), using an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA), coupled to a high-performance liquid chromatography (HPLC) system (Perkin Elmer Series 200, Shelton, USA). Three μl of the sample was injected on a symmetry C18 column (3.9*150mm, 5μm; Waters, Milford, MA, USA) and eluted with a flow rate of 1ml/min of 75% acetonitrile (containing 0.4% of formic acid). Tryptophan, tyrosine and phenylalanine eluted within 1 minute and were measured using the transitions: mass-to-charge ratio (m/z) 261.2→159.2 (tryptophan), m/z 238.2→136.2 (tyrosine) and m/z 222.2→120.2 (phenylalanine). All obtained LC-MS/MS data were acquired and processed using Analyst 1.4.2 software (Applied Biosystems, Foster City, CA, USA). The blood spot concentrations of tryptophan, tyrosine and phenylalanine were expressed in μmole/L. Reliability of the LC-MS/MS was confirmed by examining the inter-assay variance (being 5–10%), intra-assay variance (being 8–10%) and recovery (being 90–112%).

Dietary protein intake. Daily protein intake was assessed during three days, using a parent-reported nutritional diary. Standardized dietary records and instructions were provided. Parents were instructed to register all consumed foods and drinks in the dietary record and to express the consumed amounts as accurate as possible. The amount of protein intake (grams/day) was calculated based on a computerized version of the Dutch food composition database [53]. The Dutch food composition database contains over 2000 food products with information about the nutritional composition of these food products [54]. The database is widely used for scientific purposes (e.g.[55, 56, 57]).

Urine. In order to examine urinary AAA concentrations, participants collected all urine excreted within 18 consecutive hours (after-school hours) in a urine collection container. During urine collection, the container was stored in a refrigerator (≤5°C). A 10ml sample was sent to a laboratory for analysis, where the sample was stored at -20°C. An HPLC technique with fluorescence detection was used for the analysis of tryptophan in urine [58]. The concentrations of tyrosine and phenylalanine in urine were determined using a Biochrom amino acid analyzer [59]. The urinary concentrations of tryptophan, tyrosine and phenylalanine were expressed by a μmole to total urine volume ratio, to rule out effects of polyuria or oliguria. Reliability of the HPLC technique was confirmed by examining the precision of the analyses, being 2.25% for tryptophan (relative standard deviation), and 1.50% for tyrosine and phenylalanine. There are high correlations between the amino acid concentrations in 12-hour samples and 24-hour samples [60], indicating that there is no diurnal variation in the excretion of amino acids, validating the use of an 18-hour sample in the current study.

Procedure
This study received approval from the local medical ethical committee of the VU University Medical Center Amsterdam, the Netherlands (#NL39922.029.12), and has been performed in
accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained of parents of all children, and of children ≥12 years, prior to participation. Children with ADHD were recruited from mental health outpatient clinics, through the parental association for children with behavioural problems, and through a university research website. The TD group was recruited from primary schools located throughout the country. Children on stimulant medication discontinued drug use one day prior to participation (day 0), to ensure complete washout, and during the assessment of blood, urine and food intake (day 1 to day 3). On day 1 the blood spot was collected in the early morning, to rule out the effects of diurnal variation of blood AAA concentrations. The same day, after school time, urine collection started and continued for the following 18 hours, until the child would return to school the next morning (day 2). In the early morning of day 1 parents received detailed instructions on how to fill out the dietary record and how to collect urine of their child. After the instruction, parents started recording their child’s dietary intake, which continued for the following three days (day 1 to day 3). Parents and teachers were invited to fill out the questionnaires on a secured website. All data were collected between February 2013 and July 2014. The ADHD and TD group were recruited simultaneously, to control for possible seasonal effects on food intake or AAA metabolism.

Data analysis

All statistical analyses were performed using R, version 3.2.1. All variables were inspected on outliers and missing values for the ADHD and TD group separately. Winsorising was applied to outliers, these were replaced with a value one unit bigger (or smaller) than the previous most extreme score in the distribution of the group [61]. Missing data in the urinary concentrations, dietary data and behavioural data were randomly distributed and replaced using group means. For the blood spots there were no missing data. All data were normally distributed, except for CD symptoms. Group differences in gender were examined using a chi-squared test, and group differences in age and behavioural functioning were examined using independent samples t-tests.

To test the first hypothesis, group differences in AAA blood spot concentrations were assessed using Analyses of Variances (ANOVAs) with group (ADHD or TD) as fixed factor. Effect sizes were calculated in terms of partial eta squared, and interpreted as small (> .01), medium (> .06) or large (> .14) [62]. In addition, odds ratios were calculated, which expressed the risk for being diagnosed with ADHD with below average AAA concentrations. Normative data for AAA concentrations were derived from a large sample of 6 to 13 years old children (N = 104, 52% males) (unpublished data, sample information and results available from the authors). For each AAA, concentrations corresponding to the lowest 16th percentile (M-1 SD) of the normative sample were used as a threshold value to define below average AAA concentrations (for tryptophan 45 μmole/L, tyrosine 39 μmole/L, and phenylalanine 47 μmole/L). Odds ratios were calculated with their 95% confidence interval and Fisher’s Exact Test was performed to examine the significance of the odds ratios.

To test the second hypothesis, Pearson product-moment correlation coefficients investigated the relationship between blood spot AAA concentrations and both parent and teacher rated symptoms of ADHD. The magnitude of correlation coefficients was interpreted as small (> .10), medium (> .30) or large (> .50) [62]. Data of the ADHD group and TD group were combined to maximize variability in the ADHD symptom measures.

To test the third hypothesis, correlation analyses between blood spot AAA concentrations and protein ingestion and urinary AAA concentrations were performed in the whole sample. We also examined whether there were group differences in protein ingestion and urinary AAA
concentrations using ANOVAs with group (ADHD or TD) as fixed factor. Lastly, correlational analyses evaluated whether blood spot AAA concentrations were related to parent- and teacher-reported symptoms of comorbid psychiatric disorders (Pearson product-moment correlation coefficients for ODD and ASD, Spearman’s rank correlation coefficients for CD). If symptoms of ODD, CD or ASD were found related to the AAA concentrations, previous analyses were rerun with these symptoms entered as covariates. To correct for multiple testing, the alpha level of the correlation analyses was adjusted according to the Bonferroni procedure per outcome domain; ADHD symptoms (12 analyses, thus \( p = .004 \)), potential determinants of AAA abnormalities in blood spots (12 analyses, thus \( p = .004 \)), and symptoms of comorbid psychiatric disorders (18 analyses, thus \( p = .003 \)). Bonferroni adjusted results are reported.

**Results**

No groups differences were found in terms of age, but groups differed in gender as well as symptoms of ADHD, ODD, CD and ASD, see Table 1. The ADHD group had a larger proportion of males and more parent- and teacher-rated symptoms of ADHD, ODD, CD and ASD than the TD group. The DISC-IV-P indicated that in our ADHD sample, 65 children met DSM-IV criteria for the combined subtype of ADHD, 12 children met DSM-IV criteria for the predominantly inattentive subtype, and six children met DSM-IV criteria for the predominantly hyperactive-impulsive subtype.

No significant group differences were observed for blood spot concentrations of tryptophan, tyrosine, or phenylalanine, see Table 2 (all \( F_s < 4.00 \) and \( p_s > 0.05 \)). A below average (<16th percentile) blood spot concentration of phenylalanine increased the risk of being diagnosed with ADHD by 2.2, although this effect just escaped conventional levels of significance (OR = 2.22, 95%CI [92–5.73], \( p = .07 \)). A below average blood spot concentration of tryptophan (OR = 2.09, 95%CI [86–5.40], \( p = .11 \)) or tyrosine (OR = 1.83, 95%CI [74–4.79], \( p = .22 \)) did not increase the risk of being diagnosed with ADHD.

In the combined group of children with ADHD and TD children, AAA blood spot concentrations were not significantly related to ratings of ADHD symptoms (all \( r_s < .24 \) and \( p_s > .004 \)). Furthermore, blood spot concentrations of tryptophan, tyrosine or phenylalanine were not significantly related to protein intake or urinary AAA concentrations (all \( r_s < .19 \) and \( p_s > .004 \)). There were no differences between the ADHD and TD group with regard to protein intake or to urinary concentrations of tryptophan, tyrosine, or phenylalanine, see Table 2.

No significant associations between AAs and symptoms of comorbid psychiatric disorders were found (all \( r_s < .20 \) and \( p_s > .003 \)) and therefore none of the previous analyses were repeated adjusting for the effects of symptoms of ODD, CD or ASD. Since the ADHD group consisted of considerably more males than the TD group, the groups analyses were rerun with gender as a covariate. These analyses did not alter the results.

**Discussion**

The main objectives of this study were to examine whether children with ADHD had decreased AAA blood spot concentrations, and whether blood spot AAA concentrations were related to symptoms of ADHD. In contrast to our hypothesis and some earlier studies on this topic [21–23], we did not find any differences in the AAA blood spot concentrations between the ADHD and TD group or associations between AAA blood spot concentrations and symptoms of ADHD. The finding that AAA alterations are not related to ADHD, argues against nutritional interventions with amino acid supplements for children with ADHD. In past years, some studies have examined the effects of AAA supplementation in children and adults with ADHD, with inconsistent results [28, 32–34]. The apparent lack of AAA deficiencies in ADHD might
Table 1. Group characteristics of the ADHD group (N = 83) and TD group (N = 72).

|                          | ADHD group | TD group | Statistic |
|--------------------------|------------|----------|-----------|
|                          | M (SD)     | M (SD)   |           |
| Age in months            | 116.71 (19.86) | 119.17 (20.69) | t = -.75, NS |
| Males N (%)              | 62 (74.70)  | 37 (51.39) | χ² = 9.08** |
| Parent-rated symptoms    |            |          |           |
| Inattentiona             | 17.47 (4.82) | 3.31 (3.07)  | t = 22.11** |
| Hyperactivity/Impulsivitya| 16.31 (5.97) | 3.26 (2.71)  | t = 17.92** |
| Inattentionb             | 1.20 (.80)  | -.46 (.72)  | t = 13.64** |
| Hyperactivity/Impulsivityb| 1.30 (.90)  | -.39 (.90)  | t = 11.58** |
| ODDa                     | 9.72 (4.93) | 3.04 (2.71) | t = 10.63** |
| CDa                      | 2.45 (2.46) | .49 (.84)   | t = 6.83**  |
| ASDc                     | 61.77 (25.93) | 29.26 (14.23) | t = 9.84**  |
| Teacher-rated symptoms   |            |          |           |
| Inattentiona             | 14.71 (6.23) | 1.85 (2.29)  | t = 17.49** |
| Hyperactivity/Impulsivitya| 13.80 (7.39) | 1.57 (2.29)  | t = 14.30** |
| Inattentionb             | 1.04 (.85)  | -.74 (1.02) | t = 11.66** |
| Hyperactivity/Impulsivityb| 1.08 (.95)  | -.83 (1.08) | t = 11.60** |
| ODDa                     | 7.82 (6.04) | .89 (1.93)  | t = 9.89**  |
| CDa                      | 2.20 (3.03) | .18 (.66)   | t = 5.93**  |
| ASDc                     | 85.58 (21.84) | 25.07 (14.18) | t = 20.71** |

Notes.

a Disruptive Behaviour Disorder Rating Scale,
b Strengths and Weaknesses of ADHD-symptoms and Normal Behaviour rating scale,
c Social Responsiveness Scale.

**p < .01. ADHD, Attention-Deficit/Hyperactivity Disorder; ASD, Autism Spectrum Disorder; CD, Conduct Disorder; NS, not significant; ODD, Oppositional Defiant Disorder; TD, Typically Developing

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Table 2. Blood spot and urine concentrations of AAAs and protein ingestion in the ADHD and TD group.

|                          | ADHD group (N = 83) | TD group (N = 72) | Statistic |
|--------------------------|---------------------|-------------------|-----------|
|                          | M (SD)              | M (SD)            |           |
| Blood spots              |                     |                   |           |
| Tryptophan (μmole/L)     | 52.10 (10.06)       | 53.54 (9.10)      | .87, NS   | <.01 |
| Tyrosine (μmole/L)       | 50.37 (15.92)       | 55.33 (15.88)     | 3.75, NS  | .02  |
| Phenylalanine (μmole/L)  | 56.40 (14.01)       | 57.46 (11.10)     | .27, NS   | <.01 |
| Dietary intake           |                     |                   |           |
| Protein intake (g/day)   | 65.57 (16.05)       | 63.72 (13.86)     | .58, NS   | <.01 |
| Urine                    |                     |                   |           |
| Tryptophan (μmole/total urine) | 38.09 (17.23)    | 38.26 (17.02)     | <.01, NS  | <.01 |
| Tyrosine (μmole/total urine) | 73.93 (33.25)     | 66.11 (29.72)     | 2.35, NS  | .02  |
| Phenylalanine (μmole/total urine) | 48.36 (17.90)    | 46.74 (20.78)     | .27, NS   | <.01 |

Notes. AAA, Aromatic Amino Acid; ADHD, Attention-Deficit/Hyperactivity Disorder; NS, not significant; TD, Typically Developing

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explain the conflicting results of amino acid supplementation on reducing ADHD symptoms [63]. It might be that the association between AAA blood concentrations (being precursors of serotonin and dopamine) and the expression of ADHD symptoms is too indirect to detect. There are several other factors involved in the metabolism of dopamine and serotonin that could be aberrant in ADHD. It might be that an altered transport of tryptophan across the blood-brain barrier [64] or an abnormal reuptake of dopamine and serotonin [12], moderate the association between AAA concentrations in blood and ADHD symptoms. Another explanation might be that AAA concentrations should be below a certain threshold before affecting the behaviour and functioning of children. In depletion studies, tryptophan concentrations drop by 60 to 90 percent [65] and tyrosine and phenylalanine concentrations by 74 percent [66], and are therefore much lower than baseline AAA concentrations, as measured in the current study. Clearly, the studies that focused hitherto on baseline concentrations of AAAs in ADHD were scarce and the results were inconsistent. Given our larger sample size, careful screening and correction for multiple comparisons, the current results challenge the hypothesis of AAA abnormalities in ADHD. Future studies regarding the serotonin and dopamine hypothesis in ADHD [12] may focus on other aspects of the serotonin and dopamine metabolism, such as the transport of AAAs [64], rather than on decreased AAA concentrations in blood.

We did not find evidence for altered protein intake in ADHD or for an association between protein intake and blood spot AAA concentrations. Further, we did not find any evidence for an aberrant AAA excretion, since no increased urinary AAA concentrations were found in children with ADHD and urinary AAA concentrations were not significantly related to blood spot AAA concentrations, in line with the results of previous studies on urinary AAA concentrations in ADHD [21, 22, 28]. Therefore, we believe that abnormal AAA metabolism, due to a failure of the intestines to absorb AAAs into the bloodstream or increased excretion into the urine, are no plausible causes of ADHD symptoms. Lastly, we did not find a confounding role of ODD, CD or ASD symptoms in the association between AAAs and ADHD.

There are some limitations to the current study that should be noted. Firstly, we measured AAA concentrations in blood and urine, which represent only two factors in the metabolic pathway of serotonin and dopamine. Therefore, we can only draw conclusions regarding the presence of AAAs in the blood, which is a first prerequisite for an adequate biosynthesis of serotonin and dopamine. Secondly, while our study is the largest of its kind and had an adequate power to detect medium-sized effects, our study was not sufficiently powered to detect small-sized effects. We found a relatively high odds ratio of 2.2 for being diagnosed with ADHD when having low phenylalanine concentrations, but this result just escaped conventional levels of significance. Therefore, our study does not definitively rule out that low phenylalanine concentrations are present in (a subgroup of) children with ADHD. For instance, it might be that only children with severe deficiencies in executive functioning have decreased phenylalanine concentrations, as an altered dopamine functioning in the prefrontal cortex and the striatum is thought to impair executive functions, including sustained attention and interference control in ADHD [8, 67]. Despite the limitations, our study is the first to explore AAA concentrations in children with ADHD using a well-defined and relatively large sample, showing that AAA abnormalities are not related to ADHD.

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**Author Contributions**

Conceived and designed the experiments: CEB ML HJB JO. Performed the experiments: CEB. Analyzed the data: CEB. Contributed reagents/materials/analysis tools: CEB ML HJB JO. Wrote the paper: CEB ML HJB JO.

**References**

1. American Psychiatric Association. The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. Arlington: American Psychiatric Association; 2013.
2. Banerjee TD, Middleton F, Faraone SV. Environmental risk factors for attention-deficit hyperactivity disorder. Acta Paediatr. 2007; 96(9):1269–74. PMID: 17718779
3. Faraone SV, Perlis RH, Doyle AE, Smoller JW, Goralnik JJ, Holmgren MA, et al. Molecular genetics of attention-deficit/hyperactivity disorder. Biol Psychiatry. 2005; 57(11):1313–23. PMID: 15950004
4. Madras BK, Miller GM, Fischman AJ. The dopamine transporter and attention-deficit/hyperactivity disorder. Biol Psychiatry. 2005; 57(11):1397–409. PMID: 15950014
5. Swanson J, Kinsbourne M, Nigg J, Lanphear B, Stefanatos GA, Volkow N, et al. Etiologic subtypes of attention-deficit/hyperactivity disorder: brain imaging, molecular genetic and environmental factors and the dopamine hypothesis. Neuropsychol Rev. 2007; 17(1):39–59. PMID: 17318414
6. Spenser TJ, Biederman J, Faraone SV, Madras BK, Bonab AA, Dougherty DD, et al. Functional genomics of Attention-Deficit/Hyperactivity Disorder (ADHD) risk alleles on dopamine transporter binding in ADHD and healthy control subjects. Biol Psychiatry. 2013; 74(2):84–9. doi: 10.1016/j.biopsych.2012.11.010 PMID: 23273726
7. Oades RD. Dopamine–serotonin interactions in attention-deficit hyperactivity disorder (ADHD). Prog Brain Res. 2008; 172:543–65. doi: 10.1016/S0079-6123(08)00092-6 PMID: 18772050
8. Kiive E, Harro J. The effect of serotonin transporter gene promoter polymorphism on adolescent and adult ADHD symptoms and educational attainment: A longitudinal study. Eur Psychiatry. 2013; 28(6):372–8. doi: 10.1016/j.eurpsy.2012.04.004 PMID: 22986128
9. Oades RD. The Role of Serotonin in Attention-Deficit Hyperactivity Disorder (ADHD). In: C M, B J, editors. Handbook of the Behavioral Neurobiology of Serotonin. 21. Amsterdam: Elsevier; 2010. p. 565–84.
10. Krege JI, Herzing LB, Cook EH, LeBailly SA, Gouze KR, Hopkins J, et al. Gene×environment effects of serotonin transporter, dopamine receptor D4, and monoamine oxidase A genes with contextual and parenting risk factors on symptoms of oppositional defiant disorder, anxiety, and depression in a community sample of 4-year-old children. Dev Psychopathol. 2013; 25(2):555–75.
11. Van Goozen SH, Fairchild G, Sacco R, Persico AM. Blood serotonin levels in autism spectrum disorder: A systematic review and meta-analysis. Eur Neuropsychopharmacol. 2014; 24(6):919–29. doi: 10.1016/j.euroneuro.2014.02.004 PMID: 24613076
12. O’Mahony S, Clarke G, Borre Y, Dinan T, Cryan J. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav Brain Res. 2015; 277:32–48. doi: 10.1016/j.bbr.2014.07.027 PMID: 25078296
13. Felger JC, Li L, Marvar PJ, Woolwine BJ, Harrison DG, Raison CL, et al. Tryptophan metabolism during interferon-alpha administration: association with fatigue and CSF dopamine concentrations. Brain Behav Immun. 2013; 31:153–60. doi: 10.1016/j.bbi.2012.10.010 PMID: 23072272
14. Shatlock P, Whiteley P. The role of tryptophan in autism and related disorders. Nutrition. 2006: 2.
Keszthelyi D, Troost F, Masclee A. Understanding the role of tryptophan and serotonin metabolism in gastrointestinal function. Neurogastroenterology & Motility. 2009; 21(12):1239–49.

Hamer CJ, McTavish SFB, Clark L, Goodwin GM, Cowen PJ. Tyrosine depletion attenuates dopamine function in healthy volunteers. Psychopharmacology (Berl). 2001; 154(1):185–11. doi: 10.1007/s002130000613 WOS:000167350400014.

Baker GB, Bornstein RA, Rouget AC, Ashton SE, Van Muyden JC, Coutts RT. Phenylethylaminergic mechanisms in attention-deficit disorder. Biol Psychiatry. 1991; 31(1):15–22. PMID: 2001444

Bornstein RA, Baker GB, Carroll A, King G, Wong JT, Douglass AB. Plasma amino acids in attention deficit disorder. Psychiatry Res. 1990; 33(3):301–6. Epub 1990/09/01. PMID: 2243904.

Comings DE. Blood serotonin and tryptophan in Tourette syndrome. Am J Med Genet. 1990; 36(4):418–30. Epub 1990/08/01. doi: 10.1002/ajmg.1320360410 PMID: 2389798.

Hoshino Y, Ohno Y, Yamamoto T. Plasma free tryptophan concentration in children with attention deficit disorder. Folia Psychiatr Neurol Jpn. 1985; 39(4):531–6. PMID: 3833631

Oades RD, Dauvermann MR, Schimmelmann BG, Schwarz MJ, Myint AM. Attention-deficit hyperactivity disorder (ADHD) and glial integrity: S100B, cytokines and kynurenine metabolism—effects of medication. Behavioral and Brain Functions. 2010; 6:29. doi: 10.1186/1744-9081-6-29 PMID: 20509936.

Bender DA. Biochemistry of tryptophan in health and disease. Mol Aspects Med. 1983; 6(2):1–97. PMID: 6371429

Kopple JD. Phenylalanine and tyrosine metabolism in chronic kidney failure. The Journal of nutrition. 2007; 137(6):1586S–90S. PMID: 17513431

Zametkin AJ, Karoum F, Rapoport JL, Brown GL, Wyatt RJ. Phenylethylamine excretion in attention deficit disorder. J Am Acad Child Psychiatry. 1984; 23(3):310–4. PMID: 6736496

Dolina S, Margulit D, Malitsky S, Rabinov A. Attention-deficit hyperactivity disorder (ADHD) as a pyridoxine-dependent condition: Urinary diagnostic biomarkers. Med Hypotheses. 1982; 11(1):111–6. doi: 10.1016/0301-2153(82)90029-4.

Mette C, Zimmermann M, Grabemann M, Abdel-Hamid M, Uekermann J, Biskup CS, et al. The impact of acute tryptophan depletion on attentional performance in adult patients with ADHD. Acta Psychiatr Scand. 2013; 128(2):124–32. 2013-24538-005. doi: 10.1111/acps.12090 PMID: 23419004

Zepf FD, Holtmann M, Starder C, Demisch L, Schmitt M, Woeckel L, et al. Diminished serotonergic functioning in hostile children with ADHD: Tryptophan depletion increases behavioural inhibition. Pharmacopsychiatry. 2008; 41(2):60–5. doi: 10.1055/s-2007-1004593 WOS:000254356200004. PMID: 18311686

Nemzer ED, Arnold LE, Votolato NA, McConnell H. Amino acid supplementation as therapy for attention deficit disorder. J Am Acad Child Psychiatry. 1986; 25(4):509–13. PMID: 3528266

Reimherr FW, Wender P, Wood D, Ward M. An open trial of L-tyrosine in the treatment of attention deficit disorder, residual type. Am J Psychiatry. 1987; 144(8):1071–3. PMID: 3300376

Wood DR, Reimherr FW, Wender PH. Treatment of attention deficit disorder with DL-phenylalanine. Psychiatry Res. 1985; 16(1):21–6. PMID: 3903813

Zametkin AJ, Karoum F, Rapoport JL. Treatment of hyperactive children with [d]-phenylalanine. The American Journal of Psychiatry. 1987; 144(6):792–4. 1987-32260-001. PMID: 3296793

Zimmermann M, Grabemann M, Mette C, Abdel-Hamid M, Uckermann J, Kraemer M, et al. The effects of acute tryptophan depletion on reactive aggression in adults with attention-deficit/hyperactivity disorder (ADHD) and healthy controls. 2012.

Stadler C, Zepf F, Demisch L, Schmitt M, Landgraf M, Poustka F. Influence of rapid tryptophan depletion on laboratory-provoked aggression in children with ADHD. Neuropsychobiology. 2007; 56(2–3):104–10. doi: 10.1159/000112951 PMID: 18182830

Hoshino Y, Yamamoto T, Kaneko M, Tachibana R, Watanabe M, Ono Y, et al. Blood serotonin and free tryptophan concentration in autistic children. Neuropsychobiology. 1984; 11(1):22–7. PMID: 6204248

Shaffer D, Fisher P, Lucas CP, Dulcan MK, Schwab-Stone ME. NIMH Diagnostic Interview Schedule for Children Version IV (NIMH DISC-IV): description, differences from previous versions, and reliability of some common diagnoses. J Am Acad Child Adolesc Psychiatry. 2000; 39(1):28–38. PMID: 10638065

Pelham WE, Gnagy EM, Greenslade KE, Milich R. Teacher Ratings of DSM-III-R Symptoms for the Disruptive Behavior Disorders. J Am Acad Child Adolesc Psychiatry. 1992; 31(2):210–8. PMID: 1564021

Pelham WE, Aronoff HR, Midlam JK, Shapiro CJ, Gnagy EM, Chronis AM, et al. A comparison of Ritalin and Adderall: efficacy and time-course in children with attention-deficit/hyperactivity disorder. Pediatrics. 1999; 103(4):e43. PMID: 10103335
42. Oosterlaan J, Baeyens D, Scheres A, Antrop I, Roeyers H, Sergeant J. VvGK6-16: Vragenlijst voor Gedragsproblemen bij Kinderen 6 tot en met 16 jaar. Amsterdam: Harcourt Publishers; 2008.

43. Hay DA, Bennett KS, Levy F, Sergeant J, Swanson J. A twin study of attention-deficit/hyperactivity disorder dimensions rated by the strengths and weaknesses of ADHD-symptoms and normal-behavior (SWAN) scale. Biol Psychiatry. 2007; 61(5):700–5. PMID: 16962074

44. Swanson J, Schuck S, Mann M, Carlson C, Hartman K, Sergeant J, et al. Categorical and Dimensional Definitions and Evaluations of Symptoms of ADHD: The SNAP and the SWAN Ratings Scales 2005 [cited 2012 March 15th]. Available from: http://www.adhd.net/SNAP_SWAN.pdf.

45. Lakes KD, Swanson JM, Riggs M. The Reliability and Validity of the English and Spanish Strengths and Weaknesses of ADHD and Normal Behavior Rating Scales in a Preschool Sample Continuum Measures of Hyperactivity and Inattention. Journal of attention disorders. 2012; 61(5):700–6. doi: 10.1177/1087054711413550 PMID: 21807955

46. Constantino J, Davis SA, Todd RD, Schindler MK, Gross MM, Brophy SL, et al. Validation of a brief quantitative measure of autistic traits: comparison of the social responsiveness scale with the autism diagnostic interview-revised. J Autism Dev Disord. 2003; 33(4):427–33. PMID: 1299421

47. Constantino J, Gruber C. Social Responsiveness Scale (SRS) Los Angeles, CA: Western Psychological Services. 2005.

48. Bölte S, Poustka F, Constantino JN. Assessing autistic traits: cross-cultural validation of the social responsiveness scale (SRS). Autism Research. 2008; 1(6):354–63. doi: 10.1002/aur.49 PMID: 19360690

49. Rashed MS, Bucknall MP, Little D, Awad A, Jacob M, Alamoudi M, et al. Screening blood spots for inborn errors of metabolism by electrospray tandem mass spectrometry with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles. Clin Chem. 1997; 43(7):1129–41. PMID: 9216448

50. Chace DH, Sherwin JE, Hillman SL, Lorey F, Cunningham GC. Use of phenylalanine-to-tyrosine ratio determined by tandem mass spectrometry to improve newborn screening for phenylketonuria of early discharge specimens collected in the first 24 hours. Clin Chem. 1998; 44(12):2405–9. PMID: 9836704

51. Kandár R, Zákóva P. Determination of phenylalanine and tyrosine in plasma and dried blood samples using HPLC with fluorescence detection. Journal of Chromatography B. 2009; 877(30):3926–9.

52. Peccè R, Scolamiero E, Ingenito L, Parenti G, Ruoppolo M. Optimization of an HPLC method for phenylalanine and tyrosine quantization in dried blood spot. Clin Biochem. 2013; 46(18):1892–5. doi: 10.1016/j.clinbiochem.2013.08.022 PMID: 24028903

53. National Institute for Public Health and the Environment. Nederlands Voedingsstoffenbestand. NEVO-online versie 2013/4.0. 2013.

54. Westenbrink S, Jansen-van der Vliet M. NEVO-online 2013: achtergrondinformatie. 2013.

55. Altorf-van der Kuiw W, Engberink MF, De Neve M, van Rooij FJ, Hofman A, van’t Veer P, et al. Dietary amino acids and the risk of hypertension in a Dutch older population: the Rotterdam Study. The American journal of clinical nutrition. 2013; 97(2):403–10. doi: 10.3945/ajcn.112.053737 PMID: 23283504

56. Van der Zwaluw NL, Van de Rest O, Tieland M, Adam JJ, Hiddink GJ, Van Loon LJ, et al. The impact of protein supplementation on cognitive performance in frail elderly. Eur J Nutr. 2014; 53(3):803–12. doi: 10.1007/s00394-013-0584-9 PMID: 24045855

57. Van Kernebeek H, Oosting S, Feskens E, Gerber P, De Boer I. The effect of nutritional quality on comparison environmental impacts of human diets. Journal of Cleaner Production. 2014; 73:88–99.

58. Kema IP, Schellings AM, Hoppenbrouwers CJ, Rutgers HM, de Vries EG, Muskiet FA. High performance liquid chromatographic profiling of tryptophan and related indoles in body fluids and tissues of carcinoid patients. Clin Chim Acta. 1993; 221(1):143–58.

59. Fekkes D, Voskuilen-Kooyman A, Jankie R, Huijmans J. Precise analysis of primary amino acids in human urine by an automated high-performance liquid chromatography method: comparison with ion-exchange chromatography. Journal of Chromatography B: Biomedical Sciences and Applications. 2000; 744(1):183–8. PMID: 10985580

60. Khalaf A-N, Böcker J, Kerp L, Petersen K-G. Urine screening in outdoor volunteers: day versus night versus 24 hour collection. Clin Chem Lab Med. 1991; 29(3):185–8.

61. Tabachnick BG, Fidell LS. Using multivariate statistics. 4th ed. Boston: Allyn and Bacon; 2001.

62. Cohen J. Statistical power analysis for the behavioral sciences. 2nd ed. New York: Academic Press; 1988.

63. Hurt EA, Arnold LE, Lothhouse N. Dietary and nutritional treatments for attention-deficit/hyperactivity disorder: current research support and recommendations for practitioners. Current psychiatry reports. 2011; 13(5):323–32. doi: 10.1007/s11920-011-0217-z PMID: 21779024
64. Johansson J, Landgren M, Fernell E, Vumma R, Ahlin A, Bjerkenstedt L, et al. Altered tryptophan and alanine transport in fibroblasts from boys with attention-deficit/hyperactivity disorder (ADHD): an in vitro study. Behavioral and Brain Functions. 2011; 24(7):40.

65. Booij L, Van der Does A, Riedel W. Monoamine depletion in psychiatric and healthy populations: review. Mol Psychiatry. 2003; 8(12):951–73. PMID: 14647394

66. Montgomery AJ, McTavish SF, Cowen PJ, Grasby PM. Reduction of brain dopamine concentration with dietary tyrosine plus phenylalanine depletion: an [11C] raclopride PET study. Am J Psychiatry. 2014.

67. del Campo N, Chamberlain SR, Sahakian BJ, Robbins TW. The roles of dopamine and noradrenaline in the pathophysiology and treatment of attention-deficit/hyperactivity disorder. Biol Psychiatry. 2011; 69(12):e145–e57. doi: 10.1016/j.biopsych.2011.02.036 PMID: 21550021