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Paediatric reference values for total homocysteine, tryptophan, tyrosine and phenylalanine in blood spots

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ABSTRACT

Determining blood concentrations of the amino acids homocysteine, tryptophan, tyrosine and phenylalanine in children is of value in the clinical practice. Over the past decades, the use of blood spot samples to examine amino acid concentrations is increasing rapidly. In children, the use of blood spot samples is especially of relevance, as this method is much less invasive than venous blood sampling. Currently, no paediatric reference values for amino acids in blood spots are available. The aim of the current study was to establish reference values for blood spot concentrations of total homocysteine, tryptophan, tyrosine and phenylalanine in school-age children. Dried blood spots were obtained in a community sample of 104 healthy children, aged 6–12 years old (52% males). Blood spot concentrations of total homocysteine, tryptophan, tyrosine and phenylalanine were determined by positive electrospray liquid chromatography-tandem mass spectrometry. Parents of participants completed questions regarding demographic characteristics. Our sample consisted of healthy children from various ethnic backgrounds, with varying levels of socioeconomic status, in line with the composition of the Dutch society. Blood spot concentrations of total homocysteine, tryptophan, tyrosine and phenylalanine were similar in males and females, and independent of age. In conclusion, paediatric reference values for blood spot concentrations of total homocysteine, tryptophan, tyrosine and phenylalanine were established, which could be of use in the clinical practice.

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Introduction

Analysis of amino acid concentrations in blood of children is often used in clinical practice; for various diagnostic purposes and to make decisions regarding treatment [1]. Adequate reference values are required in clinical decision-making. Abnormal amino acid concentrations may reflect an imbalanced diet or infections [2]. For instance, high levels of homocysteine may reflect a vitamin B12 or folate deficiency [3]. Further, aberrant concentrations of amino acids can be indicative of metabolic disorders in children, such as decreased concentrations of tryptophan in plasma being indicative of Hartnup disease [4], and increased concentrations of phenylalanine and decreased concentrations of tyrosine in plasma being biomarkers for the various forms of hyperphenylalaninemia [1]. One way to examine amino acid concentrations, is by analyzing blood samples, obtained by venipuncture [5]. However, in children this method is sub-optimal, given that this invasive procedure can invoke pain, anxiety and distress. Over the past decades, the use of blood spot samples to examine amino acid concentrations is increasing [6,7]. Collecting blood spots, by means of a finger prick, is less invasive for children than venous blood

sampling, and the dried blood spot technique is sufficiently robust and stable for diagnostic purposes [8–10]. In addition, blood spots can be assessed at home, as they can be stored at room temperature and be sent by regular mail. Blood spot amino acid concentrations correlate well with amino acids concentrations obtained in venous blood (*r*s ranging from 0.86–0.96) [11,12]. However, there is some bias in blood spot concentrations and its causes are not completely clear. Factors like extraction efficiency, dilution, differences in amino acid concentrations between the plasma and red blood cells will likely contribute. It is therefore important to create reference values specifically for blood spot concentrations. There are some reference values published on blood spot amino acid concentrations in infants, required in newborn screening [6,8]. However, to our current knowledge, there are no normative values for blood spot concentrations of amino acids in school-age children available.

A challenge in providing reference values for amino acids in school-age children is to establish normative data obtained from a representative sample of healthy children. Papers describing reference values often lack information

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regarding recruitment methods and demographic characteristics of the samples, such as ethnicity and socioeconomic status (SES). Due to the ethical challenge of acquiring samples from healthy children from the community, many paediatric reference values are obtained from hospitalized children [5,13]. Another challenge in establishing reference values is to take into account the potential effects of age [5,14–16] and gender [17] on individual differences in amino acid metabolism.

The current study was aimed at establishing reference values for blood spot amino acid concentrations in healthy school-age children. In addition, we examined whether gender and age affected amino acid concentrations.

Materials and methods

Participants

Subjects were a community sample of healthy school-age children ($N=104$; 52% males). The mean age of the sample was 9 years and 9 months (range: 6–12 years). Parents of participants were asked to complete questions regarding age, gender, ethnicity and medical condition of the child, as well as questions regarding family status, education and occupation of the parents. Exclusion criteria were (a) a history of chronic metabolic, renal, hepatic and cardiac diseases, (b) prescribed medication use that could interfere with amino acid metabolism, and (c) acute illness, as reported by parents. Children were recruited from primary schools located throughout the Netherlands. Recruitment throughout the country resulted in a heterogeneous sample, consisting of children living in different geographic environments (rural and urban areas). The level of SES was determined based on the highest level of education and the current occupation of parents on 6-point scales [18,19]. SES was calculated as the average of the education and occupation scores of both parents in the case of two carers and as the average of the education and occupation scores of one parent in the case of a single carer [20]. Part of this sample ($n=72$) were used in another study by our research group [21]. For that study only typically developing children were selected. The children described in the current study with a clinical diagnosis of any developmental or behavioural disorder were excluded from the other study.

Amino acid concentrations

To investigate blood concentrations of total homocysteine, tryptophan, tyrosine and phenylalanine, a dried blood spot technique was used. A blood spot of each child was collected using a disposable safety lancet. Blood sampling was performed by trained researchers. Three blood drops were spotted onto a blood stain card and analyzed by the clinical chemistry laboratory of our academic medical center within 1 month after blood sampling. Disks with a diameter of 5.5 mm were punched out of the blood spots, each containing about 8.1 μL blood. Concentrations of total homocysteine in blood spots (in $\mu\text{mol/L}$) were determined using liquid chromatography-mass spectrometry (LC-MS/MS),

based on the protocol of Gempel, Gerbitz [22]. Reliability of the LC-MS/MS was confirmed by examining the inter-assay variance (being 11%), intra-assay variance (being 10%) and recovery (being 105.5%). While blood spot concentrations of tryptophan, tyrosine and phenylalanine were expressed in whole units, blood spot concentrations of total homocysteine were expressed in one decimal, since the LC-MS technique allowed for a more precise measure. See reference [21] for a more detailed description of the analysis of tryptophan, tyrosine and phenylalanine concentrations in blood spots (in $\mu\text{mol/L}$).

Procedure

This study was part of a research project on the relation between amino acids (total homocysteine, tryptophan, tyrosine and phenylalanine) and ADHD, and received approval from the local medical ethical committee (#NL39922.029.12). The study was 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 12-year-old children, prior to participation. Blood spots were collected in the early morning of the test day, to rule out possible effects of diurnal variation of amino acid blood spot concentrations. Blood spots were collected <2 h after breakfast.

Data analysis

All statistical analyses were performed using R, version 3.2.1. Outliers were replaced using winsorizing [23]. For nine children, blood spot total homocysteine concentrations were missing due to insufficient blood spots collected in some participants. These data were not replaced. In none of the other variables there were missing values.

Firstly, it was investigated whether there were interactions between gender and age on amino acid concentrations, using multiple linear regression analyses. In the case of non-significant interactions, the main effects of gender and age on amino acid concentrations were examined, using analysis of variance or by calculating Pearson product-moment correlation coefficients, respectively. When appropriate, reference values were partitioned.

Secondly, reference values were established for the total group, using percentile scores (5th, 50th and 95th). The 5–95 percentile interval represents the central 90% of the sample distribution, which is common in a community sample [24]. Further, the 10th and 90th percentiles were presented, which are more stable measures in a sample of circa 100 participants [5]. In case of significant effects of gender or age, stratified reference values were defined.

Results

Inspection of our sample revealed that the children represented diverse cultural backgrounds; of our sample 81 children (78%) had a Dutch background, eight children (8%) had a non-Dutch Western background and 15 children

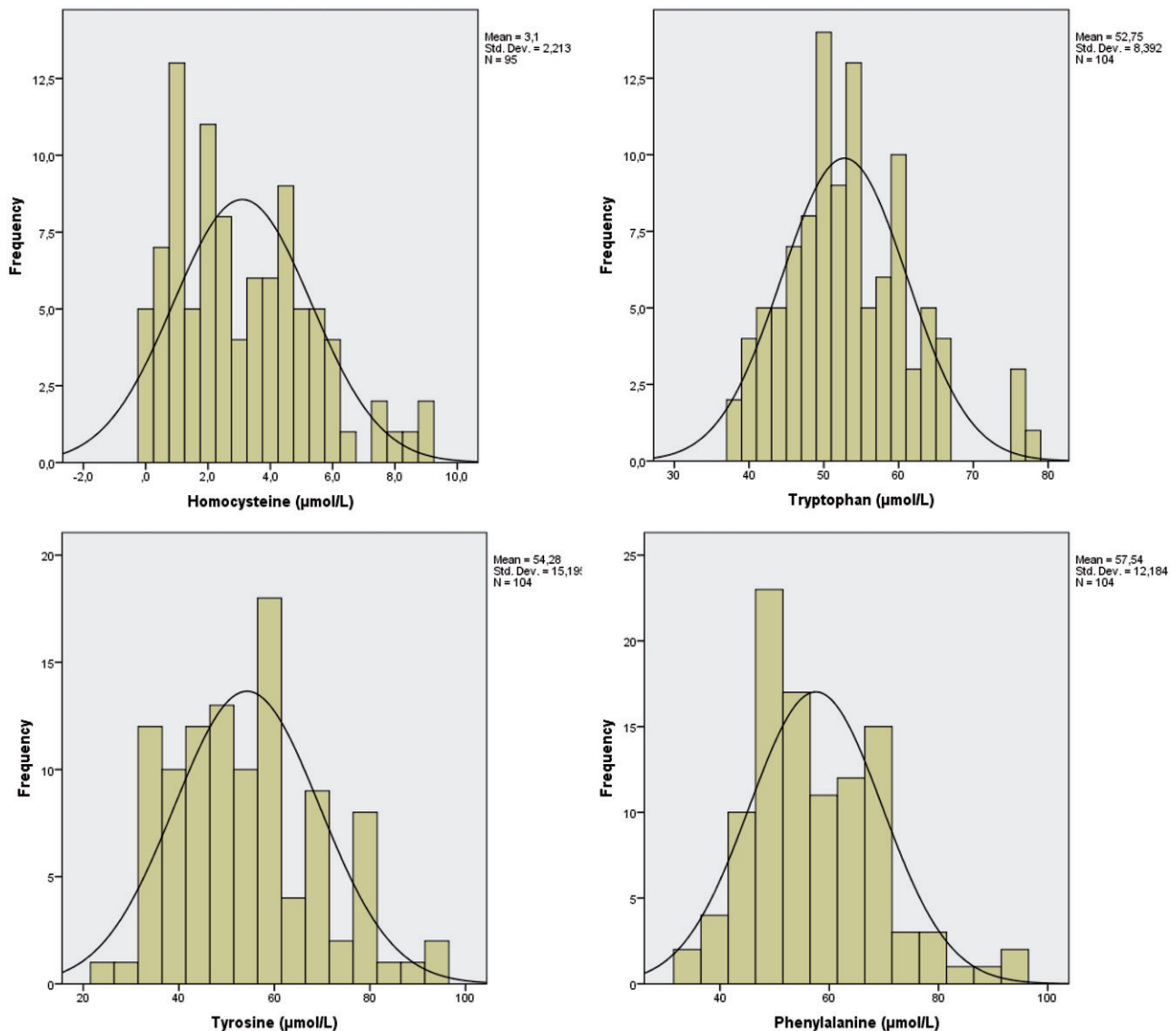


Figure 1. Histograms of blood spot amino acid concentrations in a sample of 104 healthy children.

Table 1. Reference values for blood spot amino acid concentrations, based on a sample of 104 healthy children.

	5th percentile	10th percentile	50th percentile	90th percentile	95th percentile
Total homocysteine (µmol/L) ^a	0.3 (CI -0.5–1.1)	0.5 (CI -0.3–1.3)	2.5 (CI 1.7–3.3)	6.0 (CI 5.2–6.8)	7.5 (CI 6.7–8.3)
Tryptophan (µmol/L)	40 (CI 36.4–43.6)	42 (CI 38.4–45.6)	52 (CI 48.4–55.6)	63 (CI 59.4–66.6)	65 (CI 61.4–68.6)
Tyrosine (µmol/L)	34 (CI 28.3–39.7)	34 (CI 28.3–39.7)	52 (CI 46.3–57.7)	77 (CI 71.3–82.7)	81 (CI 75.3–86.7)
Phenylalanine (µmol/L)	41 (CI 35.6–46.4)	44 (CI 38.6–49.4)	55 (CI 49.6–60.4)	71 (CI 65.6–76.4)	80 (CI 74.6–85.4)

^aBased on blood spot concentrations of a smaller sample ($N=95$). CI, 95% confidence interval.

(14%) had a non-Western background. The children in our sample had varying levels of SES; of our sample 38 children (37%) were classified as having low SES, 41 children (39%) as medium SES and 25 children (24%) as high SES.

Inspection of histograms (see Figure 1) showed that all four amino acid concentrations were normally distributed. Regression analyses showed no interaction effect between gender and age for any of the amino acid concentrations (all β -values <0.10 , all p -values >0.55). Also, we did not find main effects of gender (all F -values <1.00 all p -values >0.30) and age (all r -values <0.20 all p -values >0.05) on blood spot amino acid concentrations. Therefore, reference values for blood spot concentrations of total homocysteine,

tryptophan, tyrosine and phenylalanine were presented for the whole group of school-age children, see Table 1.

Discussion

The main purpose of the current study was to establish paediatric reference values for blood spot concentrations of total homocysteine, tryptophan, tyrosine and phenylalanine. A key finding of our study is that we did not find an effect of age on amino acid blood spot concentrations, which was contrary to our expectations based on previous studies [5,16]. The absence of age differences might be explained by

the age range studied in the present study, which was limited to 6–12-year-olds. Age differences might emerge during more critical developmental stages, such as infancy and puberty [5,16]. We furthermore did not find any gender effects on blood spot amino acid concentrations, and therefore our results do not support the hypothesis of differences in amino acid concentrations between males and females in this age range. As gender effects may reflect variations in amino acid concentrations during different stages of the menstrual cycle in females, our study does not rule out the need for gender-partitioned reference values in samples including children in puberty.

In the current study, we obtained reference values for amino acid concentrations in blood spots that differ from reference values in blood samples, obtained by venipuncture [5,24]. There does not seem to be a clear trend line formula, linking concentrations in blood spots to those obtained in plasma. While some of our reference values in blood spots are lower than those obtained in plasma [5,24], other reference values in blood spots are higher than the reference values of plasma levels [5,24]. This underlines the necessity of using normative data of blood spots, instead of calculating reference values based on normative data of plasma levels.

There are some limitations to the current study that should be noted. While our study provides reference values for school-age children (a group of children that is often seen in clinical practice), in future studies the age range of our study (6–12 years) should be expanded, to provide reference values for children under 6 years old and for adolescents. An age range of 0–18 years old would allow for examining gender and age effects on amino acids concentrations more thoroughly.

The current study also has several strengths. Normative data were obtained in a community sample of healthy children, who were recruited through schools located in diverse geographical areas. This recruitment method decreased the possibility of a biased sample, that might be present in studies selecting hospitalized children [5,13]. Further, our sample consisted of children from various ethnic backgrounds, with proportions of immigrants in line with the composition of the Dutch society, as there are 10% Western and 12% non-Western immigrants in the Netherlands [25]. An ethnically representative sample can be of importance when establishing reference values, as it has been suggested that there are differences in amino acid metabolism between ethnic groups, due to genetic or nutritional factors [16]. It would be interesting to examine whether amino acid concentrations vary between ethnic groups of children, but our study was insufficiently powered to compare the amino acid concentrations between different ethnic groups of children. The obtained reference values allow clinicians to use blood spots more often, as alternative to venipuncture for the analysis of amino acid concentrations in school-age children.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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