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RESEARCH ARTICLE

RETROSPECTIVE EVALUATION OF DIET COMPLIANCE ON PLASMA AMINO ACID AND VITAMIN LEVELS IN PATIENTS WITH PHENYLKETONURIA

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ABSTRACT

Background: In this study, it was aimed to compare the plasma amino acid and blood vitamin/mineral levels in patients with classical phenylketonuria and healthy controls.
Methods: 54 patients with classical phenylketonuria and 22 healthy controls (76 children, 47 boys, 61.8%) were included in the study. The patient group was divided into two subgroups as high adherence to phenylalanine-restricted diet (HAD, 16 patients) and low adherence to this diet (LAD, 38 patients) according to the mean plasma phenylalanine level of the patients of the previous year. Anthropometric measurements (body weight and height and standard deviation score values), plasma phenylalanine and other amino acid levels, hemoglobin, vitamin B12, folic acid, vitamin D, zinc, ferritin levels of all groups were recorded.
Results: The mean age of the entire study group was 10.1 ± 3.6 (minimum: 3.5 - maximum: 17) years. There was no significant difference between the phenylketonuria group and the control group in terms of age, gender distribution and anthropometric data. There was a significant difference between the three groups in terms of plasma phenylalanine levels (plasma phenylalanine levels 299.0 ± 77.2; 813.7 ± 356.6 and 47.5 ± 15.9 µmol/L in HAD, LAD and control groups respectively, p= 0.001). Tryptophan was significantly lower in the HAD group than in the LAD and control groups (p= 0.001 and p= 0.006, respectively). Lysine was found to be significantly higher and histidine was lower in the HAD group than the control group (p= 0.016 and p= 0.008, respectively). Hemoglobin, vitamin B12, folic acid and 25-OH vitamin D levels were found to be significantly higher in the PKU patient group compared to healthy children and no difference between zinc and ferritin levels.
Conclusion: As a result, in patients with phenylketonuria who comply with the diet and whose anthropometric data are in the normal range, no significant deterioration in vitamin/mineral and amino acid values is observed. Compliance of the patients with a diet restricted from phenylalanine will both reduce the neurological effects and ensure that the patient is nutritionally balanced.

Keywords: Classical phenylketonuria, phenylalanine-restricted diet, hemoglobin, plasma amino acids, vitamins

INTRODUCTION

Phenylketonuria (PKU, OMIM # 261600) is an autosomal recessive inherited metabolic disease that occurs as a result of deficiency of the phenylalanine hydroxylase (PAH) enzyme that catalyzes the conversion of phenylalanine (Phe) to tyrosine,

cofactor metabolism disorder or activator protein DNAJC12 metabolism disorder [1]. Untreated PKU is characterized by irreversible intellectual disability, microcephaly, motor disorders, eczematous rash, autism, seizures, developmental and psychiatric problems [3].

The goal of treatment of hyperphenylalaninemia is to reduce the blood Phe level to acceptable limits in order to prevent central nervous system damage [4]. All infants with plasma Phe levels above 360 µmol/L should be treated with diet or other options and treatment should be started in the first week after birth [2]. This is achieved by severely reducing protein-containing foods, providing a protein substitute that includes amino acids other than Phe as well as needed macro and micronutrients, and frequent monitoring of blood Phe levels [4]. The low-Phe diets prescribed for patients with classic PKU are limited to food groups, including fruits, most vegetables, sugars, pure fats, and medically modified, low-protein food products [5]. Due to the limited intake of natural protein, micronutrients need to be supplemented in protein substitutes to prevent nutritional deficiencies. Deficiencies in selenium, zinc, and iron have been reported in patients with PKU [6]. Many complications can occur if a Phe-restricted diet is not well managed. During dietary therapy, if the patient does not get enough Phe, an essential amino acid, the blood Phe level drops and neurological symptoms may occur. Problems such as growth retardation, protein-energy malnutrition, skin lesions, anemia, osteopenia or osteoporosis, diarrhea, hair loss may occur when the diet content is not sufficiently balanced, the patient does not follow the diet or does not consume the recommended amino acid formulas regularly. There is also a risk of death in severe cases. Deficiency of nutrients such as selenium, zinc, iron, retinol, long-chain polyunsaturated fatty acids and omega-3 can sometimes be seen in patients following a strict diet [7]. Vitamin B12 deficiency may also occur in patients, which may contribute to the worsening of the neurological picture [8]. This study aimed to investigate the relationship between diet compliance and plasma amino acid and blood vitamin/mineral levels in patients with classical phenylketonuria who were receiving diet therapy.

MATERIALS AND METHODS

Study Design

This study is a cross-sectional descriptive study planned to investigate the relationship between the adherence to Phe-restricted diet and plasma amino acid and blood vitamin/mineral levels in patients with PKU, who are being followed up at Dokuz Eylul University Faculty of Medicine, Division of Pediatric Metabolism and Nutrition. Fifty-four patients aged between 3.5 and 17 years who were being followed up and treated with the diagnosis of classical PKU were included in the study. All patients were referred to our hospital with high Phe levels in the national newborn screening program.

Having a chronic disease that requires other treatment in addition to PKU, having taken vitamin/mineral therapy in the last three months due to micronutrient deficiency, having a disease that will cause malabsorption (lactose intolerance, giardiasis, cystic fibrosis, celiac disease), growth hormone deficiency or hypothyroidism, had severe mental retardation, had uncontrolled epilepsy, and did not regularly come to their outpatient control visits not included in the study. In addition, 3 patients with BH4 metabolism disorder, 50 patients with hyperphenylalaninemia who were followed without diet, 20 patients on large neutral amino acid therapy, and 90 patients with PKU who were receiving sapropterin dihydrochloride in addition to Phe-restricted diet were excluded from the study. Patients who did not give consent to participate in the study were also excluded from the study.

The PKU patient group (54 patients) was divided into two subgroups according to the mean plasma Phe level of the patients in the last year. Those with a mean Phe level between 120 and 360 µmol/L in the last year were considered high adherence to diet (HAD, 16 patients), and those over 360 µmol/L were considered low adherence to diet (LAD, 38 patients). Twenty-two healthy children between the ages of 3.5 and 17 who applied to our clinic for family screening and did not receive any treatment were included in the study as the control group. Demographics (age, gender), anthropometric measurements (body weight and height and standard deviation score values), plasma Phe level and amino acid levels, hemoglobin, vitamin B12, folic acid, vitamin D, zinc, ferritin levels of all groups were measured.

Laboratory Analyses

All measurements were made on a blood sample taken in the morning after an overnight fast. Plasma amino acid levels were determined by HPLC, serum vitamin B12, folic acid and ferritin levels were determined by immunoassay method (Unicel DxI800,

Beckman Coulter, CA. USA), serum zinc levels and hemoglobin levels were determined by spectrophotometry (AU5800 analyzer, Beckman Coulter, CA. USA), 25-OH vitamin D level was analyzed by immunoassay method (Advia Centaur XP Analyzer, Siemens Healthcare Diagnostics, Erlangen, Germany). Serum limit values of micronutrients were determined as follows: B12 vitamin: 200-1900 pg/mL, folic acid: 3-17.5 ng/mL, ferritin: 14-325 ng/mL, 25-OH vitamin D: 20-42 ng/mL, zinc: 65-140 µg/dL (9,10,11).

Ethical Approval

The study protocol was designed in compliance with the Declaration of Helsinki, 1964. Non-interventional Research Ethics Committee of Dokuz Eylul University approved this study (Date: 19.10.2022, Decision No: 2022/33-03). Informed consent was obtained from the families before enrollment in the study.

Statistical analyses

Statistical analysis was performed in SPSS Software 22.0. Categorical data were expressed as numbers and percentages (%), whereas numeric data were expressed as arithmetic mean ± standard deviation. The conformity of the variables to the normal distribution was analyzed with the Kolmogorov-Smirnov test. In comparison of group ratios, Fischer's exact test was used instead of chi-square test when expected values in the cells were below 5. Student t-test was used to compare group means, and Mann Whitney-U test was used if the distribution of groups did not show normal distribution. The difference between the three groups was analyzed with the Kruskal Wallis test, and the Mann Whitney-U test was used as a post hoc test. In all statistical analyzes, a p value below 0.05 was considered significant.

RESULTS

A total of 76 children (47 boys, 61.8%; mean age 10.1 ± 3.6 [3.5-17] years) were included in the study, 54 of whom were PKU patients and 22 healthy controls. There was consanguinity between the parents of 19 patients (35.2%) in the phenylketonuria group and 6 patients (27.2%) in the control group (p = 0.597). There was no significant difference between the PKU group and the control group in terms of age, gender distribution and anthropometric data (Table 1). Physical examination of all patients was performed and no macro or micronutrient deficiency findings such as edema, skin wounds, hair loss, delayed wound healing, rickets, frequent infections

Table 1. Demographic, anthropometric features and, hemoglobin, vitamin and mineral levels of the phenylketonuria group and the control group

Parameter	Phenylketonuria group (n= 54)	Control group (n=22)	p value
Age (years)	10.7 ± 3.7	8.9 ± 2.9	0.056
Gender (M/F)	30/24	17/5	0.118
Body weight (kg)	42.7 ± 17.0	35.9 ± 17.6	0.132
Body weight SDS	0.02 ± 1.02	0.24 ± 1.21	0.460
Height (cm)	145.7 ± 20.2	135.6 ± 21.5	0.068
Height SDS	-0.08 ± 1.11	0.11 ± 1.18	0.521
Hemoglobin (g/dL)	13.2 ± 1.3	12.5 ± 1.3	**0.035**
Vitamin B12 (pg/mL)	388.3 ± 224.3	294.0 ± 116.2	**0.019**
Vitamin B12 (<200 pg/mL), n (%)	10 (18.5)	1 (4.5)	0.085
Folic acid (ng/mL)	15.0 ± 4.0	10.9 ± 4.8	**0.001**
Folic acid (>17.5 ng/mL), n (%)	15 (27.8)	2 (9.1)	**0.050**
25-OH vitamin D (ng/mL)	26.1 ± 9.8	21.1 ± 6.4	**0.012**
25-OH vitamin D (<20 ng/mL), n (%)	16 (29.6)	10 (45.5)	0.192
Zinc (µg/dL)	95.1 ± 16.4	98.6 ± 21.4	0.451
Zinc (<65 µg/dL), n(%)	3 (5.6)	0 (0.0)	*
Ferritin (ng/mL)	30.2 ± 16.8	24.5 ± 15.4	0.176
Ferritin (<16 ng/mL), n (%)	8 (14.8)	7 (31.8)	0.058

* Comparison could not be made because the groups had a value of 0.

Table 2. Plasma amino acid levels of the phenylketonuria group and the control group (µmol/L)

Parameter	Phenylketonuria group (n= 54)	Control group (n=22)	p value
Phenylalanine	580.1 ± 355.5	47.5 ± 15.9	**0.001**
Tyrosine	68.8 ± 48.8	64.6 ± 18.2	0.582
Tryptophan	60.1 ± 26.5	64.0 ± 16.5	0.528
Leucine	120.9 ± 86.3	107.9 ± 35.0	0.496
İzoleucine	73.2 ± 53.5	68.3 ± 26.5	0.683
Valine	258.3 ± 132.8	260.3 ± 90.4	0.947
Methionine	30.2 ± 14.8	27.1 ± 6.3	0.353
Lysine	165.6 ± 66.5	128.5 ± 44.1	**0.019**
Threonine	131.7 ± 53.6	123.3 ± 39.8	0.510
Histidine	76.0 ± 20.1	90.8 ± 18.9	**0.004**
Homocysteine	6.9 ± 3.8	8.5 ± 6.3	0.191

were detected in any patient or child in the control group. There were no signs of protein or essential aminoacid deficiency (skin lesions, diarrhea, edema) in any of the PKU patients.

Hemoglobin, vitamin B12, folic acid and 25-OH vitamin D levels were found to be significantly higher in the PKU patient group compared to healthy children (Table 1). There was no difference between zinc and ferritin levels. The ratio of patients with high folic acid levels in the phenylketonuria group was significantly higher than the control group (p=0.050). Although vitamin B12 levels were significantly higher in the PKU group, the rate of vitamin B12 deficiency was 18.5% in this group and 4.5% in the control group, but no significant difference was detected between the two groups (Table 1). Similarly, plasma homocysteine levels did not show any significant difference between the two groups (Table 2).

When amino acid values were compared between the two groups, a significant difference was found in plasma phenylalanine, lysine and histidine levels (Table 2). Other investigated amino acids were similar between the two groups.

The PKU group (54 patients) was divided into two subgroups according to the mean plasma Phe level of the patients in the last year. When the vitamin and mineral values of the two PKU groups and the control group were compared, a significant difference was found between the three groups in terms of folic acid and ferritin levels (Table 3). When paired group comparisons were made in terms of these parameters, it was found that the folic acid value was higher in the HDA and LDA groups than in the control group (p= 0.003 and p= 0.001, respectively); ferritin level was found to be significantly higher in the HDA group than in the LDA and control group (p= 0.012 and p= 0.004, respectively).

A significant difference was found between the phenylketonuria groups, which were compatible and incompatible with the diet, and the control group in terms of phenylalanine, tryptophan, lysine and histidine values (p= 0.001, p= 0.001, p= 0.008 and p= 0.007, respectively) (Table 4). When paired group comparisons were made in terms of these parameters, tryptophan was significantly lower in the HDA group than in the LDA and control groups (p= 0.001 and p= 0.006, respectively); lysine was found to be significantly higher in the HDA group than the control group (p= 0.016), while histidine was significantly lower in the HDA group than the control group (p= 0.008).

DISCUSSION

The primary approach to treating phenylketonuria (PKU) involves implementing a phenylalanine (Phe)-restricted diet. Compliance with a life-long Phe-restricted diet is difficult. This requires reducing the intake of natural proteins and replacing them with a protein source that lacks Phe, consisting of various amino acids. However, maintaining strict adherence to the PKU diet can be challenging for patients and their families, particularly over the long term. In this study, the effect of Phe-restricted diet compliance on the nutritional parameters and the amino acid of patients with phenylketonuria was investigated. The exclusion of patients who received BH4 or large neutral amino acid therapy in addition to diet therapy enabled us to better analyze whether dietary adherence and the amino acid formulas used had an effect on micronutrients and plasma amino acid levels.

Table 3. Hemoglobin, vitamin and mineral levels of two phenylketonuria groups and the control group

Parameter	PKU group (high adherence to diet) (n= 16)	PKU group (low adherence to diet) (n= 38)	Controls (n=22)	p value
Hemoglobin (g/dL)	13.1 ± 1.7	13.3 ± 1.1	12.5 ± 1.3	0.071
Vitamin B12 (pg/mL)	389.6 ± 201.8	387.8 ± 235.8	294.0 ± 116.2	0.214
Vitamin B12 (<200 pg/mL), n (%)	2 (12.5)	8 (21.1)	1 (4.5)	0.170
Folic acid (ng/mL)	14.8 ± 3.3	15.0 ± 4.3	10.9 ± 4.8	**0.001**
Folic acid (>17.5 ng/mL), n (%)	4 (25.0)	11 (28.9)	2 (9.1)	0.160
25-OH vitamin D (ng/mL)	27.5 ± 9.9	25.5 ± 9.8	21.1 ± 6.4	0.135
25-OH vitamin D (<20 ng/mL), n (%)	4 (25.0)	12 (31.6)	10 (45.5)	0.379
Zinc (µg/dL)	87.7 ± 19.4	98.3 ± 14.2	98.6 ± 21.4	0.172
Zinc (<65 µg/dL), n(%)	3 (18.8)	0 (0.0)	0 (0.0)	*
Ferritin (ng/mL)	40.7 ± 22.2	25.8 ± 11.8	24.5 ± 15.4	**0.011**
Ferritin (<16 ng/mL), n (%)	1 (6.3)	7 (18.4)	7 (31.8)	0.123

* Comparison could not be made because there was a value of 0 in the groups.

Table 4. Plasma amino acid levels of the phenylketonuria groups that were compatible and incompatible with the diet and the control group (µmol/L)

Parameter	PKU group (high adherence to diet) (n= 16)	PKU group (low adherence to diet) (n= 38)	Controls (n=22)	p value
Phenylalanine	299.0 ± 77.2	813.7 ± 356.6	47.5 ± 15.9	**0.001**
Tyrosine	69.1 ± 60.3	68.7 ± 44.0	64.6 ± 18.2	0.547
Tryptophan	44.9 ± 12.7	66.5 ± 28.3	64.0 ± 16.5	**0.001**
Leucine	104.7 ± 57.2	127.7 ± 95.9	107.9 ± 35.0	0.608
İzoleucine	60.8 ± 31.4	78.5 ± 60.1	68.3 ± 26.5	0.534
Valine	237.9 ± 101.0	266.8 ± 144.5	260.3 ± 90.4	0.640
Methionine	28.1 ± 11.1	31.0 ± 16.2	27.1 ± 6.3	0.878
Lysine	143.6 ± 63.6	174.8 ± 66.3	128.5 ± 44.1	**0.008**
Threonine	129.3 ± 46.3	132.7 ± 56.9	123.3 ± 39.8	0.956
Histidine	76.7 ± 15.3	75.7 ± 21.9	90.8 ± 18.9	**0.007**
Homocysteine	6.4 ± 2.6	7.1 ± 4.2	8.5 ± 6.3	0.485

In patients with classical PKU, a Phe-restricted diet is applied to reduce plasma Phe levels and to keep this level in a range that does not harm the brain, and this diet may cause growth retardation in patients [12,13]. In this study, no significant difference was found between the PKU patient groups and the control group in terms of weight and height SDS values. On the other hand, in recent years, it has been found that the prevalence of obesity has increased in this patient group over the years, since patients with PKU have too many Phe-restricted food options [14,15,16]. Since there may be differences in vitamin/mineral

levels in obesity and also malnutrition, there were no patients or controls with obesity as well as moderate-to-severe malnutrition in our study.

In our study, hemoglobin, vitamin B12, folic acid, and 25-OH vitamin D levels were found to be significantly higher in patients with PKU when compared to the control group. Ferritin levels were found to be significantly higher in the HDA patient group than in the LDA and control groups. Since protein-rich natural foods such as meat, chicken, fish, eggs, legumes and nuts are completely excluded from the diet in patients on a Phe-restricted diet, nutritional anemia may occur frequently in this patient group [5,12,17]. Patients with PKU must consume amino acid mixtures or formulas that do not contain Phe to meet their daily protein needs. Especially in the last decades, data on micronutrient deficiency in patients with PKU have been increasing; based on these data, micronutrients are added to these formulas and the contents of the formulas are updated [18,19]. Patients with PKU regularly consume these products, which meet approximately 80% of their daily protein needs, in three or four meals a day, and at the same time, these products complement the patients' vitamin, mineral and essential fatty acid requirements. Because diet-compliant patients consume these formulas regularly, micronutrient deficiencies now rarely occur in the compliant group [19,20,21]. In PKU patients who do not comply with the diet, diet leaks are usually made with foods that are poor in iron, zinc, essential fatty acids, calcium and vitamin B12, such as bread, potatoes, rice. Consuming these foods more than allowed causes the patients to become fat, but on the other hand, the level of phenylalanine increases, the intake of vitamins/minerals decreases and the blood values of these nutrients decrease. In studies conducted in the last decade, 5-14% deficiency rates were found in zinc levels in patients with classical PKU [17,20]. In our previous study, no difference was found between the patients with classical PKU and the healthy control group in terms of serum zinc levels; zinc deficiency was detected at a rate of 0.9% in the patient group and 5.6% in the control group [5]. In this study, zinc deficiency was detected in 3 patients, and there were no patients with deficiency in the control group. This may be due to the small number of individuals in all three groups.

In our study, hemoglobin, vitamin B12, and folic acid were found to be significantly higher in patients with PKU when compared to the control group. In the 1990s, approximately 10% of patients with PKU found folic acid deficiency and 30% vitamin B12 deficiency, while in recent studies, folic acid and vitamin B12 levels were found to be similar to the control group or higher in patients with PKU than in healthy controls [5,18,21,22]. This was due to the fact that the patients consumed more folic acid-rich vegetables and fruits than the control groups, and that the amino acid formulas were rich in folic acid and vitamin B12, resulting in the absence or frequency of folic acid and vitamin B12 deficiency in the patient group [18].

When the PKU group and the control group were compared in terms of plasma amino acid values, the plasma lysine level was found to be significantly higher and histidine level was low in the PKU group. There was no difference between other amino acid levels. When comparing the three groups, tryptophan was significantly lower in the HDA group than in the LDA and control groups; lysine was found to be significantly higher in the HDA group compared to the control group, and histidine was found to be significantly lower in the HDA group than the control group. There are few studies evaluating blood levels of amino acids other than Phe and tyrosine in patients with PKU who are on diet therapy, and there are discrepancies between the results of these studies. In a study conducted in China, tyrosine, alanine, asparagine, glutamine, methionine, arginine, glycine, glutamine, ornithine, and threonine were found to be significantly lower than the control group in the group that included patients with PKU who did and did not comply with the diet; valine, histidine and serine levels were found to be significantly higher [24]. In another study, it was found that arginine, citrulline, valine, methionine levels were high and tyrosine levels were low in patients at the time of initial diagnosis, and citrulline and valine levels remained high, but improvements were observed in other amino acid levels after Phe-restricted diet and amino acid formulas were given to the patients [23,24]. In our study, we did not measure the plasma amino acid levels of infants before treatment, but the results of both studies and our study do not fully overlap. According to another study by Matuszewska and colleagues, 28 different parameters were analyzed in 20 classic phenylketonuria (PKU) patients, and levels of kynurenine, tryptophan, asparagine, and proline were found to be significantly lower compared to the control group. This finding suggests that dietary restriction of phenylalanine in PKU patients can affect not only this amino acid but also the levels of other essential and non-essential amino acids. It is

particularly emphasized that some amino acids and metabolites, such as kynurenine, which are important for immune functions, inflammation, and niacin synthesis, may be affected in this process [26]. . These results suggest that the amino acid balance in the treatment of PKU patients should be carefully monitored.

All these differences may be caused by the treatment compliance of the patients, the content of the given amino acid formulas, the sources of natural proteins, and the differences in the general nutritional status of the patients. In our study, the amounts of amino acids and vitamins/minerals that the patients take with diet were not calculated.

There was no significant difference in plasma tyrosine levels between the whole patient group with PKU and the control group, and also between the PKU groups that were compatible and incompatible with the diet and the control group. Similarly, no correlation was found between plasma Phe and tyrosine levels in groups with PKU. Since patients with classical PKU have complete deficiency of the enzyme phenylalanine hydroxylase, which converts phenylalanine to tyrosine, tyrosine deficiency may occur as a result of failure of tyrosine synthesis in these patients [24]. In fact, since melanin cannot be synthesized from tyrosine, these patients may have light skin, hair and eye color [24,25]. As mentioned earlier, patients with phenylketonuria meet more than 80% of their protein needs from amino acid mixtures that do not contain phenylalanine. In order to prevent secondary tyrosine deficiency in patients with PKU, these amino acid mixture products are enriched with tyrosine. Therefore, tyrosine deficiency is not observed in patients with PKU today. In our study, low tyrosine was not found in patients with PKU. There was no skin or hair color change in the patients included in the study.

The first limitation of our research is that the number of cases in each group is relatively small. Phenylketonuria is a rare disease, so a small number of patients are followed in each center. In addition, since the amino acid levels and micronutrients we used in our study are affected by many conditions, the exclusion criteria were kept in large numbers in our study in order to interpret the findings more accurately. This caused the number of individuals included in the groups to be low. The second limitation is that only the follow-up parameters examined in routine controls were used in our study, since the study was conducted from file information. In prospective studies, in addition to these parameters, studies can be conducted with more micronutrients such as selenium, essential fatty acids, carnitine, vitamins A and E. The third limitation is that since our study was conducted retrospectively, the daily dietary vitamin-mineral levels of the patients could not be calculated.

As a result, in patients with phenylketonuria who comply with the diet and whose anthropometric data are in the normal range, there is no significant deterioration in vitamin/mineral and amino acid values. Therefore, patients with PKU should follow a Phe-restricted diet and regular vitamin/mineral follow-ups are required.

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