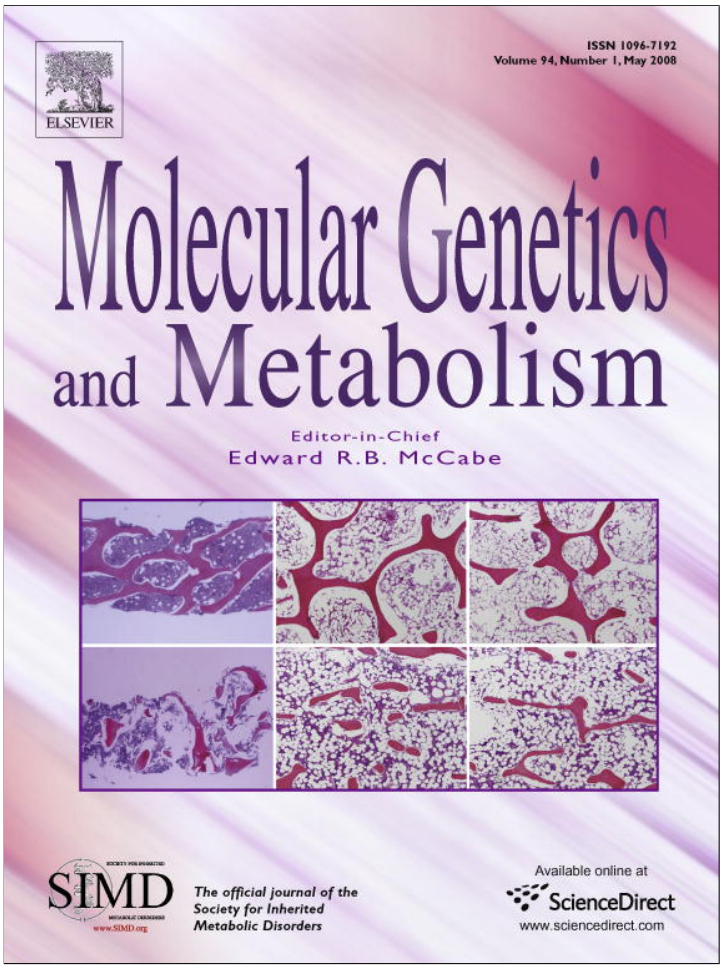

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Total homocysteine, B-vitamins and genetic polymorphisms in patients with classical phenylketonuria

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Abstract

Hyperhomocysteinemia has occasionally been reported in patients with phenylketonuria (PKU) and B-vitamin deficiency. In our study total homocysteine (tHcy) and B-vitamins were measured in treated PKU patients and healthy controls. In the patients, dietary parameters and genetic polymorphisms affecting the Hcy pathway were investigated to identify parameters modulating tHcy. A case control study including 37 PKU patients and 63 healthy controls was conducted. *t*-Tests for independent samples were used to test between groups. Multiple regressions with tHcy as dependent variable were calculated. Hardy–Weinberg expectations were tested against the observed distribution of genotypes applying the Chi-square goodness-of-fit method. THcy concentrations were not significantly different ($p = 0.059$) while folate and cobalamin (Cbl) concentrations were significantly higher in PKU patients compared to controls. However, 29.7% of patients had tHcy concentrations >97th centile. THcy did not vary with age nor correlate with folate and Cbl concentrations probably due to high saturatory levels. The presence of genetic polymorphisms had no impact on tHcy. In conclusion, in PKU patients treated with amino acid mixtures enriched with B-vitamins, tHcy is not significantly higher than in healthy controls, but tHcy concentrations exceed the 97th centile in about one third of patients. Even higher B-vitamin saturation may be required to further decrease tHcy concentrations and factors generally influencing tHcy such as betaine are to be investigated in PKU patients in the future.

Keywords: Homocysteine; MTHFR; Folate; PKU; Genetic polymorphisms

The mainstay of dietary treatment in classical phenylketonuria (PKU; McKusick 261600), is the limitation of natural, phenylalanine (Phe) containing protein. Synthetic, Phe-free L-amino acid mixtures are supplemented to provide sufficient total protein [1]. The first generation of PKU patients identified by newborn screening programs, fed with high amounts of synthetic, Phe-free food supplements for many years now reaches middle age. It is not known, whether PKU patients may—on basis of their metabolic disease and special nutrition—have a specific risk profile for cardio- and cerebrovascular disease. Hyperhomocysteinemia is an independent risk factor for thrombosis, premature vasculopathy, atherosclerosis and stroke [2,3] and in children, total homocysteine (tHcy) concentrations exceeding the 95th age specific centile result in a 4-fold increased risk for ischemic cerebrovascular disease [4]. Hyperhomocysteinemia has been observed in PKU patients with [5] and without [6–8] strict adherence to diet. While severe hyperhomocysteinemia is a hallmark of rare inborn errors of metabolism [2], the more prevalent mild to moderate hyperhomocysteinemia may be caused by nutritional deficiencies of essential cofactors [folate, cobal-

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amin (Cbl), vitamin B6], polymorphisms in genes coding for key enzymes of the Hcy pathway or the combination of these and lifestyle parameters [2,9,10]. The influence of the *MTHFR* 677TT genotype [2,11,12] and the *MTR* 2756A>G [13] and *GCP2* 1561C>T [14] polymorphisms on tHcy has repeatedly been shown in adults. From the very limited data base addressing this question in children it seems to emerge that in contrast to adults, the impact of *MTHFR* 677C>T [15], *MTRR* 66A>G [16] and the *MTHFR* 677T/1298C genotype [15,17] other polymorphisms have not yet been investigated in children on tHcy is less significant. To the best of our knowledge none of these genetic polymorphisms have yet been investigated in PKU patients.

In the present study, tHcy, genetic polymorphisms, B-vitamin concentrations, dietary parameters and body mass index (BMI)[1] were studied in PKU patients in order to screen for hyperhomocysteinemia and to identify factors influencing tHcy concentrations in this specific patient group compared to population specific centiles and healthy age and sex matched controls.

Methods

The ethics committee of Vienna Medical School approved the study. Informed written consent/assent was obtained from all participants >8 years and their parents/guardians. Venous puncture was conducted for independent medical reasons.

Subjects

Thirty-seven treated patients with classical PKU (24 males, 13 females, mean age 12.3 ± 4.5 yr; median 12.8 yr, range 4.1–20.4 yr) followed at the Department of Pediatrics, University of Vienna, Austria agreed to participate in the study. The Austrian Newborn Screening Program had identified all patients and treatment had been initiated immediately after diagnosis in the neonatal period. Protein intake was adjusted to achieve total protein intake according to Austrian recommendations [18] and to keep blood Phe levels between 40 and 240 μmol/L in children <10 years, 40 and 900 μmol/L in adolescents <15 years and below 1200 μmol/L in patients >15 years [1]. Data on tHcy, folate, Cbl concentrations and *MTHFR* 677C>T and *MTHFR* 1298A>C genotypes were available from 63 healthy (admission for elective surgery or radiological procedures) peers of comparable age and sex distribution (26 females, 37 males; mean age 11.5 ± 4.1 yr; median 12 yr, range 3.9–19.2 yr). None of the participants received vitamin supplements or any other drugs on a regular basis or had clinical evidence for acute infection, renal dysfunction, hypothyroidism, chronic inflammatory disease, inborn errors of Hcy, Cbl or folate metabolism or any other condition known to be associated with an increase or decrease in tHcy at time of sample collection. THcy, folate and Cbl concentrations in both groups were compared to centiles from the regional population [15].

Parameters

Using a standardized data sheet age, sex, weight and height were documented. The BMI was calculated using the formula (weight/height^2 [kg/m^2]). Dietary intake was recorded by the patients/their families for 3 days (one Holiday/Sunday, two working days). Average daily intake of natural and total protein, amino-acids, vitamin B6, Cbl and folate per day were calculated by an experienced dietician. All blood parameters were analysed from a 6 ml sample taken after an overnight fast. Phe concentrations were measured using a standard laboratory method (tandem mass spectrometry, automatized amino acid analyzer). THcy plasma concentrations were determined using an automated fluorescence polarization immunoassay (FPIA, Abbott IMx® analyzer). Plasma Cbl and folate concentrations were measured with Microparticle Enzyme Immunoassay (Abbott Imx® Analyzer, Abbott Laboratories, Abbott Park, Illinois, USA). RBC folate concentrations were assessed by radioimmunoassay (SimulTRAC-SNB, ICN, Costa Mesa, CA). Vitamin B6 (pyridoxal-5′-phosphate) was measured using high performance liquid chromatography. The lower range of normal for vitamin B6 was defined as 20 nmol/L and for RBC folate concentrations >120 ng/mL [centiles for our population do not exist]. For the genetic polymorphisms, genomic DNA was isolated from citrated blood samples according to standard procedures. Identification of *MTHFR* 677C>T, *MTHFR* 1298A>C, *MTR* 2756A>G, *MTRR* 66A>G and *GCP2* 1561C>T was performed by restriction length polymorphism analysis.

Statistical analysis

The Chi-square goodness-of-fit method was applied to test for differences between observed genotype frequencies and Hardy–Weinberg expectations. Univariate analysis of variance (ANOVA) was used to test effects of genetic polymorphisms, dietary parameters, serum concentrations of Phe and vitamins on tHcy. Pearson coefficients were applied to assess correlations between tHcy, B-vitamins and age, BMI and dietary parameters. Finally, multiple regression analyses were performed to evaluate independent predictors for tHcy concentrations. *t*-Tests for independent samples were used to test for differences between patients and controls and between groups with elevated and normal tHcy. Application of parametric methods was justified since variables were approximately normally distributed. *p*-values <0.05 were considered statistically significant.

Results

Polymorphisms were assessed in 33 patients and 58 controls. In the remaining patients/probands, analysis was not feasible due to blood sample collection problems. Allele and genotype frequencies in patients are depicted in Table 1. In PKU patients (and controls, data not shown), no significant differences from the Hardy–Weinberg equilibrium were present thus indicating that the distribution of genotypes was not specifically altered. The impact of different genotypes in PKU patients and controls on tHcy is summarized in Table 2. In PKU patients and controls, both in correlation and multiple regression analyses, none of the genotypes had a significant impact on tHcy, folate, RBC folate, vitamin B6 or Cbl concentrations (data not shown). Cbl and folate concentrations were significantly higher in PKU patients compared to healthy controls. Folate concentrations exceeded the 97th age specific centile in 34 out of 37 patients and were below the 3rd percentile in one patient (with normal tHcy). Cbl concentrations were below the 3rd percentile in 2 (one with normal, one with elevated tHcy) and above the 97th percentile in 6 patients [16]. All patients' vitamin B6 and RBC folate concentrations were well above the respective lower range of normal. The difference in tHcy concentrations between PKU patients and controls was not significant (Table 3). Never-

[1] *Abbreviations used:* BMI, body mass index; Cbl, cobalamin; GCP2, glutamate carboxypeptidase II; Hcy, homocysteine; *MTHFR*, methylene tetrahydrofolate reductase; *MTRR*, gene symbol for: methionine synthase reductase; *MTR*, gene symbol for: methionine synthase; THcy, total homocysteine.

Table 1
Allele and genotype frequencies and determination of Hardy–Weinberg equilibrium of genetic polymorphisms in the *MTHFR*, *MTR*, *MTRR* and the *GCP2* gene in 33 PKU patients

Polymorphisms	Allele frequencies	Genotype frequencies	Observed/ expected	Chi2
MTHFR 677C>T	C 0.64 T 0.36	CC 13 (39.4%) CT 16 (48.5%) TT 4 (12.1%)	0.99 1.05 0.93	ns
MTHFR 1298A>C	A 0.7 C 0.3	AA 15 (45.5%) AC 16 (48.5%) CC 2 (6%)	0.93 1.21 0.67	ns
Compound heterozygous		677T/1298C 7 (21.2%)		
MTR 2756A>G	A 0.73 G 0.27	AA 17 (51.5%) AG 14 (42.5%) GG 2 (6%)	0.97 1.08 0.83	ns
MTRR 66A>G	A 0.56 G 0.44	AA 10 (30.3%) AG 17 (51.5%) GG 6 (18.2%)	0.97 1.04 0.94	ns
GCP2 1561C>T	C 0.94 T 0.06	CC 29 (87.9%) CT 4 (12.1%) TT 0	0.99 1.08 0	ns

Table 2
No significant differences in tHcy concentrations between genotypes or between patients and controls[a]

Genotypes	PKU ($N = 33$) THcy (μmol/L) Mean ± SD Median Range	Controls ($N = 58$) THcy (μmol/L) Mean ± SD Median Range
MTHFR 677 C>T wild type	$N = 13$ (39.4%) 7.93 ± 5 6.6 1.3–15.3	$N = 21$ (36.2%) 7 ± 1.7 6.9 4.7–10.9
MTHFR 677 C>T het	$N = 16$ (48.5%) 8.9 ± 5 7.3 3–17.4	$N = 31$ (53.5%) 7.5 ± 2.7 7.2 3.1–13.9
MTHFR 677 C>T hom	$N = 4$ (12.1%) 9.9 ± 11 5.1 3.3–26.2	$N = 6$ (10.3%) 7 ± 2.5 7 4.1–10.8
MTHFR 677/1298 not compound heterozygous	$N = 26$ (78.8%) 8.6 ± 5.9 6.7 1.3–26.2	$N = 36$ (62.1%) 7.2 ± 2.1 7 4.1–13.9
Compound heterozygous	$N = 7$ (21.2%) 8.8 ± 5.7 6.6 3–17.4	$N = 22$ (37.9%) 7.3 ± 2.7 7.3 3.1–13.1

[a] *t*-Tests for independent samples, two-sided.

theless 11 out of 37 patients (29.7%) had tHcy concentrations exceeding the 97th age centile. We compared the two groups "high" versus "normal" tHcy but found no significant differences for other parameters (data not shown).

Table 3
Vitamin concentrations in 37 PKU patients and 63 healthy peers of comparable age and sex distribution

	PKU patients	Controls	p[a]
tHcy μmol/L			
Mean	8.9	7.2	
SD	6.5	2.3	
Median	6.6	7	
Range	1.3–29.5	3.1–13.9	$p = 0.059$
Folate ng/mL			
Mean	15.1	7.6	
SD	5.1	3.3	
Median	16.6	6.4	
Range	0.7–19.9	3.3–15.9	$p < 0.0001$
Cobalamin pg/mL			
Mean	783	478	
SD	528	180	
Median	653	466	
Range	68–2005	183–966	$p < 0.0001$

[a] *t*-Test for independent samples, two-sided.

Table 4
Intake of protein components and vitamins and concentrations of Phe, RBC folate and vitamin B6 in 37 PKU patients

Nutrient intake		% RDA	Measured concentrations
Total protein (g/kg/d)			*Phe (μmol/L)*
Mean	1.8	205	620
SD	0.3	36	425
Median	1.8	204	512
Range	0.9–2.4	98–294	61–1556
Natural protein (g/kg/d)			
Mean	0.3		
SD	0.2		
Median	0.4		
Range	0.1–0.8		
L-Amino acid mixtures (g/kg/d)			
Mean	1.5		
SD	0.3		
Median	1.5		
Range	0.6–2		
Vitamin B6 (mg/d)			*Vitamin B6 (nmol/L)*
Mean	2.2	210	146
SD	1.1	94	72
Median	1.8	193	134
Range	0.8–5.5	77–579	53–318
Vitamin B12 (μg/d)			
Mean	4	165	[a]
SD	2.6	80	
Median	3.3	158	
Range	0.2–13.5	10–451	
Folate (μg/d)			*RBC folate (ng/ml)*
Mean	356	96	559
SD	183	47	385
Median	339	86	497
Range	90–806	30–238	152–2353[a]

[a] For serum folate and vitamin B12 concentrations see Table 3.

Dietary parameters and Phe concentrations are noted in Table 4. Austrian recommended dietary allowances (RDA) for protein, vitamin B6 and Cbl intake were consistently

exceeded while folate intake approximately met the RDA [18]. Thirteen patients had Phe levels exceeding the targeted age specific values. Mean BMI was 19.1 kg/m^2 (SD 3.3, median 19.1, range 13.3–26.6 kg/m^2). No gender differences were found for any of the investigated parameters.

In the PKU patients, Pearson correlation analysis revealed a significant negative correlation between tHcy and vitamin B6 concentration ($p = 0.04$). No significant correlation was present between tHcy and folate, Cbl, BMI, Phe, gender, genetic polymorphisms, dietary parameters and age in PKU patients. In contrast, in controls tHcy correlated significantly with age ($p = 0.03$) (Fig. 1) and was inversely correlated with folate ($p < 0.01$) and Cbl ($p < 0.01$), but not with gender. Folate and Cbl both correlated significantly negative with age ($p = 0.003$ and 0.005, respectively) In PKU patients, folate and Cbl concentrations correlated significantly ($p = 0.008$) and both were inversely correlated with BMI ($p = 0.004$) and Phe levels ($p = 0.03$; $p = 0.05$). Additionally folate concentrations correlated significantly with intake of amino acids ($p = 0.004$), natural protein ($p = 0.002$), total protein ($p = 0.007$) and folic acid ($p = 0.05$). Cbl correlated positive with intake of amino acids ($p = 0.004$), vitamin B12 and total protein ($p = 0.002$). Multiple regression models with tHcy as the dependent variable including genetic polymorphisms, Phe, dietary parameters and B-vitamin concentrations showed no significant results.

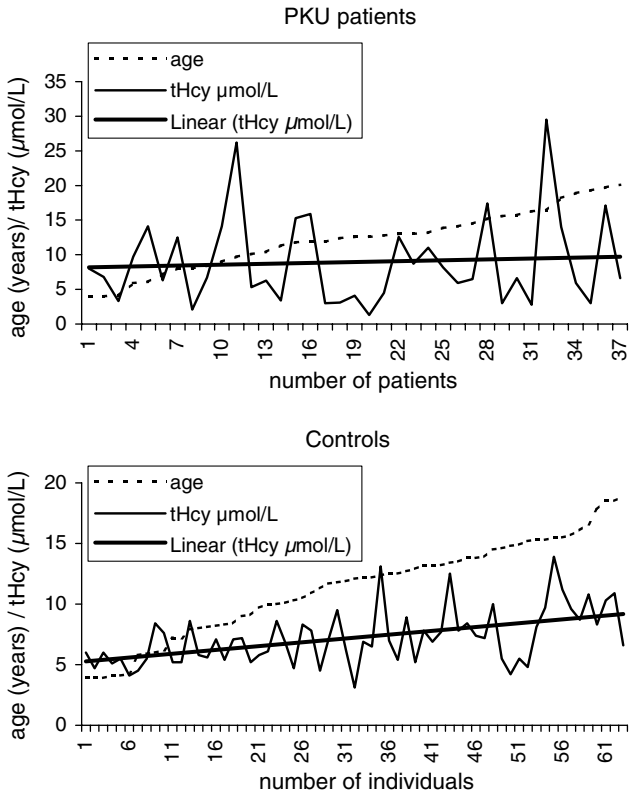

Fig. 1. tHcy concentrations and linear trend in relation to age in PKU patients and controls.

Discussion

In the PKU patients studied, tHcy concentrations exceeded the 97th percentile in 29.7% of patients, were tendentially higher than in healthy peers and showed a larger standard deviation and wider range compared to other pediatric populations [15,19–21]. Furthermore the characteristic pattern of higher tHcy and lower B-vitamin concentrations in older individuals was not present. Folate is the main determining factor for fasting tHcy concentrations in children [15,22], Cbl and vitamin B6 are of minor importance [23,24]. Folate concentrations were exceedingly high (92% above the 97th percentile) while Cbl and vitamin B6 concentrations were normal in the PKU patients [15]. We hypothesize that the consistent, age-independent B-vitamin hyper-saturation by means of intake of B-vitamin enriched amino acid mixtures results in the atypical chronological sequence of tHcy concentrations and masks the close relationship between folate and tHcy normally present in children. Furthermore other than the measured dietary parameters which are considered of minor importance for tHcy concentrations in the healthy population (such as betaine) have not been measured in our study and an influence on tHcy concentrations in this special patient group can not be excluded.

Genetic parameters do not explain the tendency towards higher tHcy concentrations in PKU patients. First of all, the accordance between Hardy–Weinberg equilibrium and observed distribution of genotypes indicates that no selection bias for these polymorphisms was present. Additionally our study supports the concept that the *MTHFR* 677TT [15,25–27], *MTHFR* 1298CC and the *MTHFR* 677T/1298C [15,17] genotypes as well as the *MTR* 2756A>G, MTRR 66A>G and *GCP2* 1561C>T polymorphisms [16,28] are not correlated with tHcy concentrations in children with sufficient folate concentrations. Nevertheless the explanatory power of our results is limited due to the small sample size.

Phe concentrations as a measure for adherence to dietary treatment as well as the daily intake of total, natural and synthetic proteins showed no correlation with tHcy values. Moreover even in patients with elevated Phe values—indicative for less strict adherence to diet, —B-vitamin concentrations were by far higher than in the normal population suggesting that the B-vitamin enriched amino acid mixtures were consumed at least to some extent.

Synthetic protein preparations have a reduced biological quality and are metabolised at a higher rate than natural protein [29,30]. To compensate for this insufficiency, the amount of L-amino acids is usually calculated to achieve a total protein intake exceeding age specific RDA [31]. In combination with the very low targeted Phe concentrations [1], only a small proportion of natural protein is ingested complemented by large amounts of synthetic protein. At the beginning of the study we had the hypothesis that the enhanced metabolism of the synthetic amino acids may resemble an intermittent protein and methionine overload,

potentially causing elevated tHcy concentrations. But in contrast to this hypothesis the amounts of daily ingested synthetic, natural or total protein did not correlate with tHcy concentrations. We did not investigate however, how amino acid mixtures were applied. It is known that ingested as a single dose, amino acid turnover is excessively enhanced and may result in protein breakdown "peaks" compared to ingestion in small portions combined with meals [32].

Considering our data against the background of earlier publications on tHcy in PKU, effects of different treatment regimens in the countries the publications originate from become evident. In Austria, natural protein restriction and supplementation with L-amino acid mixtures enriched with minerals and vitamins is recommended at least until adulthood. As none of our patients was completely non-compliant, B-vitamin deficiency as described by others [6–8] was not present.

In a Danish sample of 31 adults with PKU, 6/31 did not use amino acid supplements and 24/31 patients received a supplement containing only essential, large neutral amino acids without vitamins and minerals added. Consecutively, 29% of the patients had hyperhomocysteinemia attributed to reduced Cbl (39%) and vitamin B6 (71%) supplies in presence of a normal folate status [8]. These results are supported by data from adolescent Greek PKU patients with Phe concentrations indicating good compliance. Nevertheless, Cbl, vitamin B6 and folate intakes reached approximately only 50% of the RDA resulting in low B-vitamin blood concentrations and hyperhomocysteinemia [5]. We conclude, that not only PKU patients without strict adherence to dietary treatment are at risk for B-vitamin deficiency [6,7] but also in patients with good compliance, sufficient intake of B-vitamins is not guaranteed and must be monitored [7]. Patients must from this point of view be encouraged to take the prescribed amounts of PKU specific protein preparations enriched with vitamins and minerals but not to simply avoid natural proteins in order to keep Phe concentrations within limits.

In our study, tHcy was not significantly higher than in matched controls but approximately 1/3 of the patients had tHcy concentrations > the 97th age centile in the presence of high folate and normal Cbl and vitamin B6 concentrations. Comparing these individuals with hyperhomocysteinemia to those with normal tHcy concentrations revealed no significant differences concerning predictive factors, but a larger group would be necessary in order to compare patients with very high and not only normal but very low tHcy. In our relatively small sample a comparison of these groups was considered not advisable due to small size of the subgroups. In the only study reporting significantly lower tHcy concentrations in 42 PKU patients compared to healthy controls and had patients lower tHcy than in our sample, and folate and Cbl concentrations were even higher than in our patients [33].

We conclude that there is evidence that PKU patients may have a higher demand of folate and Cbl to keep tHcy within limits. Nevertheless it must be kept in mind that the safety of combined, high B-vitamin supplementation is currently debated [34]. Genetic polymorphisms show no specific distribution and do not exert significant influence on tHcy concentrations.

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