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Serum vitamin B_{12} concentrations within reference values do not exclude functional vitamin B_{12} deficiency in PKU patients of various ages

Inge Vugteveen [a,b], Marieke Hoeksma [a,b,*], Anne-Lise Bjorke Monsen [c], M. Rebecca Fokkema [d], Dirk-Jan Reijngoud [b,d], Margreet van Rijn [a], Francjan J. van Spronsen [a,b]

[a] Section of Metabolic Diseases, Beatrix Children's Hospital, University Medical Centre of Groningen, University of Groningen, Groningen, The Netherlands
[b] GUIDE, Graduate School for Drug Exploration, Center for Liver, Digestive and Metabolic Diseases, University Medical Centre of Groningen, University of Groningen, Groningen, The Netherlands
[c] Department of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway
[d] Laboratory of Metabolic Diseases, Department of Laboratory Medicine, University Medical Centre of Groningen, University of Groningen, Groningen, The Netherlands

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ABSTRACT

Homocysteine (Hcy) and in particular methylmalonic acid (MMA) are considered reliable parameters for vitamin B_{12} status in healthy individuals. Phenylketonuria (PKU) patients are at risk for functional vitamin B_{12} deficiency based on their diet.

Objective: The aim of this study was to investigate the prevalence of functional vitamin B_{12} deficiency in continuously treated PKU patients and the association of parameters of vitamin B_{12} and metabolic control.

Methods: In 75 continuously treated PKU patients of 1–37 years of age, serum vitamin B_{12} concentrations, plasma Hcy, MMA, and phenylalanine concentrations were studied.

Results: Eight patients had vitamin B_{12} concentrations below normal. Out of these eight patients, two had elevated MMA and/or Hcy concentrations. Ten other patients with normal vitamin B_{12} concentrations had elevated concentrations of MMA and/or Hcy.

Conclusions: A vitamin B_{12} concentration within the reference range does not automatically imply a sufficient vitamin B_{12} status. We recommend measuring serum MMA, or alternatively plasma Hcy, yearly in all PKU patients to diagnose functional vitamin B_{12} deficiency.

Introduction

Treatment of phenylketonuria (PKU; McKusick 261600) aims at lowering blood phenylalanine (Phe) concentrations by dietary Phe restriction [1]. Since Phe is part of natural protein, Phe restriction implies restriction of natural protein. Consequently, PKU patients consume individually tailored limited amounts of natural protein to maintain blood Phe concentrations within the target range for age. The requirement for the remaining amino acids and nitrogen is fulfilled with a protein substitute free of Phe. This substitute is enriched with micronutrients, normally provided for with the natural protein intake and with extra tyrosine being an essential amino acid in PKU patients.

The minor intake of natural protein in PKU patients is mainly of low quality (from vegetable rather than animal origin), and a significant number of PKU patients, especially adolescents and young adults, do not take the protein substitute adequately [2,3]. As a result, PKU patients tend to be at risk for deficiencies of nutrients that are mainly found in protein of animal origin [4,5], e.g. carnitine [6], selenium [7], and vitamin B_{12} [4,8].

A vitamin B_{12} deficiency is often diagnosed based on a combination of clinical symptoms, morphological findings, and biochemical tests [9]. The clinical picture of vitamin B_{12} deficiency is diffuse and may comprise anemia, gastrointestinal and neurological symptoms including both low and brisk tendon reflexes. Brisk tendon reflexes are often seen in PKU patients and have been related to vitamin B_{12} deficiency [10–12]. Until now, vitamin B_{12} deficiencies in PKU patients have been investigated by especially studying serum vitamin B_{12} concentrations in blood in PKU patients [4,8,10,11,13,14].

Biochemically, there seems to be a discrepancy between vitamin B_{12} concentrations in serum and vitamin B_{12} activity [15]. Vitamin B_{12} is a cofactor of two enzymatic reactions converting methylmalonyl-CoA into succinyl-CoA, and homocysteine (Hcy) into methionine [16]. In vitamin B_{12} deficiency, methylmalonyl-coA is converted into methylmalonic acid (MMA) and released into the circulation. Elevated concentrations of MMA and/or Hcy in the blood are considered measures of impaired intracellular cobalamin status [17–19] and may

Abbreviations: Hcy, homocysteine; MMA, methylmalonic acid; Phe, phenylalanine; PKU, phenylketonuria; Vit B_{12}, vitamin B_{12}.

* Corresponding author. Beatrix Children's Hospital, University Medical Centre of Groningen, PO box 30.001, CA 80, 9700 RB Groningen, The Netherlands. Fax: +31 50 3614235.
E-mail address: m.hoeksma@bkk.umcg.nl (M. Hoeksma).

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be increased even when serum vitamin B_{12} concentrations are within reference values [20,21]. Therefore, in this study, we defined functional vitamin B_{12} deficiency as an increase of MMA and/or Hcy concentration in plasma.

The present study aimed to investigate the prevalence of decreased functional vitamin B_{12} activity in continuously treated PKU patients of various ages, the prevalence of decreased vitamin B_{12} concentrations, and the association between serum vitamin B_{12} and functional vitamin B_{12} activity. Besides, the relation between vitamin B_{12} concentration in serum and biochemical vitamin B_{12} activity was investigated. We hypothesized that functional vitamin B_{12} deficiency should be considered more often than based on serum vitamin B_{12} concentrations alone.

Patients and methods

The PKU patients at the University Medical Centre of Groningen ($n=91$) were investigated retrospectively. All PKU patients but three were diagnosed within 2 weeks after birth by neonatal screening. The three patients were diagnosed at 1 to 2 years of age, either before introduction of neonatal screening ($n=2$) or missed by neonatal screening. From diagnosis onwards, patients were continuously treated with a Phe-restricted diet. None of the patients had hard clinical signs of functional vitamin B_{12} deficiency, although in most patients hyperreflexia can be found at least to some extent. From 2001 onwards, the aimed blood Phe concentrations were 120–360 μmol/L for patients below 12 years, 120–600 μmol/L for patients over 12 years, and 120–240 μmol/L for women aiming to be pregnant and during pregnancy [22]. Whole blood spots for analysis of blood Phe are controlled with frequency depending on age, e.g., twice a week for young infants and once a month for adults.

Of the 91 patients, 16 were excluded because of insufficient laboratory data ($n=15$) or pregnancy ($n=1$). No patients had severe comorbidity, leaving 75 patients (36 males and 39 females) aged 1–37 years, for statistical analysis of circulating concentrations of vitamin B_{12}, MMA, and Hcy (Table 1). All patients were taken as one group.

Starting in 2004, circulating concentrations of vitamin B_{12}, MMA and Hcy were determined in all PKU patients on a routine basis at least yearly. Laboratory data were collected from the laboratory database. Measurements of serum vitamin B_{12}, serum MMA, and plasma Hcy were performed in blood sampled taken at the same moment. Serum vitamin B_{12} concentration was measured by fluoroimmunoassay (AutoDelfia, PerkinElmer Life and Analytical Sciences, Wallac Oy, Finland). Plasma Hcy was measured by fluorescence polarization immunoassay (IMx; Abbott Laboratories, The Netherlands). Serum MMA was measured by gas chromatography-mass spectrometry. MMA and the internal standard MMA-D_3 were derivatised with pentafluorobenzylbromide and detected in the selected ion monitoring mode at target ions of m/z 233.1 (MMA) and m/z 236.1 (MMA-D3). The method uses negative ion chemical ionisation with ammonia as the reagent gas. The local laboratory did not provide reference values for children for serum vitamin B_{12} and plasma Hcy. For the outer limits of the vitamin B_{12} and Hcy concentrations, we used the $P_{2,5}$ and the $P_{97,5}$ in the population of Monsen et al. [23]. To enable comparison of values between the laboratory of Monsen (Bergen method) and our laboratory (Groningen method), 36 samples from various age groups (1–32 years) were analyzed at both laboratories. Passing and Bablok regression analysis revealed a significant difference between methods with a slope of 1.15 and an intercept of −1.29 ($r=0.974$). Corrected values were used for reference values of vitamin B_{12} in serum and Hcy in plasma (Table 1). The reference range ($P_{2,5}$–$P_{97,5}$) at the laboratory of the metabolic centre in Groningen of MMA is 90–340 nmol/L, and independent of age in children older than 1.5 years [23]. A functional vitamin B_{12} deficiency was defined as a MMA concentration above the $P_{97,5}$. Blood spot Phe is analyzed by high-performance liquid chromatography with fluorescence detection. Blood spots are punched out and left to deproteinize and elute in trichloroacetic acid containing an internal standard. Primary and secondary amino acids subsequently react with 6-aminoquinolyl-*N*-hydrozysuccinimidyl carbamate to obtain fluorescent derivatives. Metabolic control was defined as the average Phe concentration measured in the year preceding measurements of vitamin B_{12}, MMA, and Hcy.

Statistical analysis

SPSS 14 was used for statistical analyses. Descriptive statistics of the different parameters were obtained. The median and interquartile ranges were used as the data were not evenly distributed. Spearman rank was used to study the relation between vitamin B_{12} concentrations and MMA and/or Hcy concentrations. Metabolic control (as derived from mean blood Phe concentrations) was taken into account as a possible confounding factor in Spearman correlation. Analyses were considered significant at $P<0.05$.

Results

Clinical characteristics, blood Phe concentrations, and vitamin B_{12} status are presented in Table 2. Eight patients had serum vitamin B_{12} concentrations below reference ranges. One of these eight had an elevated serum MMA concentration and another had elevated concentrations of both Hcy and MMA. Ten other patients had biochemical evidence of functional vitamin B_{12} deficiency. All of these 10 patients had vitamin B_{12} concentrations within reference range. Five out of these ten patients had elevated serum MMA concentrations, and two patients had elevated concentrations of both MMA and Hcy. Three other patients had elevated plasma Hcy concentrations (Fig. 1; Table 3).

Table 2 shows that the number of patients with an increased MMA and/or Hcy was equal or higher than the number of patients with decreased serum vitamin B_{12} concentrations. Fig. 1 shows the relationship between Hcy/MMA and vitamin B_{12} concentrations in patients with increased Hcy and/or MMA concentrations.

Table 3 shows the absolute concentrations of the 18 patients with abnormal concentrations of vitamin B_{12}, MMA, and/or Hcy.

Correlations of serum vitamin B_{12} concentration with serum MMA and plasma Hcy for various age groups did not show consistently

Table 1
Age dependent reference values for vitamin B_{12} and plasma Hcy.

Age (years)	Vitamin B_{12} (pmol/L)	Plasma Hcy (μmol/L)	Blood Phe (μmol/L)	Plasma MMA (nmol/L)
1.6–11.9	194–1002	1.5–7.8	120–360	90–340
12–19.9	141–749	4.0–13.8	120–600	90–340
>20.0	170–700	5.0–15.0	120–600	90–340

Table 2
Patients characteristics and vitamin B_{12} markers.

n	75
Male (%)	48.0
Serum vitamin B_{12}, pmol/L	
Median	288.0 ($n=69$)
Interquartile range	202.0–386.0
Deficient (number of patients)	8
Serum MMA, nmol/L	
Median	229.5 ($n=68$)
Interquartile range	175.6–271.3
Elevated MMA (number of patients)	9
Plasma Hcy, μmol/L	
Median	6.0 ($n=67$)
Interquartile range	4.9–8.5
Elevated Hcy (number of patients)	6
Blood Phe, μmol/L	
Median	380.0 ($n=62$)
Interquartile range	289.8–513.8