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Original Article *Özgün Araştırma*

DIAGNOSIS OF VITAMIN B12 DEFICIENCY IN CHILDREN BY USING METHYLMALONIC ACID, HOMOCYSTEINE AND HOLOTRANSCOBALAMINE

Çocuklarda Vitamin B12 Eksikliğinin Metilmalonik Asit, Homosistein ve Holotranskobalamin ile Doğrulanması

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ABSTRACT

Objective: Vitamin B12 deficiency is a common health issue in children. Though the sensivity of serum vitamin B12 measurement for the diagnosis of vitamin B12 deficiency is low, combined methylmalonic acid, homocysteine and holotranscobalamin have been reported to be more specific biomarkers in early and accurate diagnosis. In this study, we aimed to verify the diagnosis of vitamin B12 deficiency in children by measuring methylmalonic acid, homocysteine, holotranscobalamin and to determine the accuracy of them.

Material and Methods: The study included 277 patients aged <18 years old age and with a vitamin B12 levels <200 pg/ml. The cut-off values for defining vitamin B12 deficiency were methylmalonic acid>0.4 µmol L, holotranscobalamin <21 pmol/L. Homocysteine levels were evaluated according to age.

Results: There was a positive correlation (kappa index 0.387) between vitamin B12 and holotranscobalamin and a negative correlation between vitamin B12, methylmalonic acid and homocysteine levels (kappa index -0.134, -0.258, respectively). There was a weak correlation between methylmalonic acid and holotranscobalamin; methylmalonic acid and homocysteine; homocysteine and holotranscobalamin (kappa index -0.039, 0.243, -0.377, respectively). Patients were divided into 4 groups according to holotranscobalamin, homocysteine and methylmalonic acid levels.

Seventy-three patients (26%) had low holotranscobalamin. Group 1a was consisting of 37 patients who were diagnosed vitamin B12 deficiency with low serum B12 and supported by all 3 parameters. When all groups were compared, vitamin B12 and hemoglobin levels were found to be lower in Group 1a than other groups (respectively, $p<0.05$, $p<0.001$)

Conclusion: In children, holotranscobalamin alone should not be considered an accurate indicator, and other functional markers should be combined in the diagnosis of vitamin B12 deficiency. Furthermore, most studies about vitamin B12 deficiency were carried out in adult age groups, and studies are needed in children to determine the reference intervals for holotranscobalamin and serum methylmalonic acid.

Keywords: *Vitamin B12, methylmalonic acid, holotranscobalamin*

ÖZ

Amaç: Vitamin B12 eksikliği, çocuklarda yaygın bir sağlık sorunudur. Vitamin B12 eksikliğinin tanısı için serum vitamin B12 ölçümünün duyarlılığı düşük olmakla birlikte, erken ve doğru tanıda plazma metilmalonik asit, homosistein ve holotranskobalaminin kombine kullanımının daha spesifik biyobelirteçler olduğu bildirilmiştir. Bu çalışmada çocuklarda vitamin B12 eksikliğinin tanısını metilmalonik asit, homosistein ve holotranskobalamin ölçümü ile doğrulamak ve bu belirteçlerin doğruluğunu belirlemeyi amaçladık.

Gereç ve Yöntemler: Çalışmaya <18 yaş ve vitamin B12 düzeyi <200 pg/ml olan 277 hasta dahil edildi. Tüm hastalarda vitamin B12, metilmalonik asit, homosistein, holotranskobalamin düzeyleri değerlendirildi. Vitamin B12 eksikliğini tanımlamak için cut-off değerleri metilmalonik asit >0.4 µmol L, holotranskobalamin <21 pmol/L idi. Homosistein düzeyleri yaşa göre değerlendirildi.

Bulgular: Vitamin B12 ile holotranskobalamin arasında pozitif korelasyon (kappa indeksi 0.387), vitamin B12, metilmalonik asit ve homosistein arasında negatif korelasyon (kappa indeksi -0.134, kappa indeksi -0.258) görüldü. Metilmalonik asit ve holotranskobalamin, metilmalonik asit ve homosistein, homosistein ve holotranskobalamin arasında zayıf korelasyon izlendi (sırasıyla kappa indeksi -0.039, 0.243, -0.377). Hastalar holotranskobalamin, homosistein, metilmalonik asit seviyelerine göre 4 gruba ayrıldı. Yetmiş üç hastada (%26) holotranskobalamin düzeyi düşüktü. Grup 1a, vitamin B12 eksikliği tanısı alan ve her 3 parametre ile desteklenen 37 hastadan oluşuyordu. Tüm gruplar karşılaştırıldığında Grup 1a'nın vitamin B12 ve hemoglobin düzeyleri diğer gruplardan daha düşüktü (sırasıyla, $p<0.05$, $p<0.001$).

Sonuç: Çocuklarda, holotranskobalamin tek başına doğru bir gösterge olarak kabul edilmemeli ve vitamin B12 eksikliği tanısında diğer fonksiyonel belirteçler ile birlikte değerlendirilmelidir. Ayrıca, vitamin B12 eksikliği ile ilgili çalışmaların çoğu yetişkin yaş gruplarında yapılmıştır ve çocuklarda holotranskobalamin ve serum metilmalonik asit referans aralıklarını belirlemek için yeni çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: *Vitamin B12, metilmalonik asit, holotranskobalamin*

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INTRODUCTION

Vitamin B12 "cobalamine" (Cbl) is a water-soluble vitamin, which cannot be synthesized in the human body. It can be provided by animal origined foods such as milk, cheese, egg, red meat, and can be produced partly through the gut bacteria (1). All cells require B12 and it plays roles in two major enzymatic reactions in the human body. First; methylcobalamin is used as a coenzyme in the conversion of homocysteine (Hcy) to methionine catalyzed by the methionine synthase enzyme; secondly, 5'-deoxyadenosyl cobalamin acts as a coenzyme in the reaction that converts methylmalonyl-CoA to succinyl-CoA catalyzed by the methylmalonyl-CoA mutase (1-3).

Lack of B12 may cause megaloblastic anemia, pancytopenia, glossitis, nausea, vomiting, loss of appetite, diarrhea, aphthous stomatitis, growth retardation, irritability, attention problems, low academic performance, depression/mania, demyelinating neurological disease. The neurological damage may not be fully remedied by treatment. Early diagnosis and treatment of mild vitamin B deficiency prevents anemia and neurological damage (1,4-7).

Currently, serum vitamin B12 levels are used as a standard method for the diagnosis of vitamin B12 deficiency. A total serum B_{12} level of <200 pg/mL (<249 pmol/L) is generally considered as deficient. Vitamin B12 is carried by two transport proteins; haptocorrin (HC) and transcobalamin. While 80-90% of vitamin B12 in circulation is bound to HC which is not bioavailable for the immediate delivery to cells, the remaining part is transferred to the tissue with holotranscobalamin (holoTC, bioactive B12). The moiety carried by the holoTC is the biologically active part. Patients who have no clinical and biochemical symptoms even they have low serum B12 values with normal tissue B12 were reported in some vegetarians, in patients taking mega doses of ascorbic acid and in inherited "benign" HC deficiency (8). Plasma homocysteine and methylmalonilCoA (MMA) levels that are the two functional indicators of B12 status increase in cobalamin deficiency. Homocysteine level is also influenced by many factors other than vitamin B12 deficiency, therefore serum MMA level is considered more specific marker of functional B12 deficiency (2,9-11). Recently, the assessment of holoTC has been used in the diagnosis of vitamin B12 deficiency. The low level of holoTC is accepted as the earliest sign of vitamin B12 deficiency. It is reported as more accurate in measuring bioactive form of vitamin B12 than serum vitamin B12 itself (9,12).

In our study, we assessed the utility of holoTC in comparison to serum MMA and plasma Hcy levels in children with serum vitamin B12 level <200 pg/mL for more reliable diagnosis of vitamin B12 deficiency.

MATERIALS AND METHODS

Patients with vitamin B12 deficiency symptoms referred from other departments or with low vitamin B12 levels (<200 pg/mL) in routine controls between 2013-2016 were retrospectively included. Patients with renal disease, liver disease, thyroid disease, folate deficiency, patients who had a history of multivitamin-use, and patients whose HCy level did not fall after the vitamin B12 treatment were excluded from the study.

Demographic properties of the patients were recorded. Hemoglobin (Hb), serum ferritin, serum MMA, plasma HCy and holoTC levels were assessed. Complete blood count was measured with an automated blood count device (Beckman Coulter LH 780 Hematology Analyzer). Serum ferritin was estimated by enzyme linked immunosorbent assay (Unicel Dx I 800, Becman Coulter). Serum vitamin B12 levels were analyzed by competitive-binding immunoenzymatic assay on Beckman Coulter DXI 800. The HoloTC level was measured by ELISA using an ELX 800 Biotech Brand ELISA reader at 450nm (wavelength) IBL Active B12 Holotranscobalamin ELISA results were evaluated according to the reference range of 21-123 pmol/L. The blood MMA sample analyzed using the chromatographic method (Thermo scientific instrument). The results were evaluated according to the reference range of MMA 0-0.4 µmol/L. Plasma Hcy concentrations were measured by Abbott Architect

KÜ Tıp Fak Derg 2023;25(2):200-206
Doi: 10.24938/kutfd.1263790

i2000 Automatic analyzer. Homocysteine levels were accepted normal for <1 year as 3.3-8.3 µmol / L, 1-6 years of age as 3.87±1.44 µmol/L, 7-11 years as 8.7±1.4 µmol / L and 12-17 years of age as 13.54±1.49 µmol / L (13). The reference limits for vitamin B12 deficiency were: serum MMA >0.4 µmol/L, holoTC <21 pmol/L, homocysteine higher than the upper limit of the reference range for that age. Serum MMA and holoTC cut-off values were determined by using the test manufacturer's book. Hemoglobin values more than 2 standard deviations below the mean reference value for the age and sex and serum ferritin level <12 ng/mL were accepted as iron deficiency anemia (14).

Patients were divided into 4 groups according to holoTC, Hcy and blood MMA levels:

Group 1: Low holoTC with,

a. High Hcy and MMA

b. High MMA only

c. High Hcy only

Group 2: Normal holoTC with,

a. High Hcy and MMA

b. High MMA only

c. High Hcy only

Group 3: Low holoTC, normal Hcy and normal MMA

Group 4: Normal holoTC, MMA and Hcy

The study was approved by the Clinical Research Ethics Committee of Health Sciences University Ankara Pediatrics Hematology Oncology Training and Research Hospital (approval number 2016-043). Informed written consent was obtained from the parents or caregivers of enrolled children after explanation of the study.

Hemoglobin and mean corpuscular volume values of the patients were evaluated according to age and sex.

Statistical Analysis

The Kolmogorov-Smirnov test was used to determine whether the distribution of continuous variables was normal. Descriptive statistics were expressed as mean ± standard deviation or median (minimum-maximum) for continuous variables, and categorical variables were reported as number of cases and percentage. The significance of differences between groups in terms of averages was assessed by Student t test for two independent groups. Differential significance in median values was assessed by Mann Whitney U test for independent groups. The significance of the difference between the ratios was assessed by Chi-square test or Fisher's exact test. Statistically significant correlation between continuous variables was found in normal distribution, Pearson in appropriate data and Spearman in normal data correlation test. Factors predicting treatment unresponsiveness were determined by logistic regression analysis. Results with $p<0.05$ were considered statistically significant.

RESULTS

Two hundred seventy seven patients were included in the study. One hundred eighteen patients (43%) were male and 159 patients (57%) were female. The mean age of patients were 113.24±76.14 months and the range was 1-212 months. There was no significant difference in terms of age between the sexes (p=0.959).

The hemoglobin (Hb) value of patients was 12.7±1.69 g/dl. Forty-eight patients (17%) were anemic. Fifty-six percent of anemic patients (n=27) had both B12 and iron deficiency anemia. Twenty-five percent of patients (n=68) had both iron and B12 deficiency without anemia. Eight patients (3%) had neutropenia. Thrombocytopenia was not found in any patients.

In our study population, median serum vitamin B12 level was 119 pg/mL (range: 21-198 pg/mL). Median holoTC level was 28.4 pmol/L (range 2.1-108.3 pmol/L). Median MMA level was 0.5 µmol/L (0.2-6.47 µmol/L). Median Hcy level was 9.8 µmol/L (range 2.5-47.4 µmol /L). There was no difference between genders in terms of vitamin B12, Hb, Holo TC, serum MMA and Hcy levels. Any significant difference was not detected in terms of holoTC, Hcy, MMA between patients with anemia or not, and patients with iron deficiency or not ($p>0.05$). A positive correlation (kappa index 0.387, $p<0.01$) between vitamin B12 level and holoTC, a negative correlation between vitamin B12 and MMA, Hcy (kappa index -0.134, $p<0.05$, kappa index -0.258, $p<0.01$) were found. A weak correlation was found

KÜ Tıp Fak Derg 2023;25(2):200-206
Doi: 10.24938/kutfd.1263790

between MMA and HoloTC, MMA and Hcy, Hcy and HoloTC (kappa index -0.039, p>0.05, kappa index 0.243, p<0.01, kappa index -0.377, p<0.01).

Patient groups according to HoloTC, MMA and Hcy levels were shown in Figure 1. Seventy-three patients (26%) who had low B12 also had low holoTC (Group 1). Group 1a was consisting of 37 patients (13%) who were diagnosed vitamin B12 deficiency with low serum B12 and supported by all 3 parameters (low HoloTC, high blood MMA and Hcy high) (Figure 1 and Table 1).

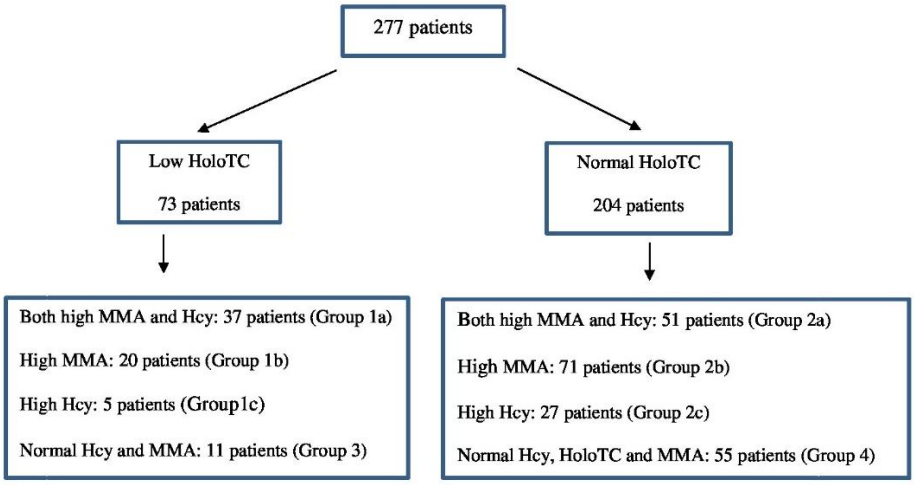

Figure 1: Classification According to HoloTC Level in Patients with Vitamin B12 Deficiency (HoloTC: Holotranscobalamin MMA: Methylmalonic acid Hcy: Homocysteine)

Table 1: HoloTC, HS, MMA, Hb, vitamin B12, platelet, WBC and neutrophil levels in patients between groups

	Group 1a n:37	**Group 2 n:149**	**Group 3 n:11**	**Group 4 n:55**	**p**
Hb(g/dL) (mean±SD)	11.30±1.51 (8.5-14.4)	12.60±1.59 (7.7-16.3)	12.95±1.90 (8.1-14.1)	13.30±1.49 (7-17.1)	<0.001
VitaminB12 (pg/mL) (mean±SD)	93±37.54 (21-184)	125±34.82 (44-198)	122.50±34.73 (50-146)	122±31.86 (64-196)	0.026
HoloTC (pmol/L) (mean±SD)	16.66±3.52 (2.07-20.51)	30.90±13.19 (21.08-90.66)	17.36±2.47 (14.07-20.69)	35.64±16.41 (21.79-108.33)	<0.001
MM (µmol/L) (mean±SD)	0.71±0.99 (0.42-6.47)	0.60±2.21 (0.16-27)	0.32±0.06 (0.22-0.39)	0.30±0.07 (0.16-0.58)	<0.001
HS (µmol/L) (mean±SD)	17.70±9.5 (9.3-47.4)	9.60±4.23 (3.61-24.47)	10.20±2.93 (5-13.80)	8.30±2.88 (2.46-15)	<0.001
WBC ($/mm^3$) (mean±SD)	8600±2538.65 (5800-17300)	7700±2375.53 (37000-20300)	6750±2629.34 (4200-12500)	6300±1770.82 (4000-10300)	<0.001
Neutrophil ($/mm^3$) (mean±SD)	2800±1338.44 (400-5800)	3200±2205.56 (600-22000)	3800±2232.11 (1400-9300)	3400±1288.06 (1500-6600)	0.130
Ferritin (ng/mL) (mean±SD)	23.20±42.1 (2-195)	18.70±61.22 (2-697)	22.95±21.95 (3-76)	19.10±13.82 (1-70)	0.228

Hb: Hemoglobin, HoloTC: Holotranscobalamin, MMA: Methylmalonic acid, Hcy: Homocysteine,WBC: White blood cell.

KÜ Tıp Fak Derg 2023;25(2):200-206
Doi: 10.24938/kutfd.1263790

Twenty patients were in Group1b and five patients were in Group1c. Eleven patients with low holoTC did not have any abnormality except low B12 (Group 3).

Group 2 consisted of 149 patients (53%) who have high/normal level of MMA and high/normal level of Hcy, but normal HoloTC.

Fifty-five patients (20%) had normal holoTC level with normal MMA and normal Hcy levels (Group 4).

When all groups were compared, vitamin B12 and Hb levels were found to be lower in Group 1a than other groups (respectively, $p<0.05$, $p<0.001$). Median Hb value of group 1a was 11.3±1.51 g/dl (range: 8.5-14.4 g/dl). Median vitamin B12 level of group 1a was 93±37.54 pg/mL (range: 21-184 pg/mL) (Table 1). When groups 2 and 3 were compared, though it was not statistically significant, vitamin B12 level was much lower in group 3 ($p>0.05$).

DISCUSSION

Serum vitamin B12 levels and vitamin B12 metabolites such as MMA and Hcy are used for the diagnosis of vitamin B12 deficiency. However, vitamin B12 levels and metabolites are affected by many conditions such as myeloproliferative diseases, transcobalamin deficiency, pregnancy, renal diseases and folate deficiency (15). Several studies in the literature indicate that the low level of holoTC is the earliest sign vitamin B12 deficiency. Bondu et al. reported that the level of holoTC was more valuable than serum vitamin B12 and might be used as an early indicator instead of vitamin B12 (16,17). However many studies, which do not support this hypothesis were also reported (10,18).

We assessed holoTC, Hcy, serum MMA to find the most reliable marker of vitamin B12 deficiency in children. A positive correlation between vitamin B12 level and holoTC, and a negative correlation between vitamin B12 and MMA and Hcy were found. A weak correlation was found between MMA and HoloTC, MMA and Hcy, Hcy and HoloTC.

Twenty-six percent of our patients had low holoTC levels. MMA and/or Hcy levels were elevated in only 50% of these patients. Moreover 15% of patients had low holoTC with normal MMA and Hcy. There are similar studies, which reported low level of serum vitamin B12 and holoTC, not accompanied by MMA and Hcy elevation (18). It might be due to holoTC being an earlier indicator than MMA and Hcy increase. Vitamin B12 levels in these patients were lower than the other groups.

Interestingly seventy-three percent of patients had normal holoTC levels while their vitamin B12 levels were deficiency state. Moreover, approximately 1/4 of these patients had high blood MMA and Hcy. Low levels of holoTC were noted in iron deficiency, folate deficiency and congenital dyserythropoietic anemia, which would reflect an increased uptake and utilization of B12 by erythroid cells (18,19). Sixty-eight patients in our study were detected combined vitamin B12 and iron deficiency. However, we did not find any significant difference in holoTC levels between patients with or without iron deficiency. Also, patients with folate deficiency were not included in the study. Low holoTC levels can also be seen in cases of TC gene polymorphism. Unfortunately, TC gene polymorphism was not evaluated in our study. This discrepancy may also be attributed to lack of reports that examine the exact holoTC cut-off in children.

Twenty percent of serum vitamin B12 deficient childrens' both MMA, Hcy and holoTC levels were within the normal reference range. It might be suggested that these patients did not have real vitamin B12 deficiency and that the serum vitamin B12 level could not able to entirely detect real vitamin B12 deficiency.

In our study, we showed that bioactive vitamin B12 deficiency was observed in only 26% of patients with serum vitamin B12 deficiency and both bioactive and functional deficiencies were observed in 13% of patients with bioactive vitamin B12 deficiency. Low holoTc levels were supported by high MMA in 80%. There are few studies in the literature that evaluate the relationship between vitamin B12 deficiency and holoTC, Hcy, and blood MMA in children (6,20,21). Studies were generally conducted with adult age group. The cut-off values of the tests that were used in those studies were

KÜ Tıp Fak Derg 2023;25(2):200-206
Doi: 10.24938/kutfd.1263790

different. This may explain the difference of our results from these studies.

Limitations of the study: There are not many studies in the literature about the relationship between holoTC, blood MMA and vitamin B12 and therefore cut-off values in children are not accurate (22). Unfortunately, we could not give a well-defined cut-off value for determining vitamin B12 deficiency since we did not have a control group. In addition, some of our patients had normal holoTC levels but low vitamin B12 levels. These patients should be evaluated in terms of TC gene polymorphism but genetic studies could not be performed in this study.

We conclude that in children, HoloTC alone should not be considered an accurate indicator, and other functional markers should be combined in the diagnosis of vitamin B12 deficiency. Further studies are needed with higher subjects to determine the cut-off values of MMA, holoTC, and reliable serum vitamin B12 level that accepted true cellular deficiency in children.

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Ethical Committee Approval: The study was approved by the Clinical Research Ethics Committee of Health Sciences University Ankara Pediatrics Hematology Oncology Training and Research Hospital (approval number 2016-043).

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