IMPORTANCE OF HOLOTRANSCOBALAMIN (HOLOTC) MEASUREMENTS IN EARLY DIAGNOSIS OF COBALAMIN DEFICIENCY, ESPECIALLY IN PATIENTS WITH BORDERLINE VITAMIN B_{12} CONCENTRATIONS

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ABSTRACT

Objective: *Subclinical vitamin B_{12} deficiency and adverse health outcomes are of general concern. Current biomarkers of vitamin B_{12} status are not always satisfactory to decide on a deficiency state. Recently, holotranscobalamin (holoTC) has been proposed as a useful alternative indicator of vitamin B_{12} status, however studies on its value in diagnosing cobalamin deficiency have not come to a conclusion yet. The purpose of this study is to investigate the usefulness of holoTC measurement together with total vitamin B_{12} measurement in diagnosing cobalamin deficiency, in a cross-sectional analysis.*

Material and Method: *Four hundred volunteers were grouped according to vitamin B_{12} levels as vitamin B_{12} deficient (vit B_{12}<193 pg/ml, n=168), borderline (vit B_{12}<193-300 pg/ml, n=100) and controls (vit B_{12}>300 pg/ml, n=132). These groups were divided into two subgroups (A and B) according to holoTC cut-off value (35 pmol/L). The diagnostic efficacy of vitamin B_{12}, holoTC and a combination of both measures were evaluated. Serum folate and homocysteine (Hcy) were used as indicators of vitamin B_{12} deficiency.*

Results: *Significantly higher Hcy and lower folate levels were observed in both vitamin B_{12} deficient and borderline B_{12} groups provided that holoTC levels were low.*

Conclusion: *Evaluation of vitamin B_{12} measures together with holoTC measures provides a more accurate diagnosis, especially in patients with borderline- B_{12} concentrations.*

Key Words: *Vitamin B12, holotranscobalamin, homocysteine, folate* **Nobel Med 2013; 9(2): 15-20**

KOBALAMİN EKSİKLİĞİNİN ERKEN TANISINDA, ÖZELLİKLE VİTAMİN B_{12} DÜZEYLERİ SINIRDA OLAN HASTALARDA, HOLOTRANSKOBALAMİN (HoloTC) ÖLÇÜMÜNÜN ÖNEMİ

ÖZET

Amaç: Subklinik vitamin B_{12} eksikliği ve neden olduğu sağlık sorunları geniş olarak ilgi gören bir konudur. Günümüzde B_{12} vitamini eksikliği için kullanılan parametreler eksiklik tanısı koymada her zaman yeterli olamamaktadır. Son çalışmalarda, B_{12} düzeylerinin daha iyi takibi için holotranskobalamin (holoTC)'in iyi bir alternatif olabileceği bildirilmektedir ancak bu konudaki çalışmalar henüz bir netlik kazanmamıştır. Bu çalışma ile total B_{12} vitamini ile holoTC düzeylerinin birlikte değerlendirilmesinin, kobalamin eksikliği tanısındaki yararının araştırılması hedeflenmiştir.

Materyal ve Metod: Bu çalışma için gönüllü olan 400 kişi, serum B_{12} vitamini düzeylerine göre B_{12} eksikliği (vit B_{12}<193 pg/ml, n=168), sınırda B_{12} (vit B_{12} <193-300 pg/ml, n=100) ve kontrol (vit B_{12}>300 pg/ml, n=132) olmak üzere gruplandırılmıştır. Gruplar holoTC cut-off değerine göre (35 pmol/L) kendi içlerinde ikişer alt gruba (A ve B) ayrılmıştır. B_{12} vitamini, holoTC ve bu iki parametrenin birlikte değerlendirilmesinin tanıdaki etkinliği araştırılmıştır. Serum folik asit ve homosistein (Hcy), B_{12} vitamini eksikliğinin göstergeleri olarak kullanılmıştır.

Bulgular: Hem B_{12} vitamini eksikliği olan hem de sınırda B_{12} düzeyine sahip kişilerde holoTC düzeyleri de düşük ise Hcy ölçümleri anlamlı olarak yüksek ve folat düzeyleri anlamlı olarak düşük bulunmuştur.

Sonuç: Vitamin B_{12} ölçümlerinin holoTC ölçümleri ile birlikte değerlendirilmesi özellikle sınırda-B_{12} düzeylerine sahip hastalarda eksiklik tanısı için daha doğru yol göstermektedir.

Anahtar Kelimeler: Vitamin B_{12}, holotranskobalamin, homosistein, folat **Nobel Med 2013; 9(2): 15-20**

INTRODUCTION

Vitamin B_{12} (cobalamin; vit B_{12}) deficiency is a major public health problem, particularly among the elderly. Vitamin B_{12} deficiency can be related to a prolonged insufficient intake, disturbed absorption, increased requirements, or an accelerated loss of the vitamin[1]. Early detection of this disorder is important for preventing probably irreversible neurological complications.

Vitamin B_{12} is required by all cells for its role in one-carbon metabolism and in DNA-synthesis and maintenance. Only two vitamin B_{12}-dependent enzymes are known in humans: methionine synthase and L-methylmalonyl-CoA mutase. The former is crucial in formation of methionine from homocysteine (Hcy) and it requires methylcobalamin and folate as cofactors, where methylfolate transfers a methyl group to vitamin B_{12}, which then transfers it to homocysteine, converting homocysteine to methionine[2]. L-methylmalonyl-CoA mutase reaction, on the other hand, needs adenosylcobalamin in the catalysis of methylmalonyl-CoA to succinyl-CoA. Serum concentrations of methyl malonic acid (MMA) and Hcy are, therefore, considered to be metabolic indicators of vitamin B_{12} status. Vitamin B_{12} is also necessary to remove the methyl from methylfolate, a circulating storage form of folate, converting it to a metabolically active form, necessary for one carbon transfers[2]. Therefore, Vitamin B_{12} and folate metabolism are closely related and serum homocysteine concentrations are affected by the concentrations of these vitamins.

Total vitamin B_{12} concentrations below 148 pmol/L (<200 pg/mL) are generally considered deficient. This range is diagnostically useful for the majority of cases of vitamin B_{12} deficiency; however, a proportion of individuals with vitamin B_{12} concentrations that would be considered deficient exhibit no clinical or biochemical evidence of deficiency[3]. Conversely, neuropsychiatric and metabolic abnormalitiescan occur with plasma vitamin B_{12} concentrations within the reference interval.[3-5]

Since vitamin B_{12} deficiency may be overlooked when using total serum B_{12} as a screening test, measurement of total serum B_{12} is considered a poor predictor of vitamin B_{12} status.[6] Measurement of serum concentration of MMA alone or in conjunction with Hcy has partly resolved the demand for a sensitive and a specific test for vitamin B_{12} deficiency.[7] On the other hand, the artificial increase of serum concentrations of MMA and Hcy in some clinical conditions is a major limitation of these parameters.[8,9] Both parameters correlate to serum concentration of creatinine and increase even in mild degrees of renal insufficiency.[8-10]

Recently, methods that measure serum concentrations of holotranscobalamin (holoTC), the transcobalamin-bound B_{12}, have become available. HoloTC assay is considered a convenient approach that measures the active B_{12} concentration, that is the only part available for the cell-use.[6] Various studies have reported the use →

of holoTC in evaluating vitamin B_{12} status in different clinical settings, however this parameter is not in routine clinical use yet. In this study, we investigated the use of serum concentrations of holoTC in predicting vitamin B_{12} status in routine blood specimens that were referred to the laboratory for total cobalamine and folic acid testing.

MATERIAL and METHOD

We carried out this analysis in participants who referred to the routine laboratory for vitamin B_{12} and folate measurements. Participant recruitment and study procedures were approved by the Ethics Committee of Uludağ University Hospital, and written informed consent was obtained from all study participants. Sera from 400 volunteers were classified into 3 groups according to the vitamin B_{12} concentrations, which were evaluated in terms of the reference range of the method used for vitamin B_{12} measurement in the study period: subjects with vitamin B_{12} levels <93 pg/ml were named low B_{12} group (Group 1, n=168), while subjects with vitamin B_{12} levels between 193-300 pg/ml were accepted as borderline B_{12} group (Group 2, n=100) and subjects with B_{12} levels >300 pg/ml consisted the controls (Group 3, n=132).

To examine the advantage of measuring vitamin B_{12} and holoTC together in evaluating vitamin B_{12} status, the study groups were further divided into two subgroups (A and B) according to the suggested cut-off value of holoTC, which was 35 pmol/L.[7,11,12] Vitamin B_{12} and holoTC levels in subgroups are defined below:

Group 1A (N=163): Vit B_{12} <193pg/ml; HoloTC<35 pmol/L
Group 1B (N= 5): Vit B_{12} <193pg/ml; HoloTC>35 pmol/L
Group 2A (N=73): Vit B_{12} =193-300 pg/ml; HoloTC<35 pmol/L
Group 2B (N=28): Vit B_{12} =193-300 pg/ml; HoloTC>35 pmol/L
Group 3A (N=37): Vit B_{12} >300 pg/ml; HoloTC<35 pmol/L
Group 3B (N=94): Vit B_{12} >300 pg/ml; HoloTC>35 pmol/L

Blood sampling and biochemical assays

Single blood samples were drawn following 12 hours fasting. Blood samples were centrifuged within 30 min at 3,000 g for 10 min to obtain serum samples. Vitamin B_{12} and folic acid levels were measured by competitive chemiluminescent enzyme immunoassay on the same day, on immulite 2500 autoanalyzer, using kits obtained from Siemens, USA. Sera that were appropriately aliquoted were stored at -80°C until they were analyzed for holoTC and Hcy measurements. HoloTC levels were measured by microparticle enzyme immunoassay, on AxSYM Systems, Abbott, USA, while Hcy levels were measured by microparticle enzyme immunoassay, on Immulite 2500 autoanalyzer, using kits obtained from Siemens, USA.

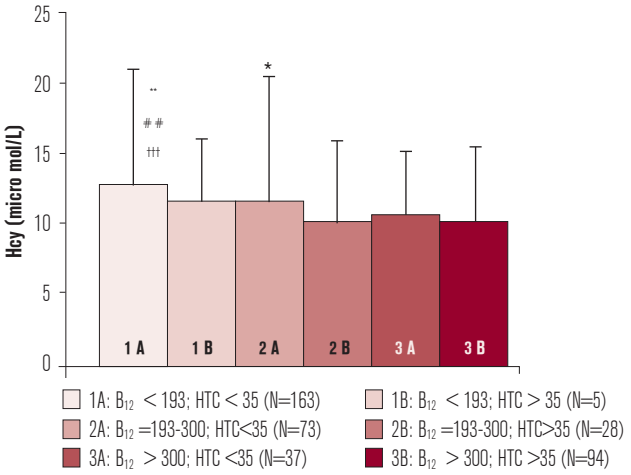

Figure 1: Homocysteine levels in subgroups organized according to vitamin B_{12} and holotranscobalamin concentrations *: p<0.05; **:p<0.01 (significantly different from 2B) ##: p<0.01 (significantly different from 3A) †††: p<0.001 (significantly different from 3B)

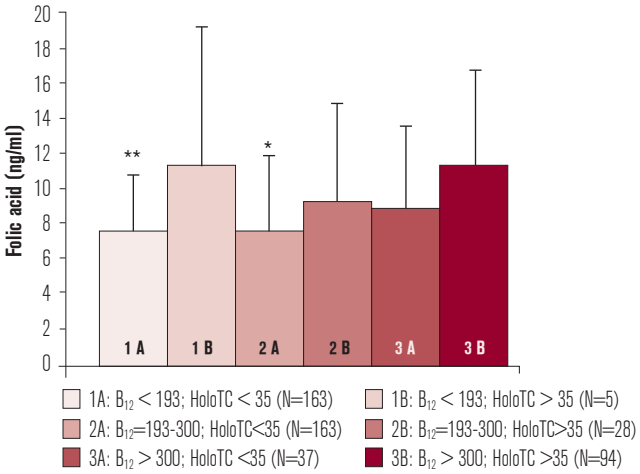

Figure 2: Folic acid levels in subgroups organized according to vitamin B_{12} and holotranscobalamin concentrations *: p<0.05; **: p<0.01 (significantly different from 3B)

Statistical Analysis

Statistical analysis was performed using statistical software (SPSS for Windows, version 13.0; SPSS; Chicago, IL). After assessing for approximate normal distribution, all continuous variables were summarized in terms of means (standard error). The difference between the groups was compared using Kruskal Wallis and Mann-Whitney U tests. Spearman correlation analysis was performed to test the relationship between the parameters. p<0.05 was considered statistically significant. →

Table 1: Serum vitamin B_{12}, HoloTC, folic acid, and Hcy concentrations of the study groups

	Groups			Kruskal Wallis
	Controls (<300 pg/ml) (n=132)	Low-B_{12} (<193 pg/ml) (n=168)	Borderline B_{12} (193-300 pg/ml) (n=100)	p
Vitamin B_{12} (pg/ml)	488±166	166±14.5***	237±34.2***††	<0.001
HoloTC (pmol/L)	50.1±25.9	18.6±7.5***	30.0±15.4*** †††	<0.001
Hcy (µmol/L)	9.6±4.9	12.8 ±7.9***	11.4±8.1	=0.001
Folic acid (ng/ml)	10.0±5.8	7.6±3.6***	8.1±4.6*	<0.001

HoloTC: Holotranscobalamin, Hcy: Homocysteine Values in the last column indicate the significance of the difference between groups by Kruskal Wallis test. Superscripts indicate the difference by Mann-Whitney U test. *: p<0.05; ***: p< 0.001 (significantly different from Controls), ††: p<0.01; †††: p<0.001 (significantly different from Low-B_{12} group)

RESULTS

Of 400 participants, 301 were women (75%) and 99 were men (25%) with mean ages 41±16 and 43±20, respectively. Serum vitamin B_{12}, holoTC, folic acid and Hcy concentrations of the three groups are shown in Table 1. According to the results, vitamin B_{12} and holoTC levels were significantly different among the three groups (p<0.001). Homocysteine levels were significantly higher in low B_{12} subjects (Group 1) compared to the borderline B_{12} subjects (Group 2) and controls (Group 3), (p<0.01 and p<0.001, respectively). Folic acid concentrations were significantly lower in Group 1 (p<0.001) and Group 2 (p<0.05) compared to the controls.

The comparison of Hcy levels of these 6 subgroups are presented in Figure 1. Hcy levels were not different between low vitamin B_{12} groups, 1A and 1B. However, in the two subgroups of Group 2, where vitamin B_{12} levels were in borderline, Hcy levels were significanly higher in Group 2A compared to that of Group 2B (p<0.05). Also, Hcy levels of Group 1A were significanly higher than those of Group 2B (p<0.01), Group 3A (p<0.01), and Group 3B (p<0.001).

The difference in folic acid concentrations of 6 subgroups are presented in Figure 2. Folic acid levels were significantly lower in Group 1A and Group 2A compared to that of Group 3B (p<0.01 and p<0.05, respectively).

The Spearman's correlation coefficients of Vit B_{12} with holoTC was found to be 0.71 (p<0.001), with Hcy -0.25 (p<0.001), and with folic acid 0.16 (p<0.001). HoloTC was negatively correlated to Hcy (p<0.001) and positively correlated to folic acid (p<0.001), while Hcy and folic acid measurements displayed negative correlations (p<0.001) (Table 2).

DISCUSSION

Undiagnosed vitamin B_{12} deficiency is quite common, therefore tests other than (or in addition to) total vitamin B_{12} measurements are needed to asses cobalamin deficiency.[1] This study was conducted to investigate the usefulness of holoTC measurement together with total vitamin B_{12} measurement in diagnosing cobalamin deficiency.

Early diagnosis of vitamin B_{12} deficiency has been widely studied and various cut-off values were reported.[13-15] In the light of the studies carried out, some investigators suggested vitamin B_{12} status to be classified as deficient, suspected deficient and undeficient (normal), however exact limits are not defined due to the methodological and population variabilities. Reference intervals can vary quite markedly between laboratories. While Nexo et al. suggest the reference interval for vitamin B_{12} as 200-650 pmol/L, Herbert et al. note a range of 148-666 pmol/L (200-900 pg/ml).[15,16] Snow states that B_{12} assays discriminate poorly at levels between 100-400 pg/mL (75-300 pmol/l), while Swain suggests that high levels rule-out deficiency, between 150-300 pmol/l require confirmation, levels below 150 pmol/l probably do not need confirmation.[17,18] Schneede suggests follow-up testing when B_{12} values fall between 150-250 pmol/l, whereas Klee suggests follow-up testing when B_{12} falls between 110-220 pmol/l and Herrmann estimates deficiency can occur up to B_{12} levels of 300 pmol/L.[6,19,20]

Studies that were carried out for assessment of cobalamin reference values in Turkey, have reported different results as well: In 2000, Tanyalçın et al., reported vitamin B_{12} reference values as 101-666.7 pg/ml for women and 100-699.57 pg/ml for men, while in 2004, İlçöl et al. stated reference values as 319-1996 pg/ml for women and 214-1544 pg/ml for men in Bursa.[21,22] Different lower limits (142-953 pg/ml) were observed in the study by Köseoğlu et al. in 2010, for Izmir region in Turkey.[23]

As is seen, even for the similar populations, reference studies are not always sufficient to establish exact limits for diagnosis of B_{12} deficiency. Of course, the methods and systems used for measurement are as important as the populational variances in terms of the factors affecting reference intervals. Therefore, it is recommended that each laboratory should establish its own reference ranges. However, assessment of reference intervals for every laboratory is not cost-effective, since it is difficult especially for small laboratories to find suitable volunteers and to study costly parameters such as vitamins with everchanging methodologies and instrumentation. At this point, use of the manufacturer's reference intervals together with some additional tests would provide reliable results.

In the present study, the reference limits of the method used for vitamin B_{12} concentrations were 193-982 pg/ml. Subjects with vitamin B_{12} levels below the reference limit were classified in the cobalamin deficient group, →

while subjects with vitamin B_{12} levels between 193-300 pg/ml (142-221 pmol/L) were in the suspected area and subjects with B_{12} levels above 300 pg/ml were controls.

According to the results, vitamin B_{12} and holoTC concentrations in the three groups were significantly different (Table 1). Also, as expected, these two related parameters were significantly correlated (Table 2). In the present study homocysteine levels were monitored as an indicator of vitamin B_{12} status and were significantly higher in the B_{12} deficient group compared to groups 2 and 3, and the significance was more pronounced compared to group 3, indicating that the level of deficiency affected the level of disturbance in the vitamin B_{12}-dependent reactions. The significantly negative correlations of Hcy with B_{12} and holoTC levels support this statement and are in accordance with the results of Loikas et al.[24]

Miller et al. treated the metabolic indicators (Hcy and MMA) of vitamin B_{12} status as continuous variables and performed data analysis in 4 groups as: both B_{12} and holoTC low; both B_{12} and holoTC normal; B_{12} low-holoTC normal and holoTC low-B_{12} normal.[11] They found that those with low concentrations of both total B_{12} and holoTC, had higher MMA and Hcy concentrations than those with low concentration of only one or neither of the measures of vitamin B_{12} status. In the present study, a similar data analysis was performed by evaluating the Hcy and folic acid levels in subjects classified into 6 subgroups as described in "Subjects and Method". Namely, the cut-off for holoTC was accepted as 35 pmol/L and each group was divided into 2 subgroups depending on the holoTC concentrations. According to this classification, Group 1A consisted of both B_{12} and holoTC low subjects, Group 1B consisted of B_{12} low-holoTC normal subjects, Group 2A consisted of B_{12} suspected-holoTC low subjects, Group 2B consisted of B_{12} suspected-holoTC normal subjects, Group 3A consisted of B_{12} normal-holoTC low subjects and Group 3B consisted of both B_{12} and holoTC normal subjects. Our results, in agreement with Miller et al.'s, reinforce that higher Hcy levels are measured in people with both total B_{12} and holoTC deficiency (Figure 1).[11] The present study provides further evidence for the literature to discuss on the borderline total B_{12} measures by evaluating the suspected-deficient group in the same manner. According to the findings of the present study, when total B_{12} concentrations are in the 193-300 pg/ml range, low holoTC concentrations indicate a deficiency state as evidenced by higher Hcy levels (Figure 1). In other words, although total B_{12} measures alone do not indicate a deficiency, an evaluation in combination with low holoTC levels may signal a deficiency state. It is well known that vitamin B_{12} and folic acid are common coenzymes of the methionine syntase reaction and that cobalamine deficiency is accompanied by decreased serum folate concentrations.[16] In the present work, folic acid levels were significantly lower in group 1 and 2 compared to group 3 (Table 1). Also, folic acid levels were significantly correlated to vitamin B_{12} and holoTC concentrations ($p<0.001$) verifying the relation between folic acid and cobalamin (Table 2). The negative and significant correlation between folic acid and Hcy was in accordance with the findings of Herrmann et al.'s study in 2000.[25] When folic acid concentrations were evaluated in subgroups organized according to vitamin B_{12} and holoTC concentrations, the results showed that folic acid concentrations were lower in low-holoTC subgroups (1A and 2A) of B_{12} deficient and suspect-deficient groups (Figure 2). This may be interpreted as serum folic acid concentrations being more sensitive to holoTC concentrations in cobalamine deficiency states.

Table 2: Correlations between the parameters[#]

n=400	Vitamin B_{12}	HoloTC	Hcy	Folic acid
Vitamin B_{12}	1	0.711***	-0.253***	0.163***
HoloTC		1	-0.254***	0.167***
Hcy			1	-0.278***
Folic acid				1

#: Data are Spearman correlation coefficients. HoloTC: holotranscobalamin, Hcy: homocysteine. ***: $p<0.001$

In summary, numerous undesirable effects caused by either vitamin B_{12} deficiency or resultant hyperhomocysteinemia, can be prevented by the early diognosis of the deficiency state. The results of this cross-sectional study in the Turkish population emphasize that evaluation of vitamin B_{12} measures together with holoTC measures would be advantageous for a more accurate diagnosis, especially in borderline-B_{12} deficiencies. Further investigations on evaluation of holoTC in cobalamine deficiency in different clinical settings would be valuable to provide holoTC for routine diagnostic use.

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DELIVERING DATE: 21 / 10 / 2011 • **ACCEPTED DATE:** 14 / 03 / 2012

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