Effects of Immune Complexes on Holotranscobalamine Assay of Vitamin B\textsubscript{12} Deficiency in Myeloproliferative Disorders

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ABSTRACT
In myeloproliferative disorders (MPDs), vitamin B\textsubscript{12} levels are measured falsely elevated with conventional methods due to increased carrier protein synthesis. Holotranscobalamin (HoloTC) assay is a first-choice method for detecting true vitamin B\textsubscript{12} deficiency in MPDs. Our aim was to determine effects of immune complexes on HoloTC assay. This is a cross-section study. Vitamin B\textsubscript{12} levels in 61 patients with myeloproliferative disorders were measured by both electrochemical immunoassay and HoloTC assay. The HoloTC cutoff was greater than 35 pmol/L. HoloTC assay for each sample were repeated after polyethylene glycol (PEG) treatment to exclude IgG, IgA and IgM type immune complexes. Also, methylmalonic acid, folate, homocysteine, liver, and kidney function tests were obtained. Methylmalonic acid test showed that 42 patients (68.9%) had vitamin B\textsubscript{12} deficiency. Vitamin B\textsubscript{12} levels by HoloTC assay decreased by 19.2±11.28% in essential thrombocytosis, 40.0±9.39% in chronic myeloid leukemia, 30.9±14.62% in myelofibrosis and 21.2±11.55% in polychromatophilic vera patients after PEG treatment. There was significant difference between the averages of groups (p<0.01). Methylmalonic Acid Test was used as the B\textsubscript{12} status variable. The comparison of ROC curves of HoloTC before and after PEG showed no statistically significant difference between area under curves. The optimum cut-off points for both HoloTC before and after PEG were 40.6 pmol/L and 32.1 pmol/L, respectively. Immune complexes may have some effect on HoloTC assay which has been recently reported to have a superior diagnostic accuracy for vitamin B\textsubscript{12} deficiency in patients with MPDs. Although exclusion of immune complexes did not improve its diagnostic performances, effects of exclusion were significantly different between subgroups of MPDs. Keywords: Immune complex, Holotranscobalamin, Vitamin B\textsubscript{12}, Myeloproliferative disorders

ÖZET
İmmün Kompleksler Myeloproliferatif Hastalıklarında B\textsubscript{12} Vitamin Eksikliğinin Holotranskobalamin Testini Etkileyebilir

Myeloproliferatif hastalıklarda (MPH), artan taşıyıcı protein sentezi nedeniyle, vitamin B\textsubscript{12} düzeylerinin, geleneksel yöntemlerle ölçümünde bazı zorluklar yarayanmakdadır Holotranskobalamin (HoloTC) testi MPH’da gerçek B\textsubscript{12} eksikliklerini tespit etmek için ilk seçilerek testlerden biridir. Bu çalışmadaki amacımız immunkomplekslerin HolotTC testi üzerindeki etkilerini tespit etmektir. Bu araştırma, Kesitsel bir çalışmadır. Hastanemizin Hematoloji Kliniği başvuran ve MPH tanısı ile takip edilen 61 hastanın Vitamin B\textsubscript{12} düzeyleri hem elektro-kimyasal immünassay hem de HoloTC testi ile çalısdı. Ayni örnekler immunkompleksleri çözütmek için polietilen glikol (PEG) ile muamele edildikten sonra HolotTC testi tekrar edildi. Ayrıca zamanda, metilmalonik asid, folat, homocystein düzeyleri ile birlikte karaciğer ve böbrek testleri yapıldı. Metilmalonik asid testi ile 42 hastanın (%68.9), vitamin B\textsubscript{12} eksikliği tespit edildi. PEG muameleinden sonra HolotTC testi ile Vitamin B\textsubscript{12} düzeyleri essanlısyel trombositos hastalarında %19.2±11.28, kronik myeloid lösemi hastalarında %40±9.39, myelofibrosis'te %30±14.62% ve polistemiya vera (PV) hastalarında ise 21.2±11.55% oranında azaldı. Grupların ortalamaları arasında istatistiksel anlamli fark bulunmu (p<0.01). B\textsubscript{12} durum değişikleri olarak Metilmalonik Asit Testi alındı. PEG uygulaması sonrası HolotTC testlerinin ROC eğrileri karşılaştırıldı. Her iki durumun eğri altında kalan alanlar istatistiksel olarak anlamli bir farklık göstermedi. Optimum “cut-off” noktaları PEG öncesi HolotTC için 40.6 pmol/L; PEG sonrası için ise 32.1 pmol/L olarak tespit edildi. Son zamanlarda myeloproliferatif hastalıklarda tanısal doğruluğu gösterimi olan HolotTC testi, bu hastalıklarda olguna immunkomplekslerden etkileyebilir. Immunkomplekslerinin ekarte edilmesi bu testin diagnostik performansını etkilememekle birlikte etkileri hastalığın gruplanmasına göre anlamlı olarak farklı göstermektedir. Keywords: Immune complex, Holotranscobalamin, Vitamin B\textsubscript{12}, Myeloproliferative disorders

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INTRODUCTION

Vitamin B₁₂ is a micronutrient that plays a vital role in some biologic functions.¹ Worldwide, moderate B₁₂ deficiency is common. Impaired vitamin B₁₂ status in human body has been associated with various diseases including megaloblastic anemia and neuropsychiatric disorders.²³ Due to serious complications of vitamin B₁₂ deficiencies such as bone marrow failure and demyelinating neurologic disease, early diagnosis and treatment of vitamin B₁₂ deficiencies is very important.³⁴

After absorption of B₁₂, it is distributed in the body by a complex set of carrier proteins, receptors and transporters. In the circulation, vitamin B₁₂ is transported as bound to haptocorrin (80%), and transcobalamin (20%).¹ Since the receptors that recognize haptocorrin-bound B₁₂ are located only in the lung, the transcobalamin-bound B₁₂ in plasma seems to be available to the body’s cells. Vitamin B₁₂ constitutes a complex with transcobalamin transport protein which is called holotranscobalamin (HoloTC), and HoloTC receptors are present in all tissues [5]. Transcobalamin-bound vitamin B₁₂, or HoloTC, is biologically active form of vitamin B₁₂.

Myeloproliferative disorders (MPDs) which consist of polycythemia vera (PV), essential thrombocythemia (ET), idiopathic myelofibrosis (IMF) and chronic myelogenous leukemia (CLL) are characterized by proliferation of hematologic cell lines.⁶ Due to rapid cell proliferation in myeloproliferative disorders folate and vitamin B₁₂ may deplete and cause hyperhomocysteinemia.⁷ However, it is not always easy to determine true levels of vitamin B₁₂ in this group of diseases. Due to the high carrier protein levels, false elevated vitamin B₁₂ results may be measured by using conventional methods.⁸ Furthermore, total serum vitamin B₁₂ concentration alone does not reliably reflect vitamin B₁₂ status. Total vitamin B₁₂ serum levels can be measured as in normal range despite low level of active vitamin B₁₂. HoloTC assay provides the advantage of measuring active B₁₂ levels.⁹ The decreased levels of methylmalonic acid (MMA), and increased levels of homocysteine are well-known biomarkers of vitamin B₁₂ deficiency.⁷⁹ Homocysteine levels may also rise in case of folate deficiency.⁸⁹ According to literature, measurement of MMA levels has been recommended as a gold standard for vitamin B₁₂ deficiency; but it should be kept in mind that the MMA levels may be affected by renal failure and testing MMA levels in serum requires more expensive techniques.⁹ Taking all these into account, HoloTC assay is good candidate for evaluating vitamin B₁₂ status in the body.⁸ In previous study, we showed that HoloTC method is one of the first-choice assay for detecting true vitamin B₁₂ deficiency in MPDs.¹⁰ In our study, prevalence of vitamin B₁₂ deficiency was 69%, despite high serum vitamin B₁₂ levels by measuring conventional methods. Our results demonstrated that 40.6 pmol/L or less vitamin B₁₂ levels by HoloTC assay and homocysteine levels greater than 14 mol/L were the best cutoff levels. At these cutoff levels, the sensitivities were 75% and 70%, the specificities were 80% and 68%, and positive predictive values were 88% and 80% for HoloTC assay and homocysteine testing, respectively.

In the literature, the increased amount of immune complexes in MPDs has been reported.¹¹,¹² However, the interference of immune complexes with HoloTC assay has not been studied in detail. It is known that polyethylene glycol (PEG) treatment precipitates immune complexes, and thus eliminates their effects. The aim of the present study was to determine effects of immune complexes on HoloTC assay.

PATIENTS AND METHODS

Subjects:

A total of 61 patients followed up in our hematology department with a diagnosis of myeloproliferative disorders were included in the study. The approval of Sakarya University School of Medicine Ethical Committee for Clinical Research was provided before the study (no. 16214662.050.01.04/39). The patients with chronic gastrointestinal disorders such as Crohn disease, prior gastric or ileal resection, using concurrent metformin, a purely vegetarian diet, or serum creatinine levels greater than 1.1 mg/dL for women and greater than 1.3 mg/dL for men were excluded.
Assays:
After fasting blood samples were obtained, they were separated and stored at –80°C. Serum MMA analysis for vitamin B\textsubscript{12} status was performed using liquid chromatography–tandem mass spectrometry (HPLC 1200 binary pump and 1200 Autosampler; Agilent, Santa Clara, CA) and a detector (API 5500; ABSciex, Framingham, MA). The lower limit of quantitation was 40 nmol/L, linearity was tested up to 10 000 nmol/L, and coefficient of variation was less than 5.2%. The insert from the manufacturer suggested that MMA concentration greater than 0.27 nmol/mL was elevated; in this study, we used the age and sex cut offs for MMA defined by the US Centers for Disease Control and Prevention: age, 20 to 39 years: greater than 0.27 nmol/mL; 40 to 59 years: greater than 0.30 nmol/mL; and older than 60 years: greater than 0.45 nmol/mL.\textsuperscript{13} The HoloTC levels were measured using an automated commercial immunoassay (Architect i2000SR, Active B\textsubscript{12}; Abbott Laboratories, Abbott Park, IL). The HoloTC cutoff was greater than 35 pmol/L according to information from the manufacturer. HoloTC assay for each sample were repeated after PEG treatment to exclude immune complexes. A total of 2.4 g of PEG 6000 was dissolved in 10 mL of 0.9% saline. PEG and samples were mixed at 1:1(V/V) ratio; incubated at room temperature for 10 minutes and then centrifuged for 20-30 minutes at 2000 rpm.\textsuperscript{14} Vitamin B\textsubscript{12} was assayed in the supernatants using auto-analyzer. Results are expressed as % difference of HoloTC result after PEG treatment.

**Table 1. General distributions of vitamin B\textsubscript{12} deficiency in myeloproliferative disorders groups**

|                | ET (n= 31) | PV (n= 7) | MF (n= 9) | CML (n= 14) |
|----------------|-----------|-----------|-----------|-------------|
| Age (years) median (min-max) | 62 (35-92) | 65 (45-68) | 65 (46-84) | 52 (36-88) |
| B\textsubscript{12} Deficient (MMA) % | 74% | 57% | 55% | 71% |
| B\textsubscript{12} Deficient (HoloTC) % | 32 % | 57% | 66% | 57% |
| B\textsubscript{12} Deficient (HoloPEG) % | 54% | 57% | 77% | 78% |
| B\textsubscript{12} Deficient (Total B\textsubscript{12}) % | - | - | - | 8% |

ET= Essential thrombocytosis; PV= Polycythemia vera; MF= Myelofibrosis; CML= Chronic myeloid leukemia

Statistical Analysis
Data analysis was performed with statistical software (SPSS, version 10.0 [SPSS Inc, Chicago, IL]; MedCalc 14.10.2 evaluation version [MedCalc Software, Ostend, Belgium]) was used for pairwise comparison of ROC curves. All differences associated with a chance probability of .05 or less were considered statistically significant.

RESULTS
A total of 61 patients included in the study, were diagnosed ET (n:31), PV (n= 7), IMF (n= 9), CML (n= 14). They were followed-up at our clinic for 24 months (median) (max-min: 3-24 months). Median duration from diagnosis of a myeloproliferative disorder was 6 years (max-min: 2 months-18 years). Median age (max-min) of patients group was 62 (35-92) years in ET, 65 (45-68) years in PV, 65 (46-84) years in MF, and 52 (36-88) years in CML (Table 1). In patients who had vitamin B\textsubscript{12} deficiency, Two patients had mild folate deficiency (folate, 2.7 - 3.0 ng/mL). Cut-off value for serum folate was 3.1 ng/mL according to the manufacturer.

Vitamin B\textsubscript{12} deficiency was diagnosed by using the MMA test as gold standard. The age and sex cut-offs of MMA defined by the US Centers for Disease Control and Prevention was used, 42 patients (68.9%) were diagnosed as vitamin B\textsubscript{12} deficient. HoloTC assay results showed vitamin B\textsubscript{12} deficiency in 27 patients (44.6%) by using cut-off value, 35 pmol/L, according to insertion of the manufacturer. HoloTC assay repeated after PEG treatment to exclude immune complexes were called
here as HoloPEG. HoloPEG assay results showed vitamin B12 deficiency in 39 patients (63.9%) by using same cutoff value, 35 pmol/L. In the MPDs groups, the vitamin B12 deficiency rates according to diagnostic test type are given in (Table 1). HoloPEG results were expressed as % difference of HoloTC (HoloPEG-HoloTC/HoloTC*100). The differences% results demonstrated that vitamin B12 levels by HoloTC assay decreased by %19.2±11.28 in ET, %40±9.39 in CML, %30.9±14.62 in MF and %21.2±11.55 in PV patients after PEG treatment (Figure 1). There was significant difference between the averages of groups with One way ANOVA (p<0.01). By using Tukey Test the % differences of HoloTC results in patients with CML and MF found significantly higher than the other diseases groups (p<0.01).

At the cutoff value of 35 pmol/L, the sensitivity was 55% and 81% for HoloTC and HoloPEG respectively; the specificity was 78.7% and 57.4% for HoloTC and HoloPEG respectively (Table 2).

The diagnostic performance of HoloTC and HoloPEG were evaluated by using Receiver Operating Characteristic (ROC) curve analysis (Figure 2). MMA test was used as the B12 status variable. The pairwise comparison of ROC curve analysis for HoloTC and HoloPEG showed no statistically significance between area under curve (AUC) values of assays. The AUCs (±standard error) were 0.804 (±0.0710) (95% CI- 0.664 to 0.904) and 0.743 (±0.0837) (95% CI- 0.596 to 0.858) for HoloTC and HoloPEG respectively. The AUC was similar for HoloTC before and after PEG treatment (p>0.05). A ROC curve analysis was performed to evaluate the optimum cut-off point for both HoloTC and HoloPEG measurements. It was found to be 40.6 pmol/L with a sensitivity of 72.5% and specificity of 78.6%; for HoloTC. The optimum cut off point was 32.1 pmol/L with a sensitivity of 75.5% and specificity of 71.4% for HoloPEG (Ta-

| Table 2. Diagnostic test estimates for measurements of HoloTC and HoloPEG levels |
|---------------------------------|----------|------------|----------------|----------|
| Cut off ≤ 35 pmol/L            | HoloTC   | Sensitivity% | 95% CI        | Specificity % | 95% CI   |
|                                |          | 55          | 38.50 to 70.70| 78.71        | 49.20 to 95.30 |
|                                | HoloPEG  | 81          | 64.80 to 92.00| 57.43        | 64.80 to 92.00 |
| Cut off ≤ 40.6 pmol/L          | HoloTC   | 72.5        | 56.10 to 85.40| 78.57        | 49.20 to 95.30 |
|                                | HoloPEG  | 75.48       | 58.80 to 88.20| 71.43        | 41.90 to 91.60 |
ble 2). The data comparison graphs of HoloTC and HoloPEG were demonstrated in (Figure 3).

We also analyzed precision of serum HoloTC assay before and after PEG treatment. For the low control, the mean HoloTC value within-run was 16.21±0.70 (mean±SD) pmol/L (intraassay coefficient of variation-CV: 4.39%) and 9.8±0.68 pmol/L (intraassay CV: 6.93%) before and after PEG treatment, respectively. The inter-assay coefficient of variation was 8.16% and 8.79% for HoloTC and HoloPEG, respectively. For the high control, the mean HoloTC value within-run was 50±3.07 (mean±SD) pmol/L (intraassay CV: 6.14%) and 31±2.74 pmol/L (intraassay CV: 8.83%) before and after PEG treatment, respectively. The inter-assay CV was 8.14% and 8.83% for HoloTC and HoloPEG, respectively.

DISCUSSION

Vitamin B\textsubscript{12} deficiency is a problem in myeloproliferative disorders due to rapid proliferation of hematologic cell lines.\textsuperscript{6} However, there are some difficulties on measuring of vitamin B\textsubscript{12} levels in MPDs because of elevated carrier protein of vitamin B\textsubscript{12}. HoloTC assay measures active form of vitamin B\textsubscript{12}. In the literature, the increased amount of immune complexes in MPDs has been reported.\textsuperscript{11} According to our results, vitamin B\textsubscript{12} levels measured by HoloTC assay. The % differences of HoloTC results were statistically higher in patients with CML and MF. However, the pair-wise comparison of ROC curves of HoloTC assay after or before PEG treatment showed no differences on their diagnostic performances.

By using conventional methods which measures total vitamin B\textsubscript{12}, the vitamin B\textsubscript{12} levels in patients with MPDs might be in normal range. Whereas vitamin B\textsubscript{12} are measured by reference methods such as MMA, the patient may have low levels of vitamin B\textsubscript{12} in this group of patients.\textsuperscript{10} The elevation of carrier protein may mask vitamin B\textsubscript{12} deficiency. Although MMA testing is reference methods for vitamin B\textsubscript{12} measurement, the MMA levels may be affected by renal failure and testing MMA levels in serum requires more expensive techniques.\textsuperscript{9} HoloTC assay is introduced as the alternative test to measure active form of vitamin B\textsubscript{12}. In previous study, we reported that HoloTC assay is a first-choice method for detecting true vitamin B\textsubscript{12} deficiency in MPDs.\textsuperscript{10}

In the literature, several studies indicating increases in the levels of immune complexes in myeloproliferative diseases have been reported.\textsuperscript{11,12} The role of immune complexes in myeloproliferative diseases has not been clarified yet. It has been demonstrated that there was not any correlation between intraplatelet PDFG values, and serum immune complex levels after removal of immune complexes by plasmapheresis.\textsuperscript{15} Immunoglobulin-TC-B\textsubscript{12} complexes causing or contributing to elevated serum B\textsubscript{12} were first reported in 1968 by a Danish group who were investigating patients with pernicious anemia treated with intramuscular B\textsubscript{12}.\textsuperscript{12} According to our results, vitamin B\textsubscript{12} levels measured by HoloTC decreased in varying degrees after PEG treatment. The most significant decrease was in patients with CLL and MF. Effect of immune complexes in CML and MF were significantly different from those in PV and ET. This condition may be attributed to relatively increased production of immune complex compared to other MPDs. Also, since MPD is a highly heterogeneous group of diseases, differences in the course of the diseases and treatment protocols may result in more production of immune complexes in some group of MPDs.
Polyethylene glycol precipitation has been extensively employed in the detection of analyte-antibody complexes which cause clinical confusion. Although PEG precipitation is a relatively crude and non-specific technique for precipitating immunoglobulin complexes from serum, we chose it as a simple and inexpensive screening test for the detection of immunoglobulin-B₁₂ complexes. In the present study, the diagnostic performance of HoloTC and HoloPEG were evaluated by using Receiver Operating Characteristic (ROC) curve analysis. By using cutoff value, 35 pmol/L, according to insertion of the manufacturer, the specificity of HoloTC was better compared to HoloPEG which means better discrimination of being not vitamin B₁₂ deficient; the sensitivity of HoloPEG was better than HoloTC which means better discrimination of being vitamin B₁₂ deficient. At the cut-off value of 35 pmol/L, the sensitivity was 55 % and 81% for HoloTC and HoloPEG respectively; the specificity was 78.7% and 57.4% for HoloTC and HoloPEG respectively. However, the comparison of ROC curves showed no statistically significance between AUC values of assays; both methods were not different in terms of their diagnostic performance. According to ROC curve analysis, the best performing cutoff for serum HoloTC and HoloPEG to determine Vitamin B₁₂ deficiency was less than 40.6 pmol/L and 32.1 pmol/L, respectively. In different reports, broad ranges of cut-off points for HoloTC assay of vitamin B₁₂ were identified ranged between 11 to 50 pmol/L. It is important to determine the true state of vitamin B₁₂ in this group of diseases. For this reason, it is important to evaluate the diagnostic performance of HoloTC assay in patients with MPDs. Thus, the deficiency is recognized earlier, and proper treatment can be provided.

This study has some limitations. First of all, the sample size of or study was small to evaluate diagnostic performance of both assays in each disease of MPDs, individually. MPDs is a heterogeneous group of diseases, the individual unique characteristics of diseases may have changed our results. Each disease of group should be assessed separately in further studies. In our study, absence of a healthy control group is another limitation. The evaluation of vitamin B₁₂ deficiency using MMA, HoloTC is the strength of our study. The MMA was tested by HPLC. The patients who had normal serum creatinine were included to minimize the effects of renal function on MMA. The effects of age and sex were taken into account when assessing MMA status. Potentially false test results were minimized. As far as we know the effects of immune complexes on HoloTC assay is studied in MPDs for the first time.

In conclusion immune complexes may have some effect on HoloTC assay in myeloproliferative diseases. Especially in CML and MF, immune complexes may interfere HoloTC assay. However, exclusion of immune complexes did not improve its diagnostic performances. It is necessary to confirm our findings in a further study with a larger sample size.

Financial disclosure statement:
This study was supported by the Scientific Research Project Support Fund of Sakarya University –No: 2013-08-06-010.

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