

# Autonomic nervous system dysfunction and serum levels of neurotoxic and neurotrophic cytokines in patients with cobalamin deficiency

*Kobalamin eksikliği olan hastalarda otonom sinir sistemi bozukluğu ve nörotoksik, nörotropik sitokinlerin serum düzeyleri*

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## Abstract

**Objective:** The imbalance between neurotoxic cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and neurotrophic cytokines epidermal growth factor (EGF) and interleukin-6 (IL-6) plays a role in the pathogenesis of cobalamin (Cbl) deficiency-induced neuropathy. The aim of this study was to evaluate autonomic nervous system dysfunction and to look for any relationship between autonomic nervous system disturbances and serum cytokine levels (TNF- $\alpha$ , EGF, IL-6) in patients with Cbl deficiency.

**Materials and Methods:** Serum levels of TNF- $\alpha$ , EGF and IL-6 were studied in patients with Cbl deficiency (n=41) and a healthy control group (n=17) and after 3 months in patients who underwent Cbl replacement therapy (n=22). All patients with Cbl deficiency underwent electrophysiological studies (EPS) for the diagnosis of neuropathy. Statistical analysis was performed using SPSS for Windows 11.5 software.

**Results:** With EPS, 29 of 41 Cbl-deficient patients (70.73%) demonstrated neurological dysfunction [3 (7.32%), 19 (46.34%) and 7 (17.07%) patients with sensorimotor peripheral neuropathy, parasympathetic, and sympathetic autonomic dysfunction, respectively]. Although there was no significant difference in serum levels of EGF and IL-6 between patients with versus without autonomic dysfunction, levels were significantly lower in Cbl-deficient patients than healthy controls.

**Conclusion:** Presence of autonomic dysfunction seems to be a frequent neurological finding in patients with Cbl deficiency. However, we could not find any relationship between serum cytokine levels and autonomic dysfunction by EPS. (*Turk J Hematol 2010; 27: 250-6*)

**Key words:** Cobalamin deficiency, autonomic dysfunction, dysautonomia, autonomic neuropathy, serum cytokine levels

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## Özet

**Amaç:** Nörotoksik sitokin tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) ile nörotrop sitokinler epidermal growth factor (EGF) ve interleukin-6 (IL-6) arasındaki dengesizlik kobalamin eksikliğine bağlı nöropati patogenezinde rol oynamaktadır. Bu çalışmanın amacı, kobalamin eksikliği olan hastalarda otonom sinir sistemi bozukluğunu değerlendirmek ve otonom sinir sistemi bozuklukları ile serum sitokin düzeyleri (TNF $\alpha$ , EGF, IL-6) arasındaki ilişkiyi araştırmaktır.

**Yöntem ve Gereçler:** Kobalamin eksikliği olan hastalar (n=41), sağlıklı kontrol grubu (n=17) ve kobalamin yerine koyma tedavisinden 3 ay sonra hastalarda (n=22) serum TNF $\alpha$ , EGF, IL-6 düzeyleri çalışıldı. Nöropati tanısı için, Cbl eksikliği olan tüm hastalarda elektrofizyolojik çalışma (EPS) yapıldı. İstatistik analizde "SPSS for Windows 11.5" yazılımı kullanıldı.

**Bulgular:** EPS ile 41 kobalamin eksikliği olan hastadan 29'unda nörolojik bozukluk gösterildi [ 3 (7.32%), 19 (46.34%) ve 7 (17.07%) hastada sırasıyla, sensorimotor periferik nöropati, parasempatik ve sempatik otonom bozukluk]. Otonom bozukluk olan ve olmayan hastalar arasında serum EGF ve IL-6 düzeylerinde anlamlı fark yoksa da, bu sitokin düzeyleri kobalamin eksikliği olan hastalarda, sağlıklı kontrollerden anlamlı derecede düşüktü.

**Sonuç:** Kobalamin eksikliği olan hastalarda otonom bozukluk, sık nörolojik bulgu olarak görünmektedir. Bununla birlikte, elektrofizyolojik çalışmayla saptanan otonom bozukluklar ve serum sitokin düzeyleri arasında ilişki bulamadık. (Turk J Hematol 2010; 27: 250-6)

**Anahtar kelimeler:** Kobalamin eksikliği, otonom bozukluk, disotonomi, otonom nöropati, serum sitokin düzeyi

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## Introduction

The clinical presentation of patients with vitamin B<sub>12</sub> (cobalamin-Cbl) deficiency varies in a spectrum ranging from hematological disorders to neuropsychiatric diseases. As the level of Cbl deficiency does not correlate with the severity of neurological disorders and the lesions may become irreversible if not treated promptly, neurological abnormalities are a matter of clinical concern in patients with Cbl deficiency. Cbl deficiency may affect both the peripheral and central nervous system, with diminished vibratory sensation as the most common abnormality [1,2]. In rare cases, autonomic nervous system dysfunction presented as orthostatic hypotension, impotence, constipation, and urinary retention have been attributed to Cbl deficiency [1,3,4]. Although the association between Cbl deficiency and neurological abnormalities is well known, the precise mechanism by which neurological impairment occurs is not yet obvious [5]. Neurological abnormalities may occur not only in patients with classic Cbl deficiency but also in subtle or atypical deficiencies in which anemia is absent [6]. In previous reports, the pathogenesis of neurological abnormalities due to Cbl deficiency has been attributed to the accumulation of methylmalonic acid (MMA) and homocysteine (Hcys). However, in totally gastrectomized Cbl-deficient rat models, Scalabrino et al. [7-9] showed that the severity of the neuropatho-

logical damage in the spinal cord white matter did not correlate with the progressive accumulation of MMA and Hcys, and it was suggested that some cytokines and/or growth factors other than MMA and Hcys might play a role in the pathogenesis of neurological damage in Cbl deficiency. Experimental studies over the last few years have demonstrated that Cbl deficiency increases the local overexpression of neurotoxic cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and decreases the synthesis of the neurotrophic epidermal growth factor (EGF) and interleukin-6 (IL-6) in the cerebrospinal fluid (CSF) of rats [10-12]. On the basis of these experimental observations, we measured the serum levels of TNF- $\alpha$ , EGF and IL-6 in adult patients with Cbl deficiency and investigated the effect of these factors on neurological abnormalities, especially on autonomic dysfunction as assessed by electrophysiological studies (EPS). We also investigated the effect of replacement therapy with Cbl in some of these patients.

## Materials and Methods

### Subjects

The study population consisted of newly diagnosed and untreated patients with Cbl deficiency (n=41), healthy volunteers as a control group (n=17) and patients after three months of treatment for Cbl deficiency (n=22).

### Patient group

The diagnosis of Cbl deficiency was based on the finding of low serum Cbl with normal serum folate levels and high plasma levels of Hcys. Cut-off value of serum Cbl was accepted as 160 pmol/L.

The patients with Cbl deficiency aged 16-80 years who had none of the exclusion criteria were enrolled into the study. Patients with a history of diabetes mellitus, uremia, chronic hepatitis, concomitant malignancies, alcohol abuse, drug use, folate deficiency, or other possible causes of polyneuropathy were excluded. None of the patients had apparent infection or any generalized/localized infection sign such as fever, leukocytosis or erythema, and none of them had malabsorption, were following a vegetarian diet, or had previous gastric surgery.

All the patients underwent careful medical history and physical and neurological examination. As a part of the medical history, all patients were queried regarding the presence of autonomic dysfunction symptoms such as orthostatic hypotension, impotence, constipation, diarrhea, and incontinence. Routine blood chemistry tests, complete blood cell count and measurements of Hcys levels, serum Cbl levels and folate concentrations were performed. Serum MMA levels could not be measured due to technical limitations in our hospital. Serum levels of TNF- $\alpha$ , EGF and IL-6 were studied in patients with Cbl deficiency, the healthy control group and after 3 months of Cbl replacement. All patients with Cbl deficiency underwent EPS for the diagnosis of neuropathy. Anti-parietal cell antibody (APA) test, gastroduodenoscopy and endoscopic biopsies were also performed in patients with Cbl deficiency.

### Control group

Healthy volunteers, who served as normal controls, were defined following a detailed clinical history and physical examination, with normal baseline blood tests including full blood count, fasting glucose, renal function, hepatic function, electrolytes, and serum Cbl. They had no acute or chronic disease such as diabetes mellitus, infections, rheumatologic disorders, immune dysfunctions, or any neurological disorders.

### Cobalamin replacement therapy group

After tests had been completed, intramuscular Cbl injections were started for patients with Cbl

deficiency. Cobalamin was given at a dose of 1000  $\mu$ g per day with intramuscular injection (week 1), 1000  $\mu$ g twice weekly (week 2), 1000  $\mu$ g/week for the third and fourth weeks, and then 1000  $\mu$ g/month. Three months after the initiation of therapy, available patients were reevaluated for the presence of neurological findings and serum cytokine levels.

All participants gave informed consent prior to participation in the study, and the study was approved by the local Ethics Committee.

## Methods

Peripheral venous blood samples of 2 ml and 8 ml were drawn after an overnight fast without stasis from an antecubital vein with a 21-gauge needle into ethylene diamine tetra-acetic acid (EDTA)-containing tubes and empty tubes, respectively. Serum samples were separated within 1-3 hours and stored at -80°C until assayed. Serum Cbl, folate and plasma Hcys concentrations were measured using commercial kits.

Serum Cbl assay was performed by chemiluminescence method using commercial kits on the ADVIA Centaur chemistry analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

Serum TNF- $\alpha$ , EGF and IL-6 assays were measured by means of an enzyme immunoassay kit (Biosource human TNF- $\alpha$  ultrasensitive ELISA, human EGF ELISA and human IL-6 ultrasensitive ELISA kit, Biosource International Inc., Camarillo, CA, USA).

### Electrophysiological study (EPS)

Electrophysiological studies (EPSs) carried out in all the patients with Cbl deficiency included motor and sensory nerve conduction studies, RR interval variation (RRIV) and sympathetic skin response (SSR).

Patients underwent motor and sensory nerve conduction studies of the right median, peroneal and sural nerves. Motor studies included wrist-elbow and knee-ankle segments of the median and peroneal nerves, respectively. Sensory nerve conduction was performed orthodromically on the digit II-wrist segment of the median nerve. The sural nerve was stimulated at midcalf, and recordings were made antidromically just lateral and posterior to the lateral malleolus. Stimuli were delivered

supramaximally by surface stimulating electrodes. Whenever nerve conduction abnormalities were found in the studied nerves, other motor and sensory nerves in the upper or lower extremities were also studied to rule out mono- or polyneuropathies. Recordings were performed by surface electrodes.

RRIV was studied by a Dantec Cantata electromyograph as described previously [13]. Recordings were made by using silver-silver chloride electrodes with a 7x4 mm recording area placed on the dorsum of each hand. Bandpass filters were set at 20-100 Hz and the sensitivity was 200-500  $\mu$ V per division. Oscilloscope was triggered at a sweep of 200 ms per division, which permitted display of 2 QRS complexes on the screen. Latency variations of the second complex were measured in each sweep. Five groups of 20 tracings were recorded at rest and two during deep breathing. The range of RR intervals (a) and the mean RR interval (b) were measured, and RRIV was calculated according to the following formula:  $a/b \times 100$ . The averages of five recordings at rest and two recordings during deep breathing were termed R% and D%, respectively.  $D\% - R\%$  and  $D\%/R\%$  were also calculated and compared to normative data of Shahani et al. [13]. Values lower than the 95% confidence limits in any of the four calculated variables were considered abnormal [*parasympathetic (cardiovagal) dysfunction*].

SSR was recorded from both hands and feet using the same equipment and electrodes described in RRIV testing. The cathode was attached either to palms or soles, referenced as the dorsum of the hand or foot. Bandpass filters were set at 0.5-2000 Hz. Sensitivity was 500  $\mu$ V per division, with a sweep of 500 ms per division for upper extremity and 1 s per division for lower extremity recording. Responses were elicited by delivering electrical stimuli to the contralateral median or tibial nerves. At least four tracings were obtained from each site. Abnormality, *sympathetic (sudomotor) dysfunction*, was defined as absence of any response in these recordings.

### Statistical analysis

Statistical analysis was performed using SPSS for Windows 11.5 software. Student's paired-*t* test and Wilcoxon ranks test were used for within-group comparison. Mann-Whitney U test was used for comparisons between groups. The relationships between the variables were assessed using either Spearman's or Pearson correlation tests. Data for TNF- $\alpha$  and IL-6 levels are expressed as

median  $\pm$  standard deviation (SD). Data for EGF levels are expressed as mean  $\pm$  SD. Differences were considered significant if  $p < 0.05$ .

### Results

A total of 41 consecutive patients with newly diagnosed and untreated Cbl deficiency (17 male, 24 female, age range: 19-75 years, median: 43 years) were included. Twenty-two of the 41 patients were re-evaluated after three months of parenteral Cbl replacement treatment. The control group consisted of 17 healthy volunteers (11 female, 6 male, age range: 16-70 years, median: 42 years).

Initial presenting symptoms and signs of the patients were: fatigue in 16/41 (39%), neurological symptoms including paresthesias, dizziness, ataxia, memory loss, and syncope in 31/41 (75.6%), anemia and/or macrocytosis in 19/41 (46.34%), and gastrointestinal symptoms in 3/41 (7.3%). Tingling (pins and needles) and/or numbness in hands and/or feet, evaluated as paresthesias, were present in 12/41 patients (29.26%). Neurological examination showed positive Romberg's sign in 15/41 patients (36.58%), extensive vibratory sense loss in 13/41 patients (31.70%), and decreased deep tendon reflex in 5/41 patients (12.19%).

Upper gastrointestinal system endoscopic examination was performed in 30/41 patients. 16/30 patients (53.3%) had *Helicobacter pylori* infection. Nine of 30 patients (30%) had atrophic gastritis. In 5 of 9 patients with atrophic gastritis (55.5%), APAs were positive.

Eleven (26.80%) patients had macrocytic anemia and in 4 of these patients, leukopenia and/or thrombocytopenia was also present. In 9 of 41 patients (21.95%), additional iron deficiency was present. Hematological variables in patients with Cbl deficiency were as follows: hemoglobin  $11,33 \pm 2,48$  g/dl, mean corpuscular volume (MCV)  $91,85 \pm 17,19$  fl, leukocyte  $5,85 \pm 1,65 \times 10^9/L$ , and platelet count  $240,20 \pm 93,84 \times 10^9/L$ . Median value of serum Cbl levels was  $114,83 \pm 39,82$  pmol/L. Median serum levels of TNF- $\alpha$  and IL-6 were  $0,17 \pm 0,27$  pg/ml (range: 0,13-1,9) and  $0,09 \pm 0,21$  pg/ml (range: 0,08 - 1,47), respectively. Mean serum level of EGF was  $1,15 \pm 0,73$  pg/ml (range: 0,06 - 2,68). Hematological variables, serum Cbl levels, and serum IL-6, TNF- $\alpha$  and EGF levels in Cbl-deficient patients and in healthy controls are summarized in Table 1.

Serum EGF and IL-6 levels were significantly lower in patients with Cbl deficiency than healthy controls [ $1.15 \pm 0.73$  vs  $1.96 \pm 0.93$  ( $p=0.001$ ) and  $0.09 \pm 0.21$  vs  $0.13 \pm 0.84$  ( $p=0.006$ ), respectively]. Serum TNF- $\alpha$  levels were not significantly different between Cbl-deficient patients and healthy controls [median  $0.17 \pm 0.27$  (range: 0.13 - 1.9) vs  $0.18 \pm 0.97$  (range: 0.15 - 3.12) pg/ml,  $p>0.05$ ]. Serum TNF- $\alpha$  and IL-6 levels were not significantly different between Cbl-deficient patients and after Cbl replacement treatment [median  $0.17 \pm 0.27$  (range: 0.13 - 1.9) vs  $0.19 \pm 0.17$  (range: 0.07 - 1.47) pg/ml, and  $0.09 \pm 0.21$  (range 0.07 - 1.47) vs  $0.09 \pm 0.08$  (range 0.04 - 0.45) pg/ml, respectively,  $p>0.05$ ]. However, serum EGF levels significantly increased after Cbl replacement treatment ( $1.13 \pm 0.73$  vs  $1.90 \pm 0.74$ ,  $p=0.005$ ). Serum levels of TNF- $\alpha$ , IL-6 and EGF did not correlate with any of the considered hematological variables in patients with Cbl deficiency or in controls. Serum Cbl, IL-6, TNF- $\alpha$ , and EGF levels were not significantly different in Cbl-deficient patients with autonomic neuropathy than in patients without neuropathy ( $p>0.05$ ). Serum levels of IL-6, EGF and TNF- $\alpha$  were not significantly different between patients whose autonomic neuropathy findings responded versus did not respond to Cbl replacement treatment ( $p>0.05$ ).

According to the EPS, 12/41 patients (29.27%) had normal EPS findings, while 29/41 patients (70.73%) demonstrated neurological abnormalities. Sensorimotor peripheral neuropathy and autonomic dysfunction according to EPS were present in 3/41 patients (7.32%) and 26/41 patients (63.41%), respectively. In detail, autonomic dysfunction by

EPS was parasympathetic (cardiovascular) dysfunction in 19 patients (46.34%) and sympathetic (sudomotor) dysfunction in 7 (17.07%) (Figure 1). Twenty-two of 41 patients were re-evaluated with hematological variables, neurological examination and serum cytokine levels after three months of parenteral Cbl replacement treatment. After Cbl replacement, a follow-up EPS could be evaluated in 13 patients with neuropathy, and improvement was achieved in only 5 patients (38.46%).

### Discussion

In our study, we demonstrated that serum EGF and IL-6 levels were significantly decreased in

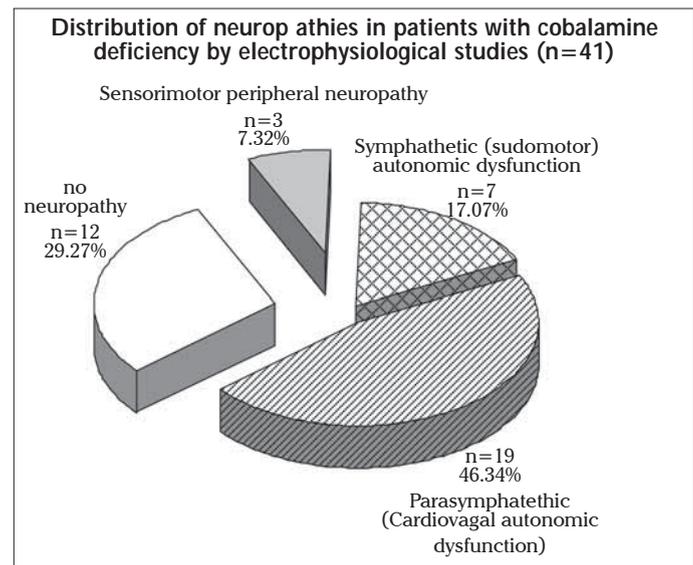


Figure 1. Distribution of neuropathies according to electrophysiological studies in cobalamin-deficient patients

Table 1. Hematological variables, serum cobalamin and cytokines levels of study groups

	Patients with Cbl deficiency (n=41)	Healthy controls (n=17)	p Value
Hematological variables (mean values)			
WBC (x10 <sup>9</sup> /L)	5.85±1.65	6.99±1.70	p>0.05
Hemoglobin (g/dl)	11.33±2.48	13.19±1.62	p<0.05
Mean corpuscular volume (fl)	91.85±17.19	84.79±9.37	p>0.05
Platelets (x10 <sup>9</sup> /L)	240.20±93.84	274.90±39.15	p>0.05
Serum cobalamin level (pmol/L) mean	114.83±39.82	217.00±48.18	p<0.001
Serum IL-6 level (pg/ml) median	0.09±0.21	0.13±0.84	p=0.006
Serum EGF level (pg/ml) mean	1.15±0.73	1.96±0.93	p=0.001
Serum TNF $\alpha$ level (pg/ml) median	0.17±0.27	0.18±0.97	p>0.05

patients with Cbl deficiency. However, serum TNF- $\alpha$  levels were not significantly different between Cbl-deficient patients and healthy controls. After Cbl replacement, only serum EGF levels increased significantly. Neurological abnormalities were demonstrated in EPS in 29 of 41 patients (70.73%) and among them, autonomic dysfunction was present in 26 of 41 Cbl-deficient patients (63.41%). There was no association between serum levels of EGF, TNF- $\alpha$ , IL-6, and autonomic dysfunction as assessed by EPS ( $p > 0.05$ ). In our study group, serum Cbl levels were  $\leq 120$  pg/ml in only 22 of 41 patients, so not all our patients had severe Cbl deficiency. This situation might be considered a limitation of our study.

Mean corpuscular volume (MCV) of our patient group with Cbl deficiency was  $91.85 \pm 17.19$  fl (mean). Generally, severe macrocytosis is expected in Cbl deficiency anemia. Nevertheless, our patient population consisted of not only megaloblastic anemia patients due to Cbl deficiency but also patients with Cbl deficiency without anemia who had neurological symptoms and signs. Furthermore, some of the patients had additional iron deficiency. Relatively moderate macrocytosis in our patients with Cbl deficiency can be explained by these factors.

It has been demonstrated that the Cbl-deficient central neuropathy of totally gastrectomized (TGX) rats is not caused by the withdrawal of the vitamin itself, but reflects a simultaneous increase in the production of the neurotoxic cytokine TNF- $\alpha$  and a decrease in the synthesis of the neurotrophic growth factor EGF and IL-6 [10-12,14]. In two studies, cytokine levels were detected in humans with Cbl deficiency [15,16]. In the first report by Peracchi et al. [15], it was demonstrated that overproduction of serum TNF- $\alpha$  and underproduction of serum EGF were present in humans with newly diagnosed severe Cbl deficiency, as in rats. Later, Scalabrino et al. [16] reported higher TNF- $\alpha$  levels and lower EGF levels in the CSF of Cbl-deficient patients with neurological manifestations of subacute combined degeneration (SCD). We could not demonstrate an increase in serum TNF- $\alpha$  levels in the Cbl-deficient patients or a relationship between serum IL-6, TNF- $\alpha$  and EGF levels and autonomic dysfunction. Severity of Cbl deficiency and the presence of clinical SCD in the study of

Peracchi et al. [15] may be the major factor explaining the discrepancy between the two studies. The duration of Cbl deficiency may also be a leading factor for the changes in serum neurotrophic and neurotoxic cytokine levels.

Although the most common clinical manifestations of Cbl deficiency are SCD of the spinal cord and peripheral neuropathy, optic atrophy, dementia, and autonomic nervous system dysfunction have been reported rarely [1-4]. In the previous studies, parameters of heart rate variability in patients with Cbl deficiency were found to be significantly lower as compared to healthy controls [17,18]. Orthostatic hypotension as a function of autonomic neuropathy was systematically assessed in patients with Cbl deficiency by Beitzke et al. [3]. In their study, a significant fall in systolic blood pressure directly after head-up tilt, stroke index, and cardiac index, and a lack of increase of total peripheral resistance index for the duration of tilt in patients with Cbl deficiency as compared to healthy controls were reported. In patients with Cbl deficiency, gastric emptying by scintigraphy has also been studied as a function of the autonomic nervous system. Mean gastric emptying  $t_{1/2}$  in patients with Cbl deficiency was reported to be prolonged. Although mean gastric emptying  $t_{1/2}$  after Cbl replacement therapy was somewhat shorter, a statistically significant difference persisted after Cbl replacement [4].

In conclusion, our present study showed that neurotrophic cytokines EGF and IL-6 serum levels decreased while neurotoxic cytokine TNF- $\alpha$  serum level did not change in patients with Cbl deficiency as compared with healthy controls. Nevertheless, we could not find a relation between autonomic neuropathy and changes in serum neurotrophic and neurotoxic cytokines levels in Cbl-deficient patients. Presence of asymptomatic autonomic neuropathy seems to be a frequent neurological finding in patients with Cbl deficiency. To determine the clinical relevance of neurotoxic and neurotrophic cytokine levels, further studies are needed.

#### Conflict of interest

No author of this paper has a conflict of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included in this manuscript.

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