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Can New Advanced Clinical Parameters Be Used in the Diagnosis of Vitamin B12 Deficiency?

B12 Vitamini Eksikliğinin Tanısında Yeni Gelişmiş Klinik Parametreler Kullanılabilir mi?

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Abstract: The measurement of vitamin B12 levels has low sensitivity for the diagnosis of vitamin B12 deficiency, therefore, clinicians may prefer to decide the deficiency with the patient's clinic. In this study, the diagnostic values of the classical and advanced clinical complete blood count parameters were investigated to find a new marker in the diagnosis of vitamin B12 deficiency. 150 adult volunteers were included in the study and volunteers were divided into two groups according to their vitamin B12 levels (with or without vitamin B12 deficiency) and hemoglobin levels (with or without anemia). The differences and correlations of laboratory test results between groups were examined. Equivalent of reticulocyte hemoglobin, equivalent of erythrocyte hemoglobin and delta-hemoglobin were found significant differences between groups with or without vitamin B12 deficiency. Hypo-hemoglobinised red cells and hyper-hemoglobinised red cells were found significant difference between groups with or without anemia. Significant positive correlations were found between vitamin B12 levels and equivalent of reticulocyte hemoglobin, equivalent of erythrocyte hemoglobin and delta-hemoglobin. Significant positive correlations were found between hemoglobin levels and macrocytic red blood cells, equivalent of reticulocyte hemoglobin, equivalent of erythrocyte hemoglobin, delta hemoglobin and hyper-hemoglobinised red cells; negative correlations with microcytic red blood cells and hypo-hemoglobinised red cells. It is suggested that these advanced clinical parameters of complete blood count analysis may be used to determine vitamin B12 deficiency.
Keywords: Vitamin B12 deficiency, Anemia, Complete Blood Count, Erythrocytes, Reticulocytes

Özet: B12 vitamini düzeylerinin ölçümünün B12 vitamini eksikliği tanısında duyarlılığı düşüktür, bu nedenle, klinisyenler eksikliğe hastanın kliniği ile karar vermeyi tercih edebilir. Bu çalışmada B12 vitamini eksikliği tanısında yeni bir belirteç bulmak amacıyla klasik ve ileri klinik tam kan sayımı parametrelerinin tanısal değerleri araştırıldı. Çalışmaya 150 yetişkin gönüllü dahil edildi ve gönüllüler B12 vitamini düzeylerine (B12 vitamini eksikliği olan veya olmayan) ve hemoglobin düzeylerine (kansızlık olan veya olmayan) göre iki gruba ayrıldı. Laboratuvar test sonuçlarının gruplar arasındaki farklılıkları ve korelasyonları incelendi. B12 vitamini eksikliği olan ve olmayan gruplar arasında retikülosit hemoglobini, eritrosit hemoglobin ve delta-hemoglobin açısından anlamlı farklar bulundu. Anemisi olan ve olmayan gruplar arasında hipo-hemoglobinize eritrositler ve hiper-hemoglobinize kırmızı hücreler arasında anlamlı fark bulundu. B12 vitamini düzeyleri ile retikülosit hemoglobini, eritrosit hemoglobin ve delta-hemoglobin arasında anlamlı pozitif korelasyon bulundu. Hemoglobin seviyeleri ile makrositik eritrositler, retikülosit hemoglobini, eritrosit hemoglobini, delta hemoglobin ve hiper-hemoglobinize eritrositler arasında anlamlı pozitif korelasyonlar; mikrositik eritrositler ve hipo-hemoglobinize eritrosit düzeyleri ile negatif korelasyonlar bulundu. Tam kan sayımı analizinin bu ileri klinik parametrelerinin B12 vitamini eksikliğini belirlemek için kullanılabileceği öne sürülmektedir.
Anahtar Kelimeler: B 12 Vitamini Eksikliği, Anemi, Tam kan sayımı, Eritrositler, Retikülositler

Ethics Committee Approval: This study was designed and conducted in accordance with the ethical guidelines set forth in the Declaration of Helsinki, and the study protocol was approved by the Eskisehir Osmangazi University Non-interventional Clinical Researches Ethics Committee (Date: 16.10.2020, Decision No:23).

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1. Introduction

Megaloblastic anemia that is in the macrocytic anemia group morphologically and often occurs due to vitamin B12 deficiency is an important hematological disorder (1). The first test to evaluate the vitamin B12 metabolism is serum vitamin B12 measurement, but metabolically active form of vitamin B12 cannot be measured by this assay, so the sensitivity is low (2, 3). Measurements of plasma homocysteine, serum/urine methyl malonic acid (MMA), and serum holo transcobalamin are used to evaluation of vitamin B12 metabolism (4). Since plasma homocysteine level also changes in the deficiency of vitamin B6 and folic acid, its diagnostic specificity is limited (5). Although serum/urine MMA measurement is more specific than the plasma homocysteine measurement, its use is limited due to the high cost and difficulty of the measurement procedure (6). Although the active form of vitamin B12, holo transcobalamin, may be measured, it is not yet a widely used as a diagnostic test since it may be affected by the transcobalamin 2 carrier protein synthesis defect, too. Therefore, new diagnostic tests are needed for the diagnosis of vitamin B12 deficiency (7, 8).

In the detection of vitamin B12 deficiency, the significance of complete blood count parameters is being investigated, but the sensitivity and specificity of classical parameters are not enough (9), therefore, the use of reticulocyte (RET) counts and indexes has been tried (10).

Sysmex XN series complete blood count analyzers measure or calculate advanced clinical parameters as percentage of microcytic and macrocytic red blood cells (MicroR and MacroR), hypo-hemoglobinised red cells (HYPO-He, percentage of RBCs with a hemoglobin content of less than 17 pg.) and hyper-hemoglobinised red cells (HYPER-He, percentage of RBCs with a hemoglobin content of more than 49 pg.), equivalent of reticulocyte hemoglobin (RET-He) and delta hemoglobin (Delta-He, subtraction between amount of mature RBC hemoglobin and RET-He) (11, 12). Measurements of these advanced clinical parameters are performed in the reticulocyte channel.

In this study, the diagnostic values of the classical and advanced clinical blood count parameters were investigated in vitamin B12 deficiency and it was aimed to find a new diagnostic marker that is more compatible with the clinic.

2. Materials and Methods

This study was conducted with the approval of the local ethics committee and 150 adult volunteers whose vitamin B12 and folic acid measurements and complete blood count analyses were performed in our hospital's biochemistry laboratory, were included in the study.

Patients with other diseases that cause macrocytic anemia, such as leukemia multiple myeloma, whose mean corpuscular volume (MCV) below 90 fL, folic acid level below 4.6 ng/mL, serum creatinine level above 1.2 mg/dL in women and 1.3 mg/dL in men and who take vitamin B12 supplements (if their vitamin B12 level higher than 400 pg/mL) and pregnant women were excluded.

Volunteers were divided into two groups firstly according to their vitamin B12 levels (with or without vitamin B12 deficiency) and then hemoglobin (Hb) levels (with or without anemia).The cut-off values of vitamin B12 for the diagnosis of deficiency was accepted as 197 pg/mL that is recommended by the manufacturer and used in our routine laboratory (Insert.Elecsys Vitamin B12 II.07028121500.V7.tr) and the cut-off values of Hb for the diagnosis of anemia were accepted as 13.5 g/dL in men and 11.9 g/dL in women that are used in our routine laboratory for adults and in accordance with the literature (13). The differences and correlations of laboratory test results between groups were examined.

Serum creatinine, vitamin B12 and folic acid levels were measured by Cobas 8000 auto analyzer (Roche Diagnostics, Mannheim, Germany). The Jaffe method was used for creatinine measurement, and the electro chemiluminescence immunoassay for vitamin B12 and folic acid measurements. Complete blood count and reticulocyte count were examined by Sysmex XN 9000 automatic blood count analyzer (Sysmex Corporation, Kobe, Japan).

Statistical analysis

Kolmogorov-Smirnov and Shapiro-Wilk tests were applied to decide the distribution of variables. Data with normal distribution were given as mean ± standard deviation, and data not with normal distribution were given as median (25th-75th quartile). Independent samples t test or Mann-Whitney U was applied as appropriate. Pearson or Spearman correlation tests were applied for showing the associations between variables. Receiver

Operating Characteristic (ROC) Curve analysis was conducted to determine the effects of the markers via areas under the ROC curve (AUC). All data analyses were performed with SPSS package program and P<0.05 level was considered statistically significant.

3. Results

150 volunteers were included in the study with the mean age of 54 years. Laboratory results of groups that were divided according to vitamin B12 levels are shown in Table 1. Hematocrit (HCT), red blood cell (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell (WBC), RET-He, RBC-He and Delta-He levels were significant difference between groups (Table 1). Laboratory results of groups that were divided according to Hb levels are shown in Table 2. Vitamin B12, HCT, RBC, MCV, RET%, Hypo-He and Hyper-He levels were significant difference between groups (Table 2).

Significant positive correlations were found between vitamin B12 levels and MCH, MCHC, absolute reticulocyte count (RET#), RET%, RET-He, RBC-He and Delta-He; negative correlations with HCT and RBC levels. Significant positive correlations were found between Hb levels and HCT, RBC, MCH, MCHC, MacroR, WBC, RET-He, RBC-He, Delta-He and HYPER-He; negative correlations with Micro-R, RET% and HYPO-He (Table 3).

ROC analysis was conducted to determine the effects of the new significant markers for each group and AUCs are shown in Table 4. All parameters were also significant and the AUCs were above 0.65 (Figure 1 and 2).

Table 1. Laboratory results of groups that were divided according to vitamin B12 levels

	Group 1 (n=46)	**Group 2** (n=104)	***P***
Age (years)	53 (40-60)	54 (44-61)	0.281
Vitamin B12 (pg/mL)	173 (153-183)	359 (248-505)	<0.001
Folic acid (ng/mL)	7.72 (6.40-10.2)	8.35 (6.16-10.8)	0.454
Hb (g/dL)	14.6 ± 1.59	14.1 ± 1.65	0.114
HCT (%)	43.2 ± 4.20	41.0 ± 4.43	**0.008**
RBC (10^6/µL)	4.69 ± 0.48	4.42 ± 0.51	**0.004**
MCV (fL)	91.3 (90.6-93.1)	92.0 (90.7-94.2)	0.140
MCH (pg)	32.0 ± 1.23	31.8 ± 1.43	**0.001**
MCHC (%)	33.7 ± 1.12	34.3 ± 1.03	**0.002**
Micro-R (%)	0.90 (0.70-1.10)	0.90 (0.70-1.10)	0.669
Macro-R (%)	3.75 (3.58-4.03)	3.70 (3.40-4.10)	0.477
Platelets (10^3/µL)	249 ± 70	239 ± 74	0.293
White blood cells (10^3/µL)	7.52 ± 2.02	6.72 ± 1.98	**0.029**
RET# (10^3/µL)	0.07 (0.06-0.08)	0.07 (0.05-0.09)	0.257
RET%	1.45 (1.22-1.70)	1.51 (1.24-1.98)	0.071
RET-He (pg)	32.7 ± 1.41	33.8 ± 1.64	**<0.001**
RBC-He (pg)	30.2 ± 1.15	30.8 ± 1.15	**0.005**
DELTA-He (pg)	2.50 (1.98-3.00)	2.85 (2.40-3.38)	**0.008**

HYPO-He (%)	0.10 (0.10-0.20)	0.10 (0.10-0.20)	0.326
HYPER-He (%)	0.70 (0.60-0.80)	0.70 (0.60-0.80)	0.120

Independent samples t test (mean ± standard) and Mann-Whitney U test (median, 25th and 75th quartile) were applied.

Table 2. Laboratory results of groups that were divided according to hemoglobin levels

	Group A (n=20)	**Group B** (n=130)	***P***
Age (years)	60 (50-62)	53 (42-59)	0.047
Vitamin B12 (pg/mL)	430 (269-528)	232 (182-408)	**0.004**
Folic acid (ng/mL)	8.60 (5.97-10.2)	7.80 (6.46-10.6)	0.668
Hb (g/dL)	11.9 ± 1.09	14.6 ± 1.41	**<0.001**
HCT (%)	35.2 ± 3.37	42.7 ± 3.71	**<0.001**
RBC (10^6/µL)	3.73 ± 0.41	4.62 ± 0.42	**<0.001**
MCV (fL)	93.6 (91.8-95.9)	91.4 (90.7-93.6)	**0.004**
MCH (pg)	32.03 ± 2.01	31.5 ± 1.30	0.146
MCHC (%)	33.9 ± 1.41	34.1 ± 1.04	0.594
Micro-R	0.90 (0.73-1.40)	0.90 (0.70-1.10)	0.166
Macro-R	3.70 (3.13-5.63)	3.70 (3.50-4.00)	0.833
Platelets (10^3/µL)	232 (187-290)	228 (193-283)	0.738
White blood cells (10^3/µL)	6.41 (4.37-8.87)	6.66 (5.70-8.12)	0.621
RET# (10^3/µL)	0.06 (0.05-0.09)	0.07 (0.06-0.08)	0.772
RET%	1.77 (1.30-2.38)	1.48 (1.21-1.75)	**0.034**
RET-He (pg)	33.6 ± 2.34	33.4 ± 1.53	0.735
RBC-He (pg)	30.7 (30.1-31.2)	30.7 (29.9-31.3)	0.954
DELTA-He (pg)	2.70 (1.93-4.20)	2.70 (2.30-3.23)	0.951
HYPO-He (%)	0.25 (0.10-0.58)	0.10 (0.10-0.20)	**0.001**
HYPER-He (%)	0.60 (0.53-0.70)	0.70 (0.60-0.80)	**0.039**

Independent samples -t test (mean ± standard) and Mann-Whitney U test (median, 25th and 75th quartile) were applied. Group A is with anemia, Group B without anemia

Table 3. Correlations of laboratory tests between vitamin B12 and Hb levels

	Vitamin B12		**Hb**	
	r	***P***	**r**	***P***
Hb	-0.156	0.056	1	
HCT	-0.222	**0.006**	0.951	**<0.001**
RBC	-0.232	**0.004**	0.913	**<0.001**
MCV	0.134	0.103	-0.128	0.117
MCH	0.188	**0.021**	0.254	**0.002**
MCHC	0.173	**0.034**	0.380	**<0.001**
MicroR	0.043	0.598	-0.264	**0.001**
MacroR	0.015	0.857	0.334	**<0.001**
Platelet	-0.018	0.831	-0.116	0.158
White blood cell	-0.097	0.238	0.169	**0.039**
RET#	0.224	**0.006**	0.150	0.068
RET%	0.272	**0.001**	-0.170	**0.038**
RET-He	0.251	**0.002**	0.317	**<0.001**
RBC- He	0.197	**0.016**	0.308	**<0.001**
DELTA- He	0.220	**0.007**	0.161	**0.048**
HYPO- He	0.139	0.091	-0.505	**<0.001**
HYPER- He	0.139	0.091	0.636	**<0.001**

Pearson or Spearman correlation test was applied as appropriate.

Table 4. Results of ROC analyses

	AUC	**P**
For the groups that were divided according to vitamin B12 levels		
RET-He	0.680	< 0.001
RBC-He	0.641	0.006
DELTA-He	0.635	0.008
For the groups that were divided according to hemoglobin levels		
RET%	0.647	0.034
HYPO-He	0.702	0.004
HYPER-He	0.639	0.046

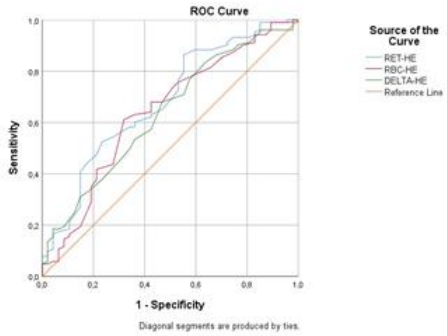

Figure 1. ROC curves groups that were divided according to vitamin B12 levels

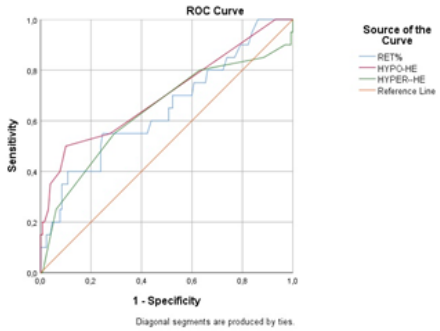

Figure 2. ROC curves the groups that were divided according to hemoglobin levels

4. Discussion

Vitamin B12 deficiency is common and causes megaloblastic anemia (14), and the first and most common laboratory test is the measurement of serum vitamin B12 level to determine vitamin B12 deficiency (2, 15). The cut-off value for vitamin B12 deficiency is accepted as 200 pg/mL. Vitamin B12 levels below this value indicate the risk of anemia (15). In this study, the cut-off value of vitamin B12 deficiency was accepted as 197 pg/mL that is used in our routine laboratory.

According to results of this study, vitamin B12 deficiency rate was 35%. The first remarkable finding was that there was no difference in Hb levels between the groups with and without vitamin B12 deficiency (Table 1). At the beginning of the study, the patients who had other causes of macrocytic anemia, with mean corpuscular volume (MCV) below 90 fL and folic acid level below 4.6 ng/mL were excluded, therefore we expected to find low Hb levels in the group with vitamin B12 deficiency, but this expect did not happen. Likewise, contrary to our expectations, RBC and HCT levels were significantly higher, MCH and MCHC levels were significantly lower in vitamin B12 deficiency group (P=0.004, P=0.008, P= 0.001, P=0.002, respectively) (Table 1). Pancytopenia, which is expected to be seen in vitamin B12 deficiency (16), was not observed in our study. All forms of vitamin B12 are measured by serum vitamin B12 assay, that's why this assay's diagnostic specificity is not high (3), therefore, the serum vitamin B12 levels may not always show parallelism with the clinicopathological findings of the vitamin B12 deficiency (17). This situation was also supported by these results of the study.

When it was determined that the clinic of vitamin B12 deficiency and serum vitamin B12 levels did not show parallelism, the participants were grouped according to their Hb levels and the groups with and without anemia were compared. As expected, HCT and RBC levels were significantly lower and MCV levels were significantly higher in the anemia group (Table 2). Contrary to expectations, vitamin B12 level was significantly higher in the anemia group

(Table 2). At the same time, it was found that vitamin B12 level had negative correlations with Hb, HCT and RBC levels, and positive correlations with MCV, MCH and MCHC (Table 3). These results also showed us that the level of vitamin B12 does not correlate accurately with clinicopathological findings of vitamin B12 deficiency.

As far as we know, this is the first study that examine advanced clinical parameters of Sysmex in vitamin B12 deficiency. According to the results of the study, it was found that RET-He, RBC-He and Delta-He levels were significantly lower in the group with Vitamin B12 deficiency (P=<0.001, P=0.005 and P=0.008, respectively). In addition, positive significant correlations were found between these three parameters and vitamin B12 levels. It was thought that these parameters, which were shown to be significant in iron deficiency anemia (18, 19) and used to differentiate between various disease-specific types of anemia (20), could also be used as new markers in vitamin B12 deficiency. On the other hand, it was found that Hypo-He level had significantly higher and Hyper-He level was significantly lower in the anemia group, and Hb level had negative correlation with Hypo-He and positive correlation with Hyper-He. These findings revealed that these parameters may be new markers for the diagnosis of vitamin B12 deficiency. All these new parameters were analysed by ROC curve and this analysis showed that these parameters can be used in the diagnosis of vitamin B12 deficiency.

Although there was no difference between the study groups, it was found that Hb level had negative correlation with MicroR, and positive correlation with MacroR. These correlations may be a focus point for other studies.

In our study, patients with other diseases that cause macrocytic anemia were excluded and patients with high MCV levels were included in the study, therefore, it was thought that the cause of anemia was vitamin B12 deficiency. This situation and the numerical difference between the groups may be a limitation of the study however it is not always possible to make a definitive diagnosis of vitamin B12.

In conclusion, the results of this study support that serum vitamin B12 levels do not accurately indicate the clinic of vitamin B12 deficiency, therefore it is suggested that the new studies may be conducted to use of easily measurable advanced clinical parameters such as RET-He, RBC-He, Delta-He, Hypo-He and Hyper-He in the diagnosis and follow-up of patients with vitamin B12 deficiency

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