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Evaluation of True Vitamin B_{12} Deficiency in a Group of Jordanians Aged 20-40 Years Visiting the Jordan University Hospital

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Abstract: A convenient study was conducted to evaluate vitamin B_{12} status among young healthy adult Jordanians and to check for the true vitamin B_{12} deficiency. One hundred sixty five subjects were recruited in the study. The subjects were chosen to be healthy aged between 20-40 years. Participants were asked to fill a detailed questionnaire that covers social and medical data as well as data on frequency consumption of food sources of vitamin B_{12}. Blood tests including CBC, blood film and serum vitamin B_{12} level were done for all volunteers. For those with serum B_{12} $\leq$300 pg/ml, plasma Methylmalonic Acid (MMA) was measured to confirm deficiency. Results showed that 27.3% had vitamin B_{12} deficiency according to standard B_{12} deficiency definition ($\leq$200 pg/ml), 41.8% had serum B_{12} between 201-300 pg/ml and 30.9% had normal B_{12} levels (>300 pg/ml). Among those with B_{12} $\leq$300 pg/ml, 47.4% had confirmed deficiency, using MMA as an indicator. Even, 44.9% of those with serum B_{12} between 201-300 pg/ml had confirmed B_{12} deficiency. Vitamin B_{12} status was found to be positively correlated with age, but negatively correlated with MCV. No significant associations were found between B_{12} status and gender, BMI, household size and total vitamin B_{12} intake. It is concluded that serum B_{12} level is not a specific test for true vitamin B_{12} deficiency. Furthermore, dietary vitamin B_{12} intake by itself is not an indicator of B_{12} deficiency.

Key words: Vitamin B_{12}, B_{12} deficiency, Jordan, methylmalonic acid (MMA)

INTRODUCTION

Vitamin B_{12} deficiency in Jordan has increased in an alerting rate in the last decade. Several studies done in Jordan reported prevalence between 16-50% (Barghouti *et al*., 2009; Abu-Samak *et al*., 2008; Habib Allah, 2008; Hakooz *et al*., 2006; Fora and Mohammad, 2005). These studies depended only on serum vitamin B_{12} test as the diagnostic tool. This test is widely used as the standard test for cobalamin deficiency (Green, 2005) and is still the standard investigation tool for vitamin B_{12} deficiency worldwide despite its limited specificity (Hvas and Nexo, 2006; Stabler *et al*., 1990). Serum vitamin B_{12} test measures total amount of circulating cobalamin, either bound to transcobalamin, which represents the functionally important fraction of plasma vitamin B_{12} (Miller *et al*., 2006) or haptocorrin, which makes the vitamin unavailable for uptake by cells (Riedel, 2007; Holleland *et al*., 1999). This makes the test unreliable to determine true vitamin B_{12} deficiency. Furthermore, dependence on serum cobalamin as a sole test of vitamin B_{12} deficiency has certain limitations, since it may miss up to one half of patients with actual tissue B_{12} deficiency (Oh and Brown, 2003; Bolann *et al*., 2000). This is because this test is a late, relatively insensitive and unspecific biomarker of deficiency (Herrmann and Obeid, 2008). Imperfection of using this classical diagnostic tool has lead to development of more reliable tests of functional cobalamin status, including plasma total homocysteine and serum methylmalonic acid (Bolann *et al*., 2000) and Holotranscobalamin (holoTC) (Hvas and Nexo, 2006). Measurements of these metabolites, methylmalonic acid and homocysteine, along with serum vitamin B_{12}, are considered more sensitive indicators than measuring serum vitamin B_{12} levels alone (Hvas and Nexo, 2006; Krautler, 2005). They can be early markers for tissue vitamin B_{12} deficiency, even before hematologic manifestations occur (Oh and Brown, 2003).

MMA is a functional vitamin B_{12} marker that increases when vitamin B_{12} status is depleted (Herrmann and Obeid, 2008). Plasma total Homocysteine (tHcy) is mentioned to be a sensitive marker of folate and vitamin B_{12} status and an increase in its level occurs long before classic deficiency of folate and vitamin B_{12} becomes evident (Fakhrazadeh *et al*., 2006). Thus, abnormal MMA or tHcy levels suggest a latent or overt tissue deficiency of vitamin B_{12} or folate (Bjorkegren and Svardsudd, 2001; Nexo *et al*., 1994).

In this study, we aimed to evaluate the percentage of true vitamin B_{12} deficiency among a group of young adult Jordanians.

MATERIALS AND METHODS

A total of more than 300 apparently healthy persons visiting the Family Medicine Clinic at Jordan University Hospital were interviewed for their principal acceptance

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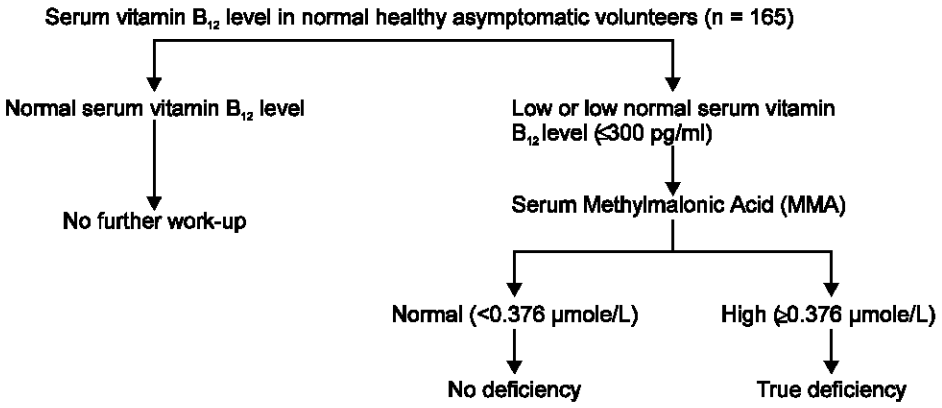

Fig. 1: Flow diagram of the diagnosis methodology used in this study

to participate in this study. However, only 165 of them aged 20-40 years (males = 66, females = 99) were recruited. The selected volunteers were apparently healthy with no previous diagnosis of vitamin B_{12} deficiency or not treated with vitamin B_{12} supplementation for at least one year. Persons with current diagnosis of liver disease, kidney problems, pregnancy and untreated hypothyroidism were excluded from the study. The protocol that was used in this study is shown in Fig. 1. Each volunteer signed a written informed consent to participate in the study which was approved by the Research Ethics Committee of the Jordan University Hospital.

Anthropometric measurements: Weight and height were measured for all volunteers and the corresponding Body Mass Index (BMI) values were calculated.

Medical and nutritional history: All volunteers were asked to fill the previously prepared and tested questionnaire (Cronbach's alpha coefficient = 0.6964) that summarizes their medical history and social information. Eating habits regarding the consumption of foods of animal sources were determined by semi-quantitative food frequency questionnaire (prepared according to Harvard University School of Public Health Food Frequency Questionnaire) (Lee and Nieman, 2007). The subjects were asked to give an approximate amount of food items eaten and the frequency of its consumption.

Blood sample collection and biochemical tests: Blood samples were collected for all volunteers. Automated Complete Blood Count (CBC) using Cell-DYN37300 (USA/Germany) and blood film reading using the Wright stain were performed. Serum vitamin B_{12} level was determined using automated micro particle enzyme intrinsic factor assay (AxSYM B_{12} Kit, Abbott, USA) at Al-Khaldi Medical Center Laboratory, Amman.

From all the volunteers, those with serum vitamin B_{12} level $\leq$300 pg/ml (n = 114) were tested for plasma Methylmalonic Acid (MMA). MMA test was done at Jordan University Hospital Laboratories, Amman. This test was done to confirm vitamin B_{12} deficiency as MMA value of $\geq$0.376 µmole/L indicates true deficiency (CDC, 2009). MMA test were done according to the procedure described by Kushnir and Komaromy-Hiller (2000) with some modifications.

Statistical analysis: Statistical analysis was performed using SPSS software, version 17.0 (Chicago, IL, USA). The association between serum vitamin B_{12} level with other parameters were assessed using Pearson correlation coefficient. The results of each variable were subjected to cross-tabulation and Chi-square test to evaluate the variables that can influence vitamin B_{12} level. The difference in means between the different study groups was subjected to Analysis of Variance (ANOVA).

RESULTS

Characteristics of the study group are shown in Table 1. The mean age of the subjects was 28.22 years with mean serum vitamin B_{12} level of 273.26 pg/ml, hemoglobin 13.79 g/dl and BMI 24.07 kg/m^2.

Participants were divided into three groups depending on serum vitamin B_{12} level (Table 2): Group 1 (Low) with serum vitamin B_{12} level $\leq$200 pg/ml; Group 2 (Low normal) with serum vitamin B_{12} level between 201-300 pg/ml and Group 3 (Normal) with serum vitamin B_{12} level >300 pg/ml. It can be noted from this table that 27.3% (n = 45) had vitamin B_{12} deficiency (defined as serum vitamin B_{12} $\leq$200 pg/ml); 41.8% (n = 69) had low-normal serum B_{12} level (defined as serum vitamin B_{12} level between 201-300 pg/ml) and 30.9% (n = 51) had normal serum B_{12} levels (defined as serum vitamin B_{12} >300 pg/ml).

Table 1: Characteristics of the study group

	Mean±SEM*	n
Age (years)	28.22±0.476	165
BMI (kg/m^2)	24.07±0.292	165
Family size (person)	5.9±0.186	165
Total B_{12} intake (µg/day)	5.857±0.370	165
Serum B_{12} (pg/ml)	273.26±8.629	165
Hemoglobin (g/dl)	13.79±0.131	164
Erythrocytes (x10^{12}/L)	4.87±0.0369	164
MCV (fl)	84.99±0.379	164
PCV (L/L)	0.41±0.004	164
MCH (pg)	28.30±0.174	164
MCHC (g/L)	332.94±0.789	164
RDW (%)	16.47±0.149	164
Platelets (x10^9/L)	259.90±4.404	164
MPV (fl)	9.23±0.117	162
WBCs (x10^9/L)	6.83±0.124	164
Neutrophils (x10^9/L)	3.85±0.103	164
Lymphocyres (x10^9/L)	2.09±0.047	164
Monocytes (x10^9/L)	0.47±0.012	164
Eosinophils (x10^9/L)	0.14±0.009	164
Basophils (x10^9/L)	0.06±0.002	164
MMA (µmole/L)	0.44±0.063	114

*SEM = Standard Error of Mean

Table 2: Distribution of subjects according to serum B_{12} levels

Serum B_{12} (pg/ml)	Frequency	Percentage (%)	Cumulative percentage (%)
<200	45	27.3	27.3
201-300	69	41.8	69.1
>300	51	30.9	100.0
Total	165	100.0	

Table 3: Distribution of subjects according to plasma MMA level*

	Frequency	Percentage
MMA<0.376 µmole/L	60	52.6
MMA≥0.376 µmole/L	54	47.4
n	114	100.0

*The cutoff point of MMA is 0.376 µmole/L

Table 4: Correlation between selected independent variables and serum vitamin B_{12} level for all subjects (n = 165)

Independent variable	Serum vitamin B_{12} level (pg/ml) Pearson correlation (r)	p-value
Age (years)	0.178	0.022*
BMI (kg/m^2)	0.063	0.424
Household size (persons)	-0.001	0.994
Hemoglobin (g/dl)	-0.048	0.545
MCV (fl)	-0.170	0.030*
MCH (pg)	-0.131	0.095
MCHC (g/L)	-0.004	0.957
MMA (µmole/L)	0.068	0.475
Total B_{12} intake (µg/day)	0.075	0.342

*Significant at p≤0.05

Table 5: Serum vitamin B_{12} levels of the study group according to different variables studied

Variable	n	Mean serum B_{12} (pg/ml) ± SEM	p-value
Age (years)			
20-29	96	258.78±11.798	0.047*
30-40	69	293.41±12.191	
Gender			
Females	99	270.20±11.093	0.666
Males	66	277.85±13.814	
Family size (persons)			
≤4	54	277.81±17.575	0.916
4-7	71	269.41±11.339	
>7	40	273.95±17.714	
BMI (kg/m^2)			
Underweight	7	253.86±30.866	0.696
Normal weight	99	272.11±11.658	
Overweight	48	270.10±15.944	
Obese	11	309.73±24.252	
Total B_{12} intake (µg/day)			
<2.4	19	265.68±24.019	0.752
≥2.4	146	274.25±09.262	

*Significant at p≤0.05

Plasma MMA concentrations were measured in those with serum B_{12} level ≤300 pg/ml. These subjects accounted for 69.1% of the total study group (Table 3). This test was done to confirm deficiency for those with serum B_{12} level ≤200 pg/ml and to check for possible hidden deficiency for those having serum B_{12} levels between 201-300 pg/ml. The cutoff point of MMA used to confirm deficiency was 0.376 µmole/L. Table 3 shows the percentage of subjects in which vitamin B_{12} deficiency is confirmed with plasma MMA.

As shown in Table 4, age was significantly correlated with vitamin B_{12} status (r = 0.178; p = 0.022). The mean vitamin B_{12} level for the age group 30-40 years was significantly higher than that for the age group 20-29 years (p =0.047) (Table 5). According to the same table, there was no significant difference in the means of vitamin B_{12} levels among males and females (p = 0.666).

Household (family) size represents the number of people living in the same house. Our results showed no significant difference between different household groups (≤4 persons, 4-7 and >7 persons) in regard to serum vitamin B_{12} level (p = 0.916) (Table 5). Also this table shows no significant difference in mean serum B_{12} levels among different BMI groups (p = 0.696).

The mean total vitamin B_{12} intake was 5.857 µg/day (Table 1). As shown in Table 4, this intake had no significant correlation with vitamin B_{12} status (r = 0.075; p = 0.342). The mean serum B_{12} level was not different in subjects with total B_{12} intake ≥2.4 µg/day and those with total B_{12} intake <2.4 µg/day (p = 0.752) (Table 5).

Different hematological parameters were compared for the three B_{12} groups. There was an inverse correlation between vitamin B_{12} status and MCV (r = -0.170; p = 0.03); whereas vitamin B_{12} levels were marginally correlated with MCH (r = -0.131, p = 0.095) (Table 4). On the other hand, as it is noticed from Table 6, there was a trend of having higher MCV values in the group of serum vitamin B_{12} ≤200 pg/ml compared to the other groups (p = 0.065). No difference in the means of other blood values were found among the B_{12} groups (Table 6).

Vitamin B_{12} status was found to have no relation with hemoglobin level ($r = -0.048$; $p = 0.545$) in the whole study sample (Table 4). Also by comparing the different B_{12} groups, no significant difference was found in hemoglobin levels among them (Table 6). No correlations were found between vitamin B_{12} status and other blood count variables ($p>0.05$) (Table 4).

Table 7 shows that 51.1% of those with serum B_{12} level ≤200 pg/ml had true B_{12} deficiency, while the percentage of true deficiency for those with low-normal vitamin B_{12} level (201-300 pg/ml) was 44.9% (as indicated by elevated MMA with values ≥0.376 μmole/L). The relatively high percentage of true deficiency in the low normal group confirmed their subclinical vitamin B_{12} deficiency.

Table 6: Mean CBC values for subjects with different serum B_{12} levels

Variable	B_{12} group	n	Mean variable ± SEM	p-value
Hemoglobin (g/dl)	≤200 pg/ml	45	13.91±0.223	0.777
	201-300 pg/ml	68	13.80±0.214	
	>300 pg/ml	51	13.67±0.244	
MCV (fl)	≤200 pg/ml	45	86.22±0.653	0.065
	201-300 pg/ml	68	84.99±0.617	
	>300 pg/ml	51	83.91±0.665	
MCH (pg)	≤200 pg/ml	45	28.79±0.296	0.141
	201-300 pg/ml	68	28.28±0.267	
	>300 pg/ml	51	27.89±0.336	
MCHC (g/L)	≤200 pg/ml	45	333.78±1.381	0.775
	201-300 pg/ml	68	332.38±1.146	
	>300 pg/ml	51	332.94±1.638	

Table 7: Percentage of subjects whom vitamin B_{12} deficiency is confirmed using plasma MMA levels

Serum vitamin B_{12} (pg/ml)	% of patients with MMA ≥0.376 μmol/L	% of patients with MMA <0.376 μmol/L
≤200	23 (51.1%)	22 (48.9%)
201-300	31 (44.9%)	38 (55.1%)
Total	54 (47.4%)	60 (52.6%)

DISCUSSION

Vitamin B_{12} deficiency in Jordan has increased to an alerting rate in the last few years (MOH, WHO and MOA, 2006). The studies conducted to evaluate the B_{12} status among Jordanians (Table 8) used serum vitamin B_{12} level as the only tool for determining the prevalence of deficiency; however, other diagnostic tools were not explored. In this study, we used Methylmalonic Acid (MMA) to confirm the deficiency when its levels are greater than 0.376 μmole/L (CDC, 2009). MMA being a metabolite in the B_{12} metabolic pathway, is considered an early marker for tissue vitamin B_{12} deficiency, even before hematologic manifestations occur (Oh and Brown, 2003).

The present study focused on the young adults (age 20-40 years) and it excluded all those older than 40 years. The reason was to limit the effect of age on increasing the prevalence of low serum vitamin B_{12} levels. Elderly patients may increase the prevalence of vitamin B_{12}, either because of low intake, or malabsorption by atrophic gastritis, or other chronic diseases such as diabetes mellitus. Age was reported to be an independent risk factor for developing vitamin B_{12} deficiency, irrespective to gastric atrophy (Gumurdulu *et al*., 2003). The results of our study showed that the mean serum B_{12} level in the younger age group (20-29 years) was significantly lower than the older group (30-40 years) ($p = 0.047$) (Table 5). These results are consistent with those of Barghouti *et al*. (2009) who found that high frequency of vitamin B_{12} deficiency (serum level ≤180 pg/ml) was observed in the age group between 18-24 years (51.8%) as compared with other age groups. These authors also found that B_{12} hypovitaminosis (serum B_{12} level between 180-300 pg/ml) was mostly in patients aged >64 years (44.4%). This prevalence of B_{12} deficiency in younger adults can be explained by their eating habits that are usually highly dependent on consumption of junk foods by the younger group.

High frequencies of vitamin B_{12} deficiency among young adults were also reported in studies conducted in several countries. In India, Yajnik *et al*. (2006) found that 67% of the participating healthy men (aged 30-50 years) had low vitamin B_{12} levels (<150 pmole/L). In Iran,

Table 8: Vitamin B_{12} deficiency in Jordan

Jordan	Cutoff point of vitamin B_{12} deficiency	n	Age (years)	Prevalence (%)
Fora and Mohammad (2005)	222 pg/ml	216	19-50	48.1%
Hakooz *et al*. (2006)	200 pg/ml	290	16-72	50.8% in Arabs
		118 Arabs		46.9% in Circassians
		172 Circassians		
Abu-Samak *et al*. (2008)	200 pg/ml	120	18-24	16%
Habib Allah (2008)	210 pg/ml	460	20-24	18.0%
Barghouti *et al*. (2009)	180 pg/ml	837	18-79	44.7%
		403	18-39	47.1%
The present study	180 pg/ml	165	20-40	≤180 pg/ml 18.8%
	200 pg/ml			≤200 pg/ml 27.3%
	222 pg/ml			≤222 pg/ml 36.4%
				≤300 pg/ml 69.1%

Shams *et al*. (2009) studied vitamin B_{12} deficiency in 984 healthy subjects aged 20-80 years. From them, 408 subjects were between 20-40 years. Shams and his colleagues found that among this young age group, 28.3% (156 of 408) had serum B_{12} level less than 200 pg/ml and this percentage increased to reach 53.9% in those who had serum B_{12} <250 pg/ml (220 of 408). All these results come in consistence with our results presented previously (Table 8). However, in a study done in Spain, serum vitamin B_{12} was reported to be significantly higher in younger age (25-39 years) than in older age groups (40-49 years) (Planells *et al*., 2003).

Gender was shown to have no effect on the occurrence of low vitamin B_{12} level. There was no significant difference in the mean serum vitamin B_{12} level between males and females ($p = 0.666$). Similar results were found in the studies done by other authors (Fora and Mohammad, 2005; Planells *et al*., 2003; Carmel *et al*., 1999). These authors reported no significant differences in the mean serum B_{12} between both sexes. However, Barghouti *et al*. (2009) and Hakooz *et al*. (2006) found that males had significantly lower vitamin B_{12} level than females ($p<0.05$). In contrast, Habib Allah (2008) found that males had marginally higher serum B_{12} concentration than females ($p = 0.072$). The difference on results could be related to eating habits of the study samples.

Household size had no association with vitamin B_{12} status of the volunteers ($p>0.05$) and the mean values of serum vitamin B_{12} level of the three groups of family size categories were not significantly different in this study. The average household size in Jordan according to Department of Statistics (DOS, 2008) is 5.2 persons. It was expected that upon increasing household size, the total intake of food would decrease. Moreover, bigger household size is usually associated with lower economic status; this also will contribute to lower dietary intake of food.

Obesity was reported to be associated with increasing risk of vitamin B_{12} deficiency (Pinhas-Hamiel *et al*., 2006). Abu Samak *et al*. (2008) found that 50% of those having vitamin B_{12} deficiency (<200 pg/ml) (16% of their study group), were overweight. However, in this study no association was found between BMI and vitamin B_{12} status ($r = 0.067$; $p = 0.424$). Moreover, there was no difference in the mean serum B_{12} levels among the different BMI categories ($p = 0.696$).

The only source of vitamin B_{12} is food from animal origin like meats, organ meats, dairy products, fish and poultry. The average daily intake of vitamin B_{12} in the study group was 5.857 µg/day, which is much higher than the daily requirement (2.4 µg/day). In contrast to what was suggested before by Fora and Mohammad (2005) and Barghouti *et al*. (2009) that low vitamin B_{12} intake is associated with vitamin B_{12} deficiency, we found no significant association between total vitamin B_{12} intake and vitamin B_{12} deficiency. In the previous studies, the average daily amount of vitamin B_{12} consumed was not calculated, thus it would be difficult to conclude that Jordanians have low consumption of meat products in their daily meals. Furthermore, it was also reported that low intake of vitamin B_{12} was not common in Europe and was reported to only be seen in one small Greek study. Vitamin B_{12} intake was not associated with vitamin B_{12} inadequate status in Netherlands and Germany; this explains the inadequate vitamin B_{12} status despite sufficient intake levels in these countries (Dhonukshe-Rutten *et al*., 2009).

High Mean Cell Volume (MCV) (above 100 fl) was reported to be an indicator of vitamin B_{12} deficiency (Galloway and Hamilton, 2007). In the present study, although MCV was within normal range, it showed an inverse correlation with vitamin B_{12} level ($r = -0.170$; $p = 0.03$). Moreover, there was a marginal higher MCV values among those with vitamin B_{12} <200 pg/ml (Table 6). In spite of this finding, we cannot conclude that MCV alone can give an idea of having risk of vitamin B_{12} deficiency. Other blood indices showed no association with total serum B_{12} level.

Serum vitamin B_{12} level is widely used as the standard method for diagnosing vitamin B_{12} deficiency despite its limited specificity and controversy about its sensitivity (Hvas and Nexo, 2006). Previous studies done in Jordan relied only on serum vitamin B_{12} level to determine the prevalence of vitamin B_{12} deficiency (Table 8). To check for the true prevalence, MMA test was used as a confirmatory test for vitamin B_{12} deficiency using the cutoff point defined by CDC (CDC, 2009) (which is 0.376 µmole/L). Any value above this level is considered a positive result confirming deficiency.

It was previously reported that approximately 50% of patients with serum vitamin B_{12} levels >200 pg/ml have elevated tHcy or MMA. This explain that using low serum vitamin B_{12} level as the sole means of diagnosis may miss up to one half of patients with actual tissue B_{12} deficiency (Oh and Brown, 2003). In our study, we found that 44.9% of those with low-normal vitamin B_{12} levels have elevated MMA confirming their subclinical vitamin B_{12} deficiency (Table 7). It was reported that serum MMA and/or tHcy were elevated in a considerable number of apparently healthy volunteers who had low-normal cobalamin level (up to 300 or 350 pg/ml) (Carmel *et al*., 2003). Therefore, levels between 200-350 pg/ml represents subclinical deficiency. Moreover, in the Framingham study, it was found that 32% of those with cobalamin levels up to 350 pg/ml had abnormal tHcy and/or MMA levels (Carmel *et al*., 2003).

In the present study, 51.1% of those with low serum B_{12} level ($\leq$200 pg/ml) had elevated MMA ($\geq$0.376 µmole/L) (Table 7). It was reported that at least 25% of such low serum B_{12} levels are not associated with elevated metabolite levels and may not indicate B_{12} deficiency.

Some of these are caused by partial deficiency of the transport protein transcobalamin (Wickramasinghe, 2006).

Conclusion: Serum B_{12} level is not a specific test for true vitamin B_{12} deficiency. Furthermore, dietary vitamin B_{12} intake by itself is not an indicator of B_{12} deficiency.

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