Are vitamin B-12 measurements adequate for evaluating its deficiency in individuals?

doi: 10.6133/apjcn.202411/PP.0006
Published online: November 2024

Running title: Vitamin B-12 precision: Adequacy of measurements

Selda Demirtaş MD[1], Sedat Özdemir MD[2]

[1]Ufuk University, Dr. Rıdvan EGE Training and Research Hospital, Mevlana Bulvarı (Konya Yolu), Ankara, Turkey
[2]Ankara Atatürk Sanatorium Training and Research Hospital, Ankara, Turkey

Corresponding Author: Dr Sedat Özdemir. Ankara Atatürk Sanatorium Training and Research Hospital, G blok Kat 1 Biyokimya Laboratuvarı Kuşcağız Mah. Sanatoryum Cad. No: 271 06280, Keçiören, Ankara, Turkey. Tel: (0312) 567 70 00. Email: drsedatozdemir@gmail.com, sedat.ozdemir@saglik.gov.tr

ABSTRACT

Background and Objectives: Measurement of vitamin B-12 deficiency using different methods may cause diagnostic difficulties. In order to rapidly and safely diagnose vitamin B-12 deficiency, it is important to determine the reference ranges of serum B-12 and its related biomarkers such as homocysteine, holotranscobalamine (holo-TC) and methylmalonic acid (MMA). This study aimed to determine reference interval (RI)s for serum vitamin B-12 and related markers. **Methods and Study Design:** Samples were collected from 404 young-to-middle-aged healthy adults aged 18-65 years. Vitamin B-12, homocysteine, holotranscobalamin, folate were analyzed using the Arcitect i2000 device. Plasma MMA was analyzed by LC/MS. RIs were then evaluated accordingly. **Results:** Vitamin B-12, folate, homocysteine, holotranscobalamin and plasma MMA were 139.1-619.1 pg/mL, 3.0-14.7 ng/mL, 5.6-18.4 μmol/L, 10.7-101.4pmol/L, and 0.01-0.8 μmol/L, respectively. Age group-specific RIs were also generated. **Conclusions:** This study revealed that the diagnosis of vitamin B-12 deficiency should not only be based on serum vitamin B-12 levels, but also of folate, homocysteine, holotranscobalamin and MMA levels; all which are related to vitamin B-12 metabolism.

Key Words: vitamin B-12, folate, holotranscobalamin II, methylmalonic acid, homocysteine

INTRODUCTION

Vitamin B-12 (cobalamin) is an essential nutrient required for normal cell activity in the human body. It acts as a coenzyme for hematopoiesis and neuropsychiatric processes, and plays an important role in synthesizing key molecules, including hormones, neurotransmitters, and DNA (Figure 1). Vitamin B-12 is also essential for the nervous system, particularly in nerve metabolism, myelin synthesis, and neuronal regeneration.[1]

Vitamin B-12 deficiency often leads to hematologic disorders, nervous system symptoms, and cognitive dysfunction.[2] Clinically symptomatic cobalamin deficiency is widely regarded as a condition primarily affecting older individuals, with symptoms observed in 1–2% of this population.[3] Vitamin B-12 deficiency in young and middle-aged individuals is diagnosed based on subnormal levels of biochemical biomarkers, often without clinical symptoms. However, studies suggest that such deficiency may lead to declines in cognitive and neurological functions.[4,5] This condition is often overlooked or mistaken for other disorders and is typically confirmed only after detecting a subnormal vitamin B-12 level. This

condition, known as subclinical cobalamin deficiency (SCCD), accounts for 10–20% of vitamin B-12 deficiency cases. Diagnosis of SCCD based on clinical findings is challenging and relies solely on biochemical biomarkers.[3] A definitive diagnosis of vitamin B-12 deficiency requires multiple biomarkers. While circulating vitamin B-12 levels are commonly measured, variability in these measurements can lead to diagnostic errors. As a result, some experts recommend using functional biomarkers, such as homocysteine or preferably methylmalonic acid (MMA), for diagnosis.[6] Holo-transcobalamin (holo-TC), also called active vitamin B-12 due to its role in cobalamin transport into cells, has recently been proposed as a more effective marker for diagnosing B-12 deficiency.[7] Vitamin B-12 deficiency impacts the proper metabolism of folic acid, as the enzyme MTHFR is required to convert folate into its active form, N5-methyl-THF. This conversion is essential for processes like DNA synthesis, neurotransmitter production, and red blood cell formation. Therefore, sufficient vitamin B-12 levels are crucial for the proper metabolism of folic acid.[8]

The primary purpose of establishing reference intervals (RIs) for the blood levels of biochemical parameters is to define the range of normal physiological variation and to distinguish between healthy individuals and patients. RIs are crucial for accurately interpreting patient results during clinical assessments, offering essential guidance for diagnosis, treatment monitoring, and risk evaluation. Tailored to the demographic and biological characteristics of the population, these intervals enhance the precision and reliability of clinical decision-making.

It should be emphasized that the RIs for assessing vitamin B12 levels in this study should only be applied to older adults and not in younger and middle-aged individuals. Research suggests that, contrary to common belief, vitamin B12 deficiency observed in older adults can also occur in younger and middle-aged individuals. Therefore, establishing appropriate reference intervals for this age group is essential for accurate diagnosis.

In the present study, we analyzed all biomarkers involved in vitamin B-12 metabolism, including vitamin B-12, holo-TC, homocysteine, folate, and plasma MMA, in healthy individuals aged 18–65 years. This study aimed to demonstrate that vitamin B-12 levels should be assessed not only through serum vitamin B-12 but also by using a panel of markers involved in vitamin B-12 metabolism. The present study also categorized young and middle-aged individuals (18–65 years) into several age groups to establish age-specific reference intervals for vitamin B-12 and related markers. This approach may help reveal how biomarkers change with age and the correlations between these biomarkers.

MATERIALS AND METHODS

This study included healthy volunteers aged 18–65 years who visited the blood bank and outpatient clinics of Ufuk University, Rıdvan Ege Hospital. Blood samples were collected between October 2014 and May 2015 and were analyzed after obtaining informed consent from the volunteers. The study involved young and middle-aged healthy individuals who attended Ufuk University Hospital for routine check-ups or aesthetic procedures. We ensured that all participants were free from significant health conditions. Additionally, there are no conflicts of interest among the individuals who consented to participate in the study. Each group was analyzed based on sex and age range.

Vitamin B-12, folate, homocysteine, and holo-TC samples were collected in BD brand gel separator tubes (BD Vacutainer; Becton Dickinson, Meylan, France). Plasma MMA samples were collected in Li-heparin tubes (BD Vacutainer) for plasma separation. All samples were stored at −80°C until the day of analysis. At the biochemistry laboratory of Ufuk University, Dr. RıdvanEge Training and Research Hospital, serum vitamin B-12, holo-TC, homocysteine, and folate levels were measured using Abbott Architect i2000sr analyzer (Abbott Diagnostics, Abbott Park, IL, USA) based on the chemiluminescent microparticle immunoassay (CMIA). Periodic internal and external quality checks were performed to identify errors that could potentially affect the test results. Plasma MMA levels were measured using liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Agilent Technologies®, Santa Clara, California, USA). The assay demonstrated a minimum detection limit of approximately 0.005 µM for plasma MMA. The analytical sensitivity of the LC-MS/MS system enabled precise and accurate measurement of MMA concentrations within this detection range, ensuring reliable evaluation of metabolic status. MMA, succinic acid, tert-butyl methyl ether, and hydrochloric acid n-butanol (3 M) were procured from Sigma-Aldrich (St. Louis, MO). Deuterium-labeled MMA (d3MMA) was purchased from CDN Isotopes (Quebec, Canada). ACS-grade methanol, phosphoric acid (H3PO4), glacial acetic acid, and acetonitrile were obtained from Thermo Fisher Scientific (Pittsburgh, PA). Purified water (with18 MΩ resistance) was prepared using CLRW Clinical Laboratory Reagent Water Systems (Bergama Tıp, Izmir, Turkey) and was used for all samples, calibrators, and reagents. Quality control samples were analyzed at low-, medium-, and high-level classification. All samples were screened for MMA and spiked with MMA standard solution to achieve the desired concentrations.

The study included volunteers who were selected from healthy individuals visiting our hospital. Healthy individuals aged 18–65 years who had no systemic disease, were not on any

medication including herbal or vitamin preparations, and had not received vitamin B-12 therapy over the last 6 months.

The study excluded participants who administered any medication affecting vitamin B-12 metabolism over the last 2 weeks (including those taking oral multivitamin tablets), women using oral contraceptives, individuals who had an acute infection over the last week, those with chronic diseases (systemic inflammatory diseases and liver and kidney diseases), pregnant women, women who gave birth in the last 3 months, those with low blood count and anemia criteria, those with positive hepatitis markers, and those who underwent surgery in the last 6 months.

Serum B12, folate, homocysteine, holotranscobalamin and serum MMA levels of the volunteers participating in the study were evaluated in accordance with CLSI EP09-A3 and IFCC-CLSI C28-A3 guidelines.

Ethics

The protocol for this study has been approved by the Ethics Committee of Ufuk University with the protocol number 27.12.2013/1. The research was conducted in compliance with the principles of the Declaration of Helsinki as revised in 1995 and 2000 in Edinburgh.

Statistical analysis

Statistical analysis was performed using IBM Statistical Package for Social Sciences (SPSS software v.21, Chicago, IL, USA),and reference interval analysis was performed using Reference Value Advisor v2.0 software program. 9-12 Numerical data were analyzed for normality of distribution using the Kolmogorov–Smirnov test. As the measured vitamin B-12 and all related markers were non-normally distributed, pair wise group comparisons were performed using the Mann–Whitney U test and whereas multiple group comparisons were performed using the Kruskal–Wallis test. Intergroup comparisons were conducted using the Conover–Iman test. p-values of $p<0.05$ were considered to indicate statistical significance.

Also the study parameters were analyzed using the Mann – Whitney U test to determine if there were any differences in reference intervals between males and females. The results showed that no statistically significant difference ($p \geq 0,05$) was observed in reference intervals for vitamin B-12, folate, homocysteine, holo-TC, and plasma MMA between males and females.

RESULTS

A total of 6524 patients who visited the outpatient clinics of Ufuk University Dr. Rıdvan Ege Training and Research Hospital were considered for inclusion and selected based on the inclusion and exclusion criteria described in Methods. Overall, 460 patients were considered eligible. After face-to-face interviews, 438 volunteered to participate in the study. After sample review, 11 lipemic and 16 hemolyzed samples were excluded. Samples from seven volunteers could not be analyzed due to inadequate volume. The mean age of 404 volunteers who participated in the study was 25 (18-65) years. Among them, 156 (38.61%) volunteers were male and 248 (61.38%). There are no significant differences in vitamin B12 (p=0.899) and associated markers, including folate (p=0.624), homocysteine (p=0.911), Holo-TC (p=0.534), plasma MMA (p=0.970), between genders. Age and sex distribution of the volunteers, number of volunteers, and reference intervals by sex are presented as shown in Table 1.

The present study revealed a reference interval of 139.1–619.1 pg/mL for vitamin B12, 3.0–14.7 ng/mL for folate, 5.6–18.4 µmol/L for homocysteine, 10.7–101.4pmol/L for holo-TC, and 0.01–0.8 µmol/L for plasma MMA as shown in Figure 2.

The results were classified by age groups to determine age-specific reference intervals as shown in Table 2. The results showed that reference intervals varied by age group for B-12 and related markers such as folate, holo-TC, and plasma MMA. The reference interval for homocysteine, however, did not vary by age.

Regarding vitamin B-12, the age-specific reference interval was 132.2–629.6 pg/mL for young individuals aged 18–25 years (classified as Group I), whereas that for the oldest group aged 56–65 years (Group V) was 175–653.9 pg/mL.

Regarding folate, the reference interval was 2.7–11.4 ng/mL for Group I (18–25 years) and 3.3–17.9 ng/mL for Group V (56–65 years).

Regarding holo-TC, the lowest reference interval (8.9–87.7 pmol/L) was observed in Group II comprising individuals aged 26–35 years, whereas the highest reference interval (6.9–110 pmol/mL) was observed in Group IV comprising individuals aged 46–55 years.

Plasma MMA levels did not vary by age, and the corresponding reference interval was found to be 0.01–0.8 µmol/L. The lowest interval for this parameter was 0.01–0.6 µmol/L, as observed in Group II (26–35 years) and Group IV (46–55 years).

Contrary to popular belief, there are age-related differences even among young to middle-aged healthy individuals when comparing Vitamin B-12 levels and other measured markers with different age groups.

In the present study, the correlation between parameters are examined. p-values and correlations among the tests are summarized in Table 3.

No correlation was found between homocysteine and plasma MMA (r = -0.03). Significant correlations were observed among other parameters (r > 0.05). Notably, a strong correlation was identified between vitamin B12 and Holo-TC. Additionally, correlations were found between age and all parameters. A negative correlation was noted between age and plasma MMA (r = -0.256), while positive correlations were observed with other parameters.

DISCUSSION

The reference intervals determined in the present study for vitamin B-12, folate, homocysteine, and holo-TC were compared with those used in present laboratory, which were established based on measurements using Abbott Architect i2000 analyzer. The reference intervals found in this study for all parameters were lower than the currently used reference intervals. The obtained reference interval for plasma MMA was compared with the corresponding LC–MS/MS reference interval currently used in clinics, which showed that the current reference interval based on the analyzer was higher than the reference interval found in present study. This indicates that the use of international reference intervals provided by companies for B-12 and associated markers may be inadequate for diagnosis and follow-up.

There were some limitations in the present study. As there have been relatively few studies evaluating reference intervals for all analyzed parameters, we compared our results to only a small number of studies. A second limitation was that the enzymes methionine synthase and methylmalonyl-CoA mutase was not measured due to measurement difficulties and funding issues. Also serum folate levels are considered an important biomarker for the short-term assessment of folate status. Serum folate reflects the immediate folate levels in the body and can be used for the early detection of folate deficiency. Studies have shown that low serum folate levels can reliably indicate folate deficiency. Additionally, increases in serum folate levels shortly after folate supplementation confirm its effectiveness in reflecting folate bioavailability and levels in the body.[6] These findings support that serum folate levels alone can be a valid parameter for assessing folate status.

Serum B-12 test is one of the most frequently requested tests in clinical practise. In particular, when certain neuropathic, hematologic, and cognitive symptoms are observed, clinicians determine B-12 deficiency based on serum B-12 levels. This indicates the need for establishing a reliable reference interval for the diagnosis, follow-up, and treatment of B-12

deficiency. However, the use of variety methods via different analyzers poses a problem for accurate diagnosis.

The HELENA Study included 1051, 12.5–17.9 years from 10 cities of 9 European countries and investigated vitamin B-12 levels using chemiluminescence via Siemens DPC Immulite 2000 autoanalyzer. According to the data obtained from this study, the reference interval for vitamin B-12 was 199.5–977.5 pg/mL (147.3–721.4 pmol/L).[13] Moreover, in a study conducted in Sydney, Australia, involving 302 healthy female university students aged 18–35 years, the reference interval for vitamin B-12 was determined to be 162.6–813 pg/mL (120–600 pmol/L) using chemiluminescence via Beckman Coulter UniCel DXI 800 Access autoanalyzer.[14] None of these reference intervals were consistent with those found in the present study. This may be due to the inclusion of adolescents alone and the use of different analyzers in the HELENA study. Additionally, the fact that the Helena study is multicentered that also a reason for the difference. Andersen et al. investigated vitamin B-12 reference intervals in 129 individuals using Abbott Alinity analyzer via CMIA and found a reference interval of 227.6–749.3 pg/mL (168–553 pmol/L),[15] which is similar to that found in the present study. In another study, Demirin et al. assessed 1251 individuals aged 18–79 years and used Siemens DPC Immulite 2000 autoanalyzer for measurements, which yielded a reference interval of 158–563 pg/mL for vitamin B-12.[16]

Similar findings in Andersen et al.'s study may be attributed to the use of similar methods and reagents in both studies. In Demirin at al's study, they investigated a similar age group using a different analyzer and found a reference range similar to that found in the current study. This suggests that studies conducted with different age groups may give similar reference ranges regardless of device differences. In the National Health and Nutrition Examination Survey (NHANES), conducted with a large population, a value of 139.1 pg/mL was found for vitamin B-12, compared to the lower limit of 200 pg/mL (148 pmol/L) for vitamin B-12. However, this shows that regional factors also play an important role in vitamin B-12 deficiency.

Notably, analysis of the differences between age groups in this study revealed that vitamin B-12 levels were lower in younger individuals aged 18–25 years, the group pursuing higher education, than in individuals aged 56–65 years, considered as the middle-aged group. This result might be considered an indicator of nutritional deficiency in the young group engaged in academic activities.

Regarding folate, the reference interval found in the present study was lower than that indicated by the manufacturer of the analyzer used. This led us to conclude that the Turkish

population is deficient not only in vitamin B-12 but also in folate. A retrospective study involving 723 Korean adults reported a reference interval of 2.9–38.0 ng/mL for folate.[17] Schwettmannn et al. conducted a study involving 144 volunteers using Abbott Architect i2000 analyzer and found a reference interval of 5.2–29.2 nmol/L (2.9–12.8 ng/mL) for folate, 18 whereas a study conducted by Önder et al. in 300 healthy individuals using Siemens Advia Centaur XP autoanalyzer revealed a folate reference interval of 2.87–19.49 ng/mL.[19] Considering the reference interval of 3–14.7 ng/mL for folate found in the present study, it seems that the lower limits for folate reported in these studies are consistent with each other regardless of the analyzer and method used for folate measurement. However, Schwettmann et al. found a reference range closer to the current study; this suggests that age is an important determinant of folate levels. Indeed, the comparison of reference intervals for folate in different age groups showed a significant difference between the youngest and oldest groups. This indicates, contrary to common knowledge, a deficiency in folate levels in the younger population. Considering that folate is essential for fetal neurological development, especially during the reproductive period, subnormal folate levels among young individuals of reproductive age is a significant public health problem. Folate deficiency may be coupled with B-12 deficiency, indicating the importance of a combined assessment of serum levels of both vitamins in the diagnosis of deficiency.

Elevated homocysteine levels are a risk factor for coronary heart disease and are closely associated with low levels of vitamin B-12. The reference interval for homocysteine found in the present study was higher than that provided by the manufacturer, which indicates a serious vitamin B-12 deficiency and consequently elevated homocysteine levels in the community. To compare the reference intervals found in the present study with those reported in the literature, a study involving 20880 people was conducted to determine the reference interval for homocysteine, which revealed a reference interval of 9–14.6 µmol/L.[20] Thus, the lower limit reported in the abovementioned study was higher than that reported in our study, whereas the upper limit was lower than that found in the present study. Furthermore, the same study revealed a difference in reference intervals between males and females. Present study, however, found no significant difference between males and females, which may explain the difference in these values. In a study conducted by Kweon et al. in 3150 individuals, the reference intervals for homocysteine in females and males were 5.03–13.80 and 3.95–10.19 µmol/L, respectively.[21] The reference intervals reported in the abovementioned study for both males and females were lower than those reported in our study. In males, the reference interval was reported to be 4.81–12.27 µmol/L for individuals aged 45–54 years and 5.00–

14.80 µmol/L for those aged 55–64 years, whereas in females, the interval was reported to be 3.74–8.98 µmol/L for those aged 45–54 years and 3.99–9.80 µmol/L for those aged 55–64 years. Present study, however, found no significant difference between males and females in the same age groups, with a reference interval of 3.9–16.1 µmol/L for those aged 46–55 years and 6.7–20.6 µmol/L for those aged 56–65 years. The reference interval for homocysteine found in the present study was higher than that reported by Kweon.[21] Furthermore, a study conducted by Lahiri et al. involving 1288 healthy individuals reported a homocysteine interval of 6.5–16.3 µmol/L,[22] which is similar to that found in present study. This suggests that differences in reference intervals may be due to diet and lifestyle rather than racial differences. In addition, as vitamin B-12 deficiency in the population leads to an increase in homocysteine levels, caution should be exercised in monitoring high homocysteine levels in populations with low B-12 levels.

The literature contains scarce data regarding reference intervals for holo-TC. The present study found an interval of 10.7–101.4 pmol/L for holo-TC. Among the limited number of reports, a study by Refsum et al. involving 500 healthy individuals aged 18–69 years, which recommended the measurement of holo-TC levels for confirming subnormal B-12 levels, reported a holo-TC reference interval of 42–157 pmol/L.[23] Another study involving 105 healthy individuals aged 20–80 yearsreported a holo-TC reference interval of 24–157 pmol/L.[24] Although the upper reference limits in both studies were the same, the lower reference limits were significantly different. In contrast, Al Aisari et al. revealed a holo-TC reference interval of 7.75–128 pmol/L.[25] Compared with the first two studies, our study revealed a considerably lower reference interval. The lower limit reported by Aisari et al. was lower than that reported inour study as well as other studies. However, the upper limit reported by Aisari et al. was still higher than that reported in the present study. Holo-TC measurement maybe more suitable than total vitamin B-12 measurement for diagnosing vitamin B-12 deficiency.[7]

MMA level is considered the best indicator of intracellular vitamin B-12 deficiency. In patients with vitamin B-12 deficiency, this enzyme system does not work well and causes MMA accumulation in the blood. Several studies have investigated the reference range for plasma MMA based on LC-MS/MS. Present study revealed a plasma MMA range of 0.01–0.8 µmol/L. However, the NHANES study, which included 7300 volunteers, found that the plasma MMA range was 60–210 nmol/L (0.06–0.21 µmol/L).[26] Mineva et al. Based on data from 10,020 individuals through review of 2011–2014 NHANES data, a plasma MMA reference range of 70.6–451 nmol/L (0.076–0.45 µmol/L) was obtained.[27] In another study

involving 285 healthy individuals aged 18–69 years, the plasma MMA reference range was reported to be 0.10–0.40 μmol/L.[28] These three studies yielded results below the reference range found in this study. In contrast, Obeid et al. In this study involving 138 individuals, a reference range of 0.15–0.782 μmol/L was reported for plasma MMA.[29] The upper limit of the reference range in the above-mentioned study is consistent with that reported in the present study, and the high plasma MMA level is due to the presence of individuals with low vitamin B-12 levels in the study. This suggests that the vitamin B-12 levels found in present study were low.

Relatively higher plasma MMA levels were found in this study compared to other studies, which may be attributed to B-12 deficiency in the population. Absence or lack of enzyme activity due to vitamin B-12 deficiency leads to increased plasma MMA levels, which may explain the high reference range found in this study.[30] As only a few studies in the literature have provided reference intervals for all analyzed parameters, we separately compared the reference intervals for parameters in the present study with those from different publications.

Conclusions

In addition to the fact that Vitamin B12 measurement methods have yet to be standardized, serum B12 measurements may yield falsely low or falsely normal results. This can lead to diagnostic errors.[3] Therefore, in present study, we aimed to minimize diagnostic errors by presenting the reference ranges for Vitamin B12.

Altogether, these findings suggest that low levels of vitamin B-12 in the population may lead to low or high reference ranges for relevant markers. This study revealed that, contrary to popular belief, levels of vitamin B-12 and related markers were significantly lower not only in the older group, but also in younger individuals, the most productive group with higher education. Therefore, it is recommended that vitamin B-12 levels be evaluated together with holo-TC levels, which show a strong correlation with B-12 levels, in healthy young and middle-aged individuals. In addition, in order to obtain accurate results, the diagnosis of vitamin B-12 deficiency must be made based on a panel consisting of vitamin B-12 and related markers such as folate, homocysteine, holo-TC, plasma MMA. Current methods cannot fully reveal vitamin B-12 deficiency due to methodological reasons and the lack of standard reference ranges. Such a panel may aid in the diagnosis of subclinical cobalamin deficiency, which has been described previously and is believed to be common among young adults. Signs and symptoms such as poor academic performance, cognitive changes, difficulty understanding, fatigue in young people should be taken seriously and all vitamin B-12 related

markers should be evaluated together to provide appropriate treatment. As a result, to accurately diagnose vitamin B12 deficiency and provide effective treatment, it may be beneficial to assess all markers related to vitamin B12 collectively.

CONFLICT OF INTEREST AND FUNDING DISCLOSURE

The authors declare no conflict of interests.

This study was supported by the TUBITAK Rapid Support (Project No: 114S844). Funders had no role in study design, data collection and analysis, decision to publish, or drafting of the manuscript.

REFERENCES

1. 1. Bor MV, Lydeking-Olsen E, Moller J, Nexo E. A daily intake of approximately 6 microg vitamin B-12 appears to saturate all the vitamin B-12-related variables in Danish postmenopausal women. Am J Clin Nutr. 2006;83(1):52-8.
2. Sezgin Y. Vitamin B-12 Yetersizliğine Yaklaşım. Konuralp Medical Journal. 2019;11(3):482-8.Turkish.
3. Carmel R, Sarrai M. Diagnosis and management of clinical and subclinical cobalamin deficiency: advances and controversies. Curr Hematol Rep. 2006;5(1):23-33.
4. Wang Z, Zhu W, Xing Y, Jia J, Tang Y. B vitamins and prevention of cognitive decline and incident dementia: a systematic review and meta-analysis. Nutr Rev. 2022;80(4):931-49.
5. Schrempf W, Eulitz M, Neumeister V, Siegert G, Koch R, Reichmann H, Storch A. Utility of measuring vitamin B-12 and its active fraction, holotranscobalamin, in neurological vitamin B-12 deficiency syndromes. J Neurol. 2011;258(3):393-401.
6. Yetley EA, Pfeiffer CM, Phinney KW, Bailey RL, Blackmore S, Bock JL, et al. Biomarkers of vitamin B-12 status in NHANES: a roundtable summary. Am J Clin Nutr. 2011;94(1):313S-21S.
7. Nexo E, Hoffmann-Lucke E. Holotranscobalamin, a marker of vitamin B-12 status: analytical aspects and clinical utility. Am J Clin Nutr. 2011;94(1):359S-65S.
8. Smith C, Marks A, Lieberman M. Mark's. Temel Tıbbi Biyokimyası Klinik Yaklaşım.2nd ed, Đnal ME, Atik U, Aksoy N, Haşimi A, editor. Güneş Tıp Kitabevleri Yayınları(Ankara); 2007.
9. Wilks SS. Statistical prediction with special reference to the problem of tolerance limits. The annals of mathematical statistics. 1942;13(4):400-9.
10. Tukey JW. Some thoughts on clinical trials, especially problems of multiplicity. Science. 1977;198(4318):679-84.
11. Horn PS. A biweight prediction interval for random samples. Journal of the American Statistical Association. 1988;83(401):249-56.
12. McWilliams TP. A distribution-free test for symmetry based on a runs statistic. Journal of the American Statistical Association. 1990;85(412):1130-3.

13. Gonzalez-Gross M, Benser J, Breidenassel C, Albers U, Huybrechts I, Valtuena J, et al. Gender and age influence blood folate, vitamin B12, vitamin B6, and homocysteine levels in European adolescents: the Helena Study. Nutr Res. 2012;32(11):817-26.
14. Fayet-Moore F, Petocz P, Samman S. Micronutrient status in female university students: iron, zinc, copper, selenium, vitamin B-12 and folate. Nutrients. 2014;6(11):5103-16.
15. Andersen SL, Hansen AB, Hindersson P, Andersen L, Christensen PA. Vitamin B-12 reference intervals. Dan Med J. 2023;70(6).
16. Demirin H, Memişoğullari R, Uçgun T, Yildirim HA, Celer A, Bulur Ş, et al. Batı Karadeniz Bölgesinde yaşayan Türk erişkinlerinde demir, ferritin, B 12 vitamini ve folat gibi anemi parametrelerinin referans aralıkları uygun mudur? Turkish Journal of Biochemistry/Turk Biyokimya Dergisi. 2012;37(4).Turkish.
17. Choi R, Oh Y, Park MJ, Lee SG, Lee EH. Reference Interval for Korean Serum Folate Assay Traceable to the WHO International Standard. Clin Lab. 2021 Apr 1;67(4). doi: 10.7754/Clin.Lab.2020.200714. PMID: 33865264..
18. Schwettmann L, Berbu S. Reference Interval and Status for Serum Folate and Serum Vitamin B-12 in a Norwegian Population. Clinical Laboratory. 2015;61(8):1095-100.
19. Önder Y, Tezcan S, Kadılar ÖH, Dağdelen LK, Madenci ÖÇ, Yücel N, Orçun A. Determination of vitamin B-12 and folic acid Reference intervals at Kartal district. J Turk Clin Biochem. 2014;12(3):131-6.
20. Ma C, Wang X, Wu J, Cheng X, Xia L, Xue F, Qiu L. Real-world big-data studies in laboratory medicine: current status, application, and future considerations. Clinical Biochemistry. 2020;84:21-30.
21. Kweon S-S, Lee Y-H, Jeong S-K, Nam H-S, Park K-S, Choi S-W, et al. Methylenetetrahydrofolate reductase 677 genotype-specific reference values for plasma homocysteine and serum folate concentrations in korean population aged 45 to 74 years: the Namwon study. Journal of Korean Medical Science. 2014;29(5):743-7.
22. Lahiri KD, Datta H, Das HN. Reference interval determination of total plasma homocysteine in an Indian population. Indian Journal of Clinical Biochemistry. 2014;29:74-8.
23. Refsum H, Johnston C, Guttormsen AB, Nexo E. Holotranscobalamin and total transcobalamin in human plasma: determination, determinants, and reference values in healthy adults. Clinical chemistry. 2006;52(1):129-37.
24. Ulleland M, Eilertsen I, Quadros EV, Rothenberg SP, Fedosov SN, Sundrehagen E, Orning L. Direct assay for cobalamin bound to transcobalamin (holo-transcobalamin) in serum. Clinical chemistry. 2002;48(3):526-32.
25. Al Aisari F, Al-Hashmi H, Mula-Abed W-A. Comparison between serum holotranscobalamin and total vitamin B-12 as indicators of vitamin B-12 status. Oman Medical Journal. 2010;25(1):9.
26. Pfeiffer CM, Caudill SP, Gunter EW, Osterloh J, Sampson EJ. Biochemical indicators of B vitamin status in the US population after folic acid fortification: results from the National Health and Nutrition Examination Survey 1999–2000–. The American journal of clinical nutrition. 2005;82(2):442-50.

27. Mineva EM, Sternberg MR, Zhang M, Aoki Y, Storandt R, Bailey RL, Pfeiffer CM. Age-specific reference ranges are needed to interpret serum methylmalonic acid concentrations in the US population. The American journal of clinical nutrition. 2019;110(1):158-68.
28. Abildgaard A, Knudsen CS, Hoejskov CS, Greibe E, Parkner T. Reference intervals for plasma vitamin B-12 and plasma/serum methylmalonic acid in Danish children, adults and elderly. Clinica Chimica Acta. 2022;525:62-8.
29. Obeid R, Geisel J, Herrmann W. Comparison of two methods for measuring methylmalonic acid as a marker for vitamin B-12 deficiency. Diagnosis. 2015;2(1):67-72.
30. Froese DS, Fowler B, Baumgartner MR. Vitamin B(12) , folate, and the methionine remethylation cycle-biochemistry, pathways, and regulation. J Inherit Metab Dis. 2019;42(4):673-85.

Table 1. Age, gender and reference interval table

	95% RI		p*	
	Men (n=156)	Women (n=248)		Total (n=404)
		RI%95		RI%95
Age (Mean±SD)	36.9±16.09	29.9±13.3		32.6±14.8
Vitamin B-12 (pg/mL)	134.4-674.5	142.2-581	0.899	139.1-619.1
Folate (ng/mL)	2.7-14.9	3.2-13.6	0.624	3-14.7
Homocysteine (µmol/L)	5.8-23.2	5.4-17.4	0.911	5.6-18.4
Holo-TC (pmol/L)	9.4-96.6	10.3-101.5	0.534	10.7-101.4
Plasma MMA(µmol/L)	0.01-0.8	0.01-0.8	0.070	0.01-0.8

RI: reference intervals
†95% Reference interval were calculated according to the IFCC-CLSI C28-A3 guideline.
*p < 0,05 is considered statistically significant.

Table 2. Reference intervals by age groups

Age groups	2.5-97.5% RI†					
	Group I (18-25 y/o)	Group II (26-35 y/o)	Group III (36-45 y/o)	Group IV (46-55 y/o)	Group V (56-65 y/o)	Total (18-65 y/o)
n	211	59	37	39	58	404
Vitamin B-12 (pg/ml)	132.2-629.6	143-632.5	163.6-633.4	116.8-579.4	175.2-653.9	139.1-619.1
Folate (ng/ml)	2.7-11.4	2.3-18.3	3.1-18.6	3.2-13.7	3.2-17.9	3-14.7
Homocystein (µmol/L)	5.6-23	5.3-16.7	5.3-21.2	5.7-16.2	6.6-20.9	5.6-18.4
Holo TC (pmol/L)	10.5-100.7	8.9-87.7	9.4-102.4	10.2-110	8.3-115	10.7-101.4
Plasma MMA (µmol/L)	0.01-0.8	0.01-0.6	0.01-0.7	0.01-0.6	0.01-0.5	0.01-0.8

y/o: years old; RI: reference intervals.
†95% Reference interval were calculated according to the IFCC-CLSI C28-A3 guideline.

Table 3. Correlation between parameters (r) and (*p*) values

Spearman's rho	Age	Vitamin B-12 (pg/ml)	Folate (ng/ml)	Homocysteine (µmol/L)	Holo TC (pmol/L)	Plasma MMA (µmol/L)
Age						
r	1,000	0,092*	0,198**	0,103*	0,126*	-0,256**
p		0,032	0,000	0,036	0,010	0,000
n	404	404	404	404	404	404
Vitamin B-12 (pg/mL)						
r	0,092*	1,000	0,362**	-0,478**	0,780**	-0,263**
p	0,032		0,000	0,000	0,000	0,000
n	404	404	404	404	404	404
Folate (ng/mL)						
r	0,198**	0,362**	1,000	-0,206**	0,336**	-0,184**
p	0,000	0,000		0,000	0,000	0,000
n	404	404	404	404	404	404
Homocysteine (µmol/L)						
r	0,103*	-0,478**	-0,206**	1,000	-0,372**	-0,003
p	0,036	0,000	0,000		0,000	0,952
n	404	404	404	404	404	404
Holo TC (pmol/L)						
r	0,126*	0,780**	0,336**	-0,372**	1,000	-0,198**
p	0,010	0,000	0,000	0,000		0,000
n	404	404	404	404	404	404
Plasma MMA (µmol/L)						
r	-0,256**	-0,263**	-0,184**	-0,003	-0,198**	1,000
p	0,000	0,000	0,000	0,952	0,000	
n	404	404	404	404	404	404

r: correlation coefficient
*: Correlation significant at the 0.05 level (2-tailed).
**: Correlation significant at the 0.01 level (2-tailed)

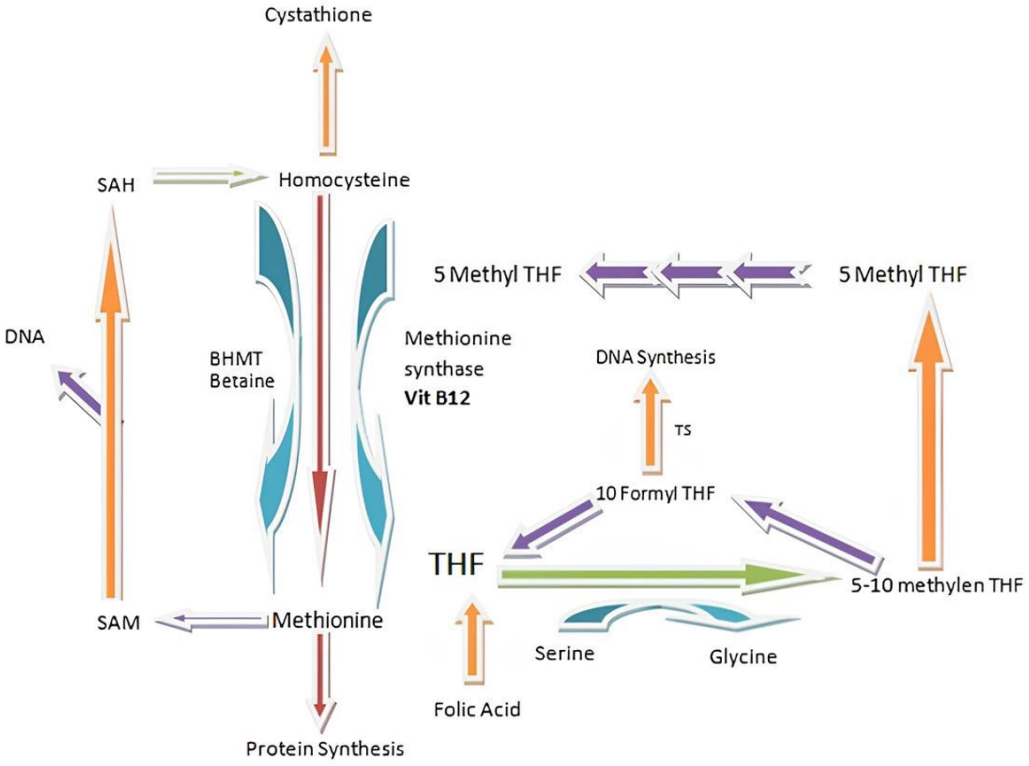

Figure 1. Formation of methionine via remethylation and role of vitamin B-12. TS: Thymidylate synthase, SAM: S-adenosyl methionine, SAH: S-adenosine homocysteine, BHMT: Betaine homocysteine methyl transferase, THF: Tetrahydrofolate, MTHFR: methylenetetrahydrofolate reductase

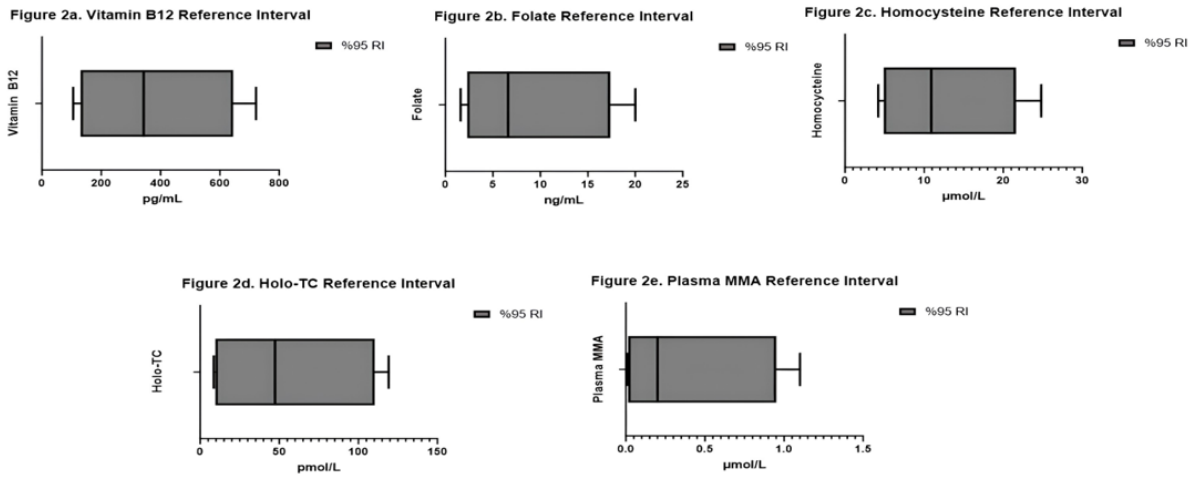

Figure 2. Our proposed 95% reference interval (RI) for serum (a) vitamin B12, (b) folate, (c) homocysteine, (d) holotranscobalamine (Holo-TC) and plasma (e) methylmalonic acid (MMA) based on blood samples from healthy individuals and patient data. Vitamin B12, folate and homocysteine were analyzed using chemiluminescent immunoassay; Holo-TC was analyzed using chemiluminescent microparticle immunoassasy; MMA was analyzed using LC-MS/MS method.