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Reference values for serum levels of vitamin B_{12} and folic acid in a population-based sample of adults between 35 and 80 years of age

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Abstract

Objectives: To examine folic acid and vitamin B_{12} status in a group of 1000 persons sampled from the community of Umeå, Sweden, and aged 35, 40, 45, 50, 55, 60, 65, 70, 75 or 80 years. Reference data for folate and age-stratified reference data for vitamin B_{12} are presented, together with an examination of potential confounders.
Measurements: All subjects participated in extensive health examinations and interviews, and laboratory blood testing was performed.
Results: A series of exclusion criteria were applied, and data from 961 subjects were analysed. Vitamin B_{12} levels were found to decrease with increasing age, whereas folate levels remained constant across the age span studied. None of the vitamins was found to vary with sex, education, smoking or alcohol consumption, body mass index, prescription-free vitamin supplements, level of haemoglobin, or mean cell volume of erythrocytes. Further, none of these factors was associated with the age-related decrease of vitamin B_{12} level.
Conclusions: The offered reference ranges should be used only in order to rule out deficiency. For B_{12} levels, the age of the subject should be considered such that, for elderly people in particular, values above the medians should be considered as indicative of normal vitamin status.

Keywords
Vitamin B_{12}
Folic acid
Reference values
Ageing

Reference values for various blood parameters are often obtained through sampling of young individuals with a high probability of good health, or a mix of both healthy and non-healthy individuals recruited in hospitals or other medical service units. Representative sampling from the general population is less common. In this article, we present reference data on vitamin B_{12} and folic acid from a community-based, random sample of 961 healthy individuals between 35 and 80 years of age living in Umeå, Sweden.

Vitamin B_{12} and folic acid are water-soluble vitamins. Both are important to nerve and brain function and are required as coenzymes related to the synthesis of serotonin and catecholamine neurotransmitters as well as *S*-adenosylmethionine[1–3]. Deficiency may cause disorders of different types, including memory disturbance[4–6], delirium and mood disorders[7,8] and psychosis[9].

Most recent reports on vitamin deficiency have acknowledged that diagnosis of B_{12} deficiency cannot be based solely on assessment of vitamin levels. It is generally accepted that, in the majority of cases, a low level of B_{12} or folic acid must be accompanied by elevated levels of the serum metabolites methylmalonic acid (MMA) and/or homocysteine in order to diagnose deficiency[10]. Further studies are needed to determine the role of these or other metabolites in folate deficiency[11]. Although folate is required for the metabolism of homocysteine it is not needed for the metabolism of MMA. However, elevated levels of MMA are sometimes found in folate-deficient patients. Moreover, several studies have shown that vitamin substitution treatment is effective only when a low vitamin B_{12} or folate level is accompanied by elevated metabolite levels[12,13]. Interestingly, there may be a time window where, at least for B_{12} deficiency, treatment of cognitive deficits resulting from vitamin deficiency is effective for a limited period only[14]. Further research is needed to examine whether this is the case also in folate deficiency.

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Prevalence rates for low serum vitamin B_{12} and folate vary considerably across studies[15]. These variations likely reflect differences among study samples in terms of age and health status, as well as differences in assessment procedures[16]. However, there are indications that the world-wide occurrence of B_{12} deficiency ranges between 5 and 15%[11], especially in older adults[17]. The corresponding figures for folate deficiency are generally thought to be somewhat lower but also to vary across cultures, where frequencies as high as 50% have been reported[18] in South American populations aged 60+. The much lower figures for folate deficiency in Western countries are thought to reflect different nutritional habits and the rather frequent use of prescription-free vitamin supplements[19].

Regular screening for vitamin B_{12} or folate deficiency by means of serum tests is often recommended, also in contemporary research[11]. Although definite diagnosis of deficiency cannot be based on serum tests, values above the means of the reference intervals obtained from healthy individuals generally rule out deficiency of vitamin B_{12}. It has been proposed that most B_{12}-deficient persons will have a level[20] equivalent to less than 260 pmol l^{-1}, whereas recommendations in terms of specific cut-offs for folic acid are less clear. However, for both vitamins it appears that low or low-normal values may be more indicative of deficiency in older adults compared with younger adults[21].

Moreover, if a high value within normal ranges rules out vitamin deficiency, it is not just important to determine what constitutes a normal range, but also to identify factors that potentially covary with vitamin levels and hence may influence the range of normality. One such factor is chronological age. The relationship between serum vitamin B_{12} level and age is yet tentative, with some studies indicating an age-related decline[17] and others showing age invariance[22]. In one of the few existing longitudinal studies[23], vitamin B_{12} levels were even found to increase slightly across a 10-year period, although older subjects remained at the lowest part of the distribution across the retest interval.

Several studies have failed to demonstrate sex differences in vitamin B_{12} levels[24], although women are at times found to demonstrate either higher levels[25] or lower levels[26]. Similarly, in some studies no sex differences in folate levels have been reported[27], whereas other studies show lower levels in females[28].

Previous research has generally documented an association between cigarette smoking, high alcohol intake and folate level, where smoking and high alcohol consumption are both related to lower levels of folic acid[29], although some research has failed to find such an association[30]. Alcohol consumption has been regarded as one of the most powerful agents responsible for secondary folate malnutrition[31], with significant deterioration of folate status in heavy drinkers[32]. However, other research indicates no such effects of more moderate alcohol consumption[33].

Finally, a negative relation between body mass index (BMI), expressed in kg m^{-2}, and vitamin B_{12} has been documented in some studies[28], although other studies have failed to confirm such an association[15]. Similarly, an association between folate level and BMI is documented in some studies[34], where risk of folate deficiency appears to increase with increasing BMI. In sum, the evidence is mixed with regard to the influence on vitamin levels of the confounders discussed. However, as most work has focused on only a few of the potential confounders, it would be of interest to include them all in the same study.

We report reference data for serum vitamin B_{12} and folic acid, obtained from a study of 1000 persons randomly sampled from the population of Umeå, a city of about 100 000 inhabitants in the north-eastern part of Sweden. The participants belong to 10 different age cohorts and although they were all reasonably healthy at the time of assessment, we applied a series of exclusion criteria to reduce interfering effects on the calculated reference values. We also examined the impact of multiple potential confounders on serum vitamin levels, namely age, sex, education, alcohol consumption, smoking, BMI, and prescription-free vitamin B supplements. In addition, blood haemoglobin (Hb) and mean cell volume of the erythrocytes (MCV) were examined as potential covariates to vitamin levels.

Methods

All procedures were approved by the ethics committee at Umeå University, and in accordance with the Helsinki Declaration of 1975.

Participants

The study sample included 100 individuals from each of 10 age cohorts (35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 years of age) who were recruited through the population registry in Umeå, Sweden. In each age cohort, equal numbers of men and women were sampled. The selected persons were taking part in the Betula project, which is a prospective study on memory, health and ageing. Participants were randomly sampled, with the restrictions that persons suffering from a severe sensory handicap, mental retardation or an organic disease known to affect brain function (e.g. dementia), as well as persons having a native tongue other than Swedish, were not included in the sample. For a detailed description of the sampling procedure and the study design, the reader may consult Nilsson *et al.*[35].

Individuals treated with vitamin B_{12} or folate substitution due to diagnosed deficiency ($n = 11$) were deleted from further analyses. We also excluded persons treated with drugs that are known to interfere with B_{12} or folate uptake, including anticonvulsant and peptic ulcer drugs, antimetabolites, trimethoprim, hydrazides and mestoranum. Twenty-six persons were excluded due to treatment

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with at least one of these types of drug. Finally, two persons who received pharmacological treatment of gastrointestinal problems were excluded from the sample. After these exclusions, 961 subjects remained for further analyses.

Health assessments

All data presented in this study were collected on the same occasion. Health examinations, blood sampling and interviews about health status were conducted by nurses and lasted 1.5 to 2 h for each participant.

Blood sampling

Collection of 40 ml of venous blood was performed between 7 a.m. and 7.30 p.m. Owing to this range of blood sampling hours, subjects were not fasting.

Biochemical assays

B_{12} and folate in serum were measured at the Department of Clinical Chemistry at Umeå University Hospital with a radioimmunoassay method, the Quantphase II B_{12}/folate Radioisotope Dilution Assay (BIO-RAD Diagnostics Group, CA, USA), according to the manufacturer's instructions. Reference values offered for this method are 96–568 pmol l^{-1} for serum vitamin B_{12} (border zone of clinical significance: 96–151 pmol l^{-1}) and 3.4–47 nmol l^{-1} for serum folate (border zone of clinical significance: 3.4–7 nmol l^{-1}). Blood cell parameters, including blood haemoglobin and MCV, were determined on a Sysmex NE8000 analyser (Toa Medical Electronics Co. Ltd, Kobe, Japan).

Substance use

Drinking behaviour was assessed by means of a structured questionnaire covering beer, wine and strong liquor consumption per month, and coded after calculating grams of alcohol consumed per week. Smoking habits were assessed using the same questionnaire and indexed by number of cigarettes smoked per day.

Anthropometric survey

Weight and height (without shoes and with light clothing) were determined using a digital electronic weighing scale (range: 0.1–136 kg) and a stationary tape measure. These data allowed the calculation of each subject's body mass index (in kg m^{-2}).

Health status

All subjects were interviewed about past and present diseases, where detailed records of both pharmacological treatment and prescription-free drugs were taken.

Results

Table 1 describes data for the demographic variables, substance use, BMI, prescription-free vitamin supplements, laboratory data and time of blood sampling. The percentage of vitamin-deficient persons according to standard laboratory criteria (i.e. $B_{12} < 151$ pmol l^{-1}; folate < 7 nmol l^{-1}) was 4.2% for vitamin B_{12}, 2.1% for folic acid, and 0.2% for combined low B_{12} and folic acid levels. These persons were equally distributed across sex. Further, the persons with low B_{12} values tended to be somewhat older (mean = 64.00 years, standard deviation (SD) = 12.15) than persons with low folate (mean = 58.15 years, SD = 13.88) and persons with normal vitamin levels (mean = 56.86 years, SD = 14.34).

Confounding factors

In order to examine whether vitamin levels were related to smoking, alcohol intake or body constitution, we performed a series of correlational analyses where smoking status as indexed by number of cigarettes smoked per day, alcohol consumption as indexed by grams of alcohol consumed per week and BMI (kg m^{-2}) were entered together with the two vitamin indices. In addition, years of age, education, time of blood sampling, Hb and MCV were included. Because men and women are not comparable with respect to BMI, Hb and vulnerability to alcohol intake, correlations were performed separately

Table 1 Summary data for demographics, alcohol and smoking habits, body mass index, prescription-free vitamin supplements, laboratory data (Hb, MCV), and time of blood sampling

		Mean	SD	Range
Years of age		57.19	14.31	35–80
Years of education		9.97	4.05	1–30
Percentage of women	53			
Number of cigarettes smoked per day		2.64	6.17	0–30
Grams of alcohol consumed per week		33.72	56.78	0–813
Body mass index, BMI (kg m^{-2})		24.98	3.56	14.66–41.26
Subjects using prescription-free vitamins		0.04	0.19	0–1
Blood haemoglobin, Hb (g l^{-1})		144.05	12.48	98–184
Mean cell volume, MCV (fl)		91.54	4.33	69–121
Serum vitamin B_{12} (pmol l^{-1})		312.88	135.98	13–1770
Hour of B_{12} blood sampling		1.16 p.m.	2.78 h	7 a.m.–7.30 p.m.
Serum folate (nmol l^{-1})		17.91	10.91	3.8–55
Hour of folate blood sampling		1.34 p.m.	2.86 h	7 a.m.–7.30 p.m.

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for the two sexes. These analyses showed, first, that B_{12} and folic acid were highly correlated in both sexes; second, that age was negatively related to B_{12} level in both men and women; and, third, that BMI was negatively correlated with folic acid level in men. Inspection of the data revealed, however, that the latter association was driven by three outliers having BMI $< 20\,kg\,m^{-2}$ and folate level $> 40\,nmol\,l^{-1}$. Elimination of these persons resulted in a non-significant correlation ($r = -0.08$, $P = 0.08$). Hence, the association was not examined further. Apart from these associations, vitamin levels were unrelated to the demographics, substance use, body constitution, other laboratory variables, and time of blood sampling (all P-values > 0.05). These correlations are shown in Table 2.

Next, because some of the variables studied were positively skewed with a skewness index of ≥ 2 (vitamin B_{12}, folate, alcohol consumption and smoking), these variables were log-transformed and the correlations recalculated. After the log-transformations, the skewness was, for all of these variables, less than 1. The new correlations revealed, however, the same pattern of results as for the correlations based on raw scores. The only substantive difference was that the correlation of age with B_{12} among women increased from -0.104 to -0.190. Also, as many subjects reported themselves as abstinent from alcohol and non-smokers (female non-drinkers 24%, non-smokers 79%; male non-drinkers 16%, non-smokers 81%), we performed separate correlations between the vitamins and levels of substance use among the alcohol users and smokers, respectively. These analyses showed, however, that amount of alcohol consumed or number of cigarettes smoked were, among users, not related to vitamin levels in either men or women (all P-values > 0.10).

In order to examine the association between age and vitamin B_{12} level further, we next performed a series of hierarchical regression analyses, separately for each of the sexes. The log-transformed variables were used for this purpose. In the first set of analyses, the amount of B_{12}-related variance accounted for by age was determined by regressing age on the log-transformed B_{12} data. These analyses revealed that age accounted for 3.6% of the variance in serum vitamin B_{12} among women ($\beta = -0.19$, $P = 0.00$) and for 3.2% among the men ($\beta = -0.18$, $P = 0.00$). Next, although none of them was individually correlated with B_{12}, we entered all potential confounders as a block first in the analyses, and then re-examined the variance accounted for by age after controlling for the confounder-related variance. The results showed that the age-related variance remained highly significant in both sexes (women $R^2 = 0.033$, $\beta = 0.24$, $P = 0.00$; men $R^2 = 0.021$, $\beta = -0.18$, $P = 0.00$). The control variables were not significantly related to B_{12} level in either of the sexes, either individually or as a block (all P-values > 0.10).

Vitamin B_{12} and folate levels across age and sex

For ease of interpretation, we next combined the 10 age cohorts into five groups (AGE3540: 35 and 40 year olds; AGE4550: 45 and 50 year olds; AGE5560: 55 and 60 year olds; AGE6570: 65 and 70 year olds; and AGE8085: 80 and 85 year olds). A 5 (age group) × 2 (sex) analysis of variance (ANOVA) was conducted on the raw scores of each of the two vitamin variables. These analyses showed no significant main effects of age group for folic acid, no significant main effects of sex and no significant age group by sex interaction effects for any of the vitamins (all P-values > 0.05). As expected, there was a significant main effect of age group on vitamin B_{12} level ($F = 3.14$, $P = 0.01$). Tukey tests showed that this effect reflected the fact that the oldest age group (AGE7580) had significantly lower levels of vitamin B_{12} than the two youngest age groups (AGE3540 and AGE4550, P-values < 0.05).

Next, for the purpose of establishing reference ranges, the cumulative frequency was computed for each of the two vitamins, and for B_{12} this was done separately within

Table 2 Correlations of vitamins with demographics, alcohol and smoking habits, body mass index, prescription-free vitamin supplements, laboratory data (Hb, MCV), and time of blood sampling for men and women

	Women		Men	
	B_{12}	Folate	B_{12}	Folate
Folate ($nmol\,l^{-1}$)	0.173**	–	0.259**	–
Years of age	−0.104*	0.045	−0.155**	0.077
Years of education	0.025	−0.054	0.067	−0.038
Alcohol consumption ($g\,week^{-1}$)	0.029	−0.066	−0.044	−0.066
Cigarette smoking ($cigarettes\,day^{-1}$)	−0.006	0.000	0.026	0.008
Body mass index, BMI ($kg\,m^{-2}$)	0.015	−0.026	−0.021	−0.123**
Prescription-free vitamins	−0.021	0.012	−0.045	−0.071
Haemoglobin, Hb ($g\,l^{-1}$)	−0.005	−0.015	0.064	−0.049
Mean cell volume, MCV (fl)	−0.053	−0.071	−0.039	0.032
Time of B_{12} blood sampling	0.048	0.001	−0.006	−0.006
Time of folate blood sampling	0.009	0.000	0.004	−0.016

*, $P < 0.05$; **, $P < 0.01$.

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Table 3 Serum vitamin B_{12}, statified by age group, and folate levels in the entire sample

	n	Mean	SD	Median	$P_{2.5}$–$P_{97.5}$*	P_5–P_{95}*
Vitamin B_{12} (pmol l^{-1})						
AGE3540	197	332.14	111.92	316	167–573	178–517
AGE4550	194	324.92	109.81	308	146–582	168–500
AGE5560	194	309.65	104.16	300	129–552	156–473
AGE6570	193	308.04	164.12	279	118–579	151–533
AGE7580	183	287.95	173.55	268	113–515	134–457
Folate (nmol l^{-1})	961	17.91	10.91	14.4	6.7–55.0	7.6–46.8

*$P_{2.5}$–$P_{97.5}$, 2.5th–97.5th percentile; P_5–P_{95}, 5th–95th percentile.

each of the five age groups. The reference interval was defined as the range between the concentrations of the cumulative frequencies (percentiles) 2.5–97.5 and 5–95, respectively. Examining the reference ranges in Table 3, it is evident that these ranges correspond quite well with standard laboratory ranges for the method used (B_{12} 152–568 pmol l^{-1}; folic acid 7–47 nmol l^{-1}), but also that the B_{12} ranges for the oldest age group are well below the standard ranges.

Discussion

The chief objective of this study was to provide reference values for serum vitamin B_{12} and folate levels across the adult life span. This was accomplished by assessing a large, community-based, random sample and comparing vitamin levels across age and sex. In addition, the influence of some potential confounders on vitamin levels was examined.

The main findings were that levels of folic acid remained constant in the age range 35–80 years, although serum vitamin B_{12} levels declined across the same age span. In addition, vitamin levels were similar for men and women irrespective of age. These results are in agreement with some previous research[27], although they differ from data reported in other studies[26]. It has been proposed that age differences in vitamin B_{12} levels may be the result of the increasing prevalence of atrophic gastritis in old age[17]. If this were to be the case, however, one would expect the variation of B_{12} levels to increase with age. Inspection of Table 3 indicates that the standard deviations are indeed increasing somewhat after 60 years of age. However, the slightly higher standard deviations in the two oldest age groups were equal to those of the three youngest age groups after exclusion of two outliers among the 65–70 year olds and three outliers in the oldest age group. The decrease of B_{12} across the age span could, however, be attributed to an age-related decrease in intake of vitamin B_{12}-containing food, or a decreased capacity to absorb cobalamin-intrinsic factor complexes in the intestine. Although we were able to control for critical drug use, and also failed to detect an association with use of prescription-free vitamin supplements, the dataset did not permit further evaluation of the impact of eating habits.

The limited effects of the potential confounders are in accordance with data from a recent, large-scale, multicentre study of individuals aged 70–75 years[28]. Similar to the present findings, that study revealed weak or nonexistent relations between smoking, BMI, or chronic diseases and vitamin status.

Alcohol is known to have a profound effect on folate metabolism[36]. However, these effects may be limited to excessive alcohol use, as research indicates that moderate consumption of alcohol does not result in lowered folate levels[33]. The absence of a significant association between alcohol use and folate in the present study may be an indication that no persons with excessive alcohol drinking participated. However, inspection of the data revealed that six women and eight men reported consumption levels that are considered to be harmful according to Swedish alcohol recommendations[37]. The vitamin data for these 14 persons were all in the middle ranges for both vitamins. As is often the case, alcohol consumption may be underreported, and the possibility that such underreporting may be a more pronounced problem among severe drinkers cannot be ruled out. Relatedly, smoking has been found to coexist with lowered levels of folic acid in a number of studies[29]. We found no such relationship, which concurs with data reported in some previous studies[30].

In defining vitamin deficiency according to standard laboratory criteria for serum vitamin levels, a rather low prevalence of untreated deficient subjects was found. In interpreting this finding, several points of caution need to be addressed. One concern relates to the diagnosis of deficiency, where it has been argued that additional information including tests of homocysteine and methylmalonic acid is necessary to confirm true deficiency[10,11]. Unfortunately, the data collection did not include such information. Using these metabolites, vitamin B_{12} deficiency can be found also in individuals with values in the lower normal range of the serum vitamin distribution, and this may be especially true for older persons[17]. This should be considered together with our finding that, at least for vitamin B_{12}, the reference intervals are not constant in the age range 35–80 years. Folate deficiency is generally considered to be rare, but high

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frequencies of deficiency have been reported, especially in older populations[18]. This might be due to homozygosity of the C677T mutation in the methylenetetrahydrofolate reductase enzyme[38,39], also at serum folate levels usually considered normal. In these individuals, approximately 10% of the population, an elevated homocysteine level may be observed at folic acid levels less than 15 nmol l^{-1}, which could be regarded as a more proper cut-off value for folate deficiency at all ages in the absence of homocysteine data. This possibility is currently under investigation. As well, the clinical recommendations for vitamin B_{12} tend to include higher cut-offs than the standard limits used by many laboratories and certainly well above the lower limit of the reference range obtained in the present study, where a standard percentile model was applied on the data.

In sum our data show that, for vitamin B_{12}, the reference intervals are not constant in the age range 35–80 years, although the corresponding intervals for folic acid remain rather stable across this age span. A conservative approach to the offered reference ranges is recommended. Against the background of the reviewed literature, the reference ranges should not be used for diagnosis of vitamin deficiency but rather in order to rule out deficiency. To be sure, values close to or well above the medians are indicative of a normal vitamin status, and this rather conservative approach may be especially valid with respect to B_{12} levels in the oldest age groups.

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