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Algorithm for the early diagnosis of vitamin B$_{12}$ deficiency in elderly people

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Abstract

Background: The elderly population is particularly at risk for developing vitamin B$_{12}$-deficiency. Serum cobalamin does not necessarily reflect a normal B$_{12}$ status. The determination of methylmalonic acid is not available in all laboratories. Issues of sensitivity for holotranscobalamin and the low specificity of total homocysteine limit their utility. The aim of the present study is to establish a diagnostic algorithm by using a combination of these markers in place of a single measurement.

Methods: We compared the diagnostic efficiency of these markers for detection of vitamin B$_{12}$ deficiency in a population (n = 218) of institutionalized elderly (median age 80 years). Biochemical, haematological and morphological data were used to categorize people with or without vitamin B$_{12}$ deficiency.

Results: In receiver operating curves characteristics for detection on vitamin B$_{12}$ deficiency using single measurements, serum folate has the greatest area under the curve (0.87) and homocysteine the lowest (0.67). The best specificity was observed for erythrocyte folate and methylmalonic acid (100% for both) but their sensitivity was very low (17% and 53%, respectively). The highest sensitivity was observed for homocysteine (81%) and serum folate (74%). When we combined these markers, starting with serum and erythrocyte folate, followed by holotranscobalamin and ending by methylmalonic acid measurements, the overall sensitivity and specificity of the algorithm were 100% and 90%, respectively.

Conclusion: The proposed algorithm, which combines erythrocyte folate, serum folate, holotranscobalamin and methylmalonic acid, but eliminate B$_{12}$ and tHcy measurements, is a useful alternative for vitamin B$_{12}$ deficiency screening in an elderly institutionalized cohort.

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Key words: *Elderly. B$_{12}$ deficiency. Diagnostic accuracy. Biomarkers. Algorithms.*

ALGORITMO PARA EL DIAGNÓSTICO PRECOZ DE LA DEFICIENCIA DE VITAMINA B12 EN ANCIANOS

Resumen

Introducción: Los mayores son una población que presenta un riesgo importante de desarrollar una deficiencia de vitamina B$_{12}$, pero las concentraciones de cobalamina en suero no reflejan necesariamente un estado abnormal en el estado de B$_{12}$. Existen biomarcadores asociados a la vitamina B$_{12}$: el ácido metilmalónico no está disponible en todos los laboratorios, la holotranscobalamina es poco sensible y la homocisteína presenta una baja especificidad. El objetivo del presente estudio es establecer un algoritmo de diagnóstico mediante el uso de una combinación de estos biomarcadores en lugar de la medición de uno sólo de ellos.

Métodos: Se comparó la eficacia diagnóstica de estos marcadores para la detección de deficiencia de vitamina B$_{12}$ en una población (n = 218) de ancianos institucionalizados (edad media 80 años). Los parámetros bioquímicos, hematológicos y morfológicos fueron utilizados para clasificar a los sujetos con o sin deficiencia de vitamina B$_{12}$.

Resultados: Se establecieron las curvas ROC (Receiver Operating Curves) para determinar la eficacia diagnóstica de cada parámetro, tomado individualmente. El folato sérico tenía la mayor área bajo la curva (0,87) y la homocisteína la más baja (0,67). Se observó que la mejor especificidad la presentaba el folato eritrocitario y el ácido metilmalónico (100% para ambos), pero sus sensibilidades eran muy bajas (17% y 53%, respectivamente). Y se observó que la sensibilidad más alta la presentaba la homocisteína (81%) y el folato sérico (74%), pero en contrapartida una especificidad baja. Cuando se combinaron estos marcadores, iniciando las determinaciones con el folato sérco y eritrocitario, seguido por holotranscobalamina y terminando por las mediciones de ácido metilmalónico, la sensibilidad y especificidad global del algoritmo fueron 100% y 90%, respectivamente.

Conclusión: El algoritmo propuesto, que combina la determinación de folato sérico y eritrocitario, holotranscobalamina y ácido metilmalónico, sin necesidad de evaluar la vitamina B$_{12}$ y la homocisteína, es una alternativa útil para la detección de un estado abnormal del estado de vitamina B$_{12}$ en una población de ancianos institucionalizados.

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Palabras clave: *Ancianos institucionalizados. Deficiencia de B$_{12}$. Eficacia diagnóstica. Biomarcadores. Algoritmos diagnósticos.*

Introduction

In developed countries, clinical vitamin deficiency has mostly disappeared and only affects marginal population groups. May be the only exception is for vitamin B_{12}, which clinical deficiency, the megaloblastic anemia, affects around 20% of the elderly people in developed countries.[1] Also at risk for clinical vitamin B_{12} deficiency are vegetarians, the more strict vegetarians the higher the risk,[2] people suffering from gastritis and chronic consumers of medication for this pathology, like H2 pump inhibitors.[3,4] Subclinical deficiency is quite more prevalent, especially in the elderly.[5-9] In the literature there are data ranging from 6 to 40%, but as there is no accepted method for a valid diagnosis of subclinical vitamin B_{12} deficiency, these data have to be taken with caution. In the general population, the most prevalent vitamin deficiency is for folate that affects both sexes and all age groups.[10,11] This means that in the elderly people, folate and vitamin B_{12} deficiency come together. In fact, the combined folate/vitamin B_{12} deficiency was supposed to be present in up to 63% of the healthy elderly subjects and up to 83% in elderly hospitalized patients.[12] As both vitamin deficiencies lead to the development of megaloblastic anemia and the clinical diagnosis based on the identification of the hypersegmentation of the nucleus of neutrophiles is common to both deficiencies, it is not possible to differentiate by this method if there is an isolated vitamin B_{12} or isolated folate deficiency or a combined deficiency, besides that it appears at a later stage.[13] But early diagnosis is decisive, as neurologic symptoms due to vitamin B_{12} deficiency at an advanced state are irreversible.[14,15] Therefore, the scientific community is searching for a better diagnostic method, which could be used in the routine clinical laboratory, considering also economical aspects. Normal serum cobalamin (B_{12}) does not necessarily reflect a normal B_{12} status. Methylmalonic acid (MMA) has been also proposed as an alternative diagnostic method for vitamin B_{12} deficiency.[16] A relatively novel marker, Holo-transcobalamin (HoloTC), has been presented to afford superior diagnostic specificity because it constitutes the biologically functional vitamin B_{12} fraction.[17,18] Total homocysteine (tHcy) has been cited as a marker for blood B vitamin levels.[16] Arguments will be discussed further in this article, but all in all, it seems that no single parameter will allow early accurate identification of vitamin B_{12} deficiency.[19] Therefore, the main objective of our study was to establish a diagnostic scheme in order to try to facilitate early diagnosis and to differentiate between isolated folate, isolated B_{12} and combined B_{12}/folate deficiency. For this aim, we have used several B_{12}-related parameters measured in Spanish institutionalized elderly among who there was a high prevalence of B_{12} deficiency.[9]

Material and methods

Study design

This study is part of a broader study that aims to establish the vitamin B status of Spanish institutionalized elderly, with special regard to vitamin B_{12} and folate in association with homocysteine. It has been supported by a grant from the Fondo de Investigación Sanitaria of the Instituto de Salud Carlos III (Spanish Ministry of Health, FIS PI021830). Briefly, this study includes nutritional assessment by means of the Mini Nutritional Assessment,[20] cognitive analysis by means of the Mini Mental State[21] and haematological and biochemical data. For this work, from all the analysed variables in the blood, those which are related directly to B_{12} and folate metabolism were selected, based on clinical criteria and correlation coefficients[9]: B_{12}, HoloTC, tHcy, serum folate, erythrocyte folate, and MMA.

Study population

Two-hundred and eighteen elderly (82 males, 136 females) with a median age (percentile 5th-95th range) of 80 years (65-90 years) living in an Elderly s Home in Granada, Spain, were recruited for the study. The residents who took part in the study were all elderly living at the residence. They were not taking any vitamin supplement. Anamnesis and blood sampling were included in the biannual medical review that is performed in all the elderly living in the residence. There were no exclusion criteria. None of the volunteers presented any problem that would have justified their exclusion from the study. The study was approved by the Human Research Review Committee of the University of Granada, School of Medicine. The study has been performed following the ethic norms of the Declaration of Helsinki (revision of Edinburgh 2000), Convention of Oviedo, the Good Clinical Practice of the CEE (document 111/3976/88 July 1990) and the Spanish legislation about clinical research in humans (Real Decreto 561/1993 sobre ensayos clínicos). Informed written consent was obtained from subjects and/or their legal representatives.

Analytical methods

After an overnight fast, blood samples were drawn between 8:00 and 10:00 h in the morning. Following venipuncture, 3 ml of EDTA-treated blood was analysed within an hour. Two 8 ml vacutainers with gel for serum were put on ice immediately and centrifuged within one hour at 2000 x g and 4° C for 10 min. Following centrifugation they were aliquoted and immediately frozen at -80° C.

Hypersegmentation of the neutrophylic granulozytes was analysed as lobe average and according to

Table I Population characteristics			
Variable	n	Median (p5th-p95th)	Percent abnormal (cutoff value)
Age, years	218	80 (65-90)	
B_{12}, pmol/l	216	231 (96-549)	17.4 (≤ 148)
Serum folate, µg/l	218	6.1 (2.8-15)	62.4 (≤ 7)
Erythrocyte folate, µg/l	218	275 (111-696)	14.2 (≤ 175)
Holo-TC, pmol/ml	214	(13-210)	23.8 (≤ 35)
MMA, nmol/l	205	281 (100-864)	46.8 (≥ 300)
tHcy, µmol/l	217	15.8 (9.1-26.3)	63.6 (> 13)

the rule of five.[21] tHcy was measured using a fluorescence polarisation immunoassay and serum folate, erythrocyte folate, and B_{12} were determined by microparticle enzyme immunoassay by using IMx analyzer (Abbott Laboratories, Abbott Park, IL). HoloTC levels were measured by radioimmunoassay provided by Axis-Shield (Oslo, Norway). MMA was analysed by gas chromatography-mass spectrometry.

Statistical methods

No statistical significant differences ($p > 0.05$) were found between sexes; therefore, all data for both men and women were analysed together. As not all the variables followed a normal distribution, after using the Kolmogorov-Smirnov test, natural log-transformed data were used for calculating Pearson's correlations coefficients to examine linear associations between the different markers.

To determine the single relative efficiency of SCbl, HoloTC, tHcy, serum folate, erythrocyte folate and MMA to discriminate among individuals with vitamin B_{12} deficiency and the others individuals, ROC curves were constructed and areas under the curve (AUCs) compared. Subjects with or without vitamin B_{12} deficiency were categorized by using biochemical (SCbl, tHcy), haematological (RBC count, haemoglobin, hematocrit, MCV, MCH, MCHC, reticulocytes count) and morphological (lobe average, rule of five) data.

Cutoff values for each metabolite were based on literature reports or standard clinical concentrations. These cutoff values were as follows: $B_{12} \leq 148$ pmol/l (standard clinical value), HoloTC ≤ 35 pmol/l[17], tHcy > 13 µmol/l[17], serum folate ≤ 7 µg/l (standard clinical value), erythrocyte folate ≤ 175 µg/l (standard clinical value) and MMA ≥ 300 nmol/l.[23]

A hierarchical step-by-step classification model was developed by using different combinations of these biomarkers. In order to decide which of these combinations presented the best diagnostic efficiency, sensitivity and specificity for detecting individuals with vitamin deficiency were determined for the algorithms proposed by using the cutoff values cited above. The diagnostic outcomes of these algorithms were divided into 4 groups: 1) isolated B_{12} deficiency, 2) combined B_{12}-folate deficiency, 3) isolated folate deficiency and 4) normal. ANOVA analysis was performed to determine if there were significant differences for each of the biomarkers in the different outcomes.

Sample size for each variable was not equal because sufficient volumes of blood were not available from all participants for each assay (table I). Statistical analyses were made with data from individuals having available measurements for all variables used in any particular analyses and they were performed using SPSS for Windows (ver.19.0, SPSS Inc) and MedCalc (ver. 11.6.1.0, MedCalc Software bvba). Statistical significance was defined for all analyses as $P < 0.05$.

Results

Table I summarizes the population characteristics. A large proportion (63.6%) of the population had increased tHcy levels (> 13 µmol/l). The prevalence for serum folate deficiency (≤ 7 µg/l) was present in 62.4% of individuals, although only 14.2% had erythrocyte folate levels below the normal range (≤ 175 µg/l). Increased level of MMA (≥ 300 nmol/l) was present in 48.8% of participants and 23.8% had holoTC levels below normality (≤ 35 pmol/l). Subnormal level of B_{12} (≤ 148 pmol/l) was present in 17.4% of population. The prevalence of the overall vitamin B_{12} deficiency (i.e. isolated B_{12}, combined B_{12}-folate and isolated folate deficiencies) determined by biochemical, haematological and morphological data, was 83.3%.

The strongest positive linear associations were observed between B_{12} and holoTC (correlation coefficient: $r = 0.65$, $P < 0.001$), B_{12} and erythrocyte folate ($r = 0.33$, $p < 0.001$), erythrocyte folate and serum folate ($r = 0.39$, $P < 0.001$), holoTC and MMA ($r = 0.31$, $P < 0.001$). Higher inverse correlations were obtained for MMA with B_{12} ($r = -0.32$, $P < 0.001$) and serum folate with tHcy ($r = -0.29$, $P < 0.001$).

To establish the best variable to start the algorithm, we constructed ROC curves that indicated the capacity of each measurement to discriminate between individuals with or without vitamin deficiency. The AUC (CI) of the ROC curve for serum folate [0.87 (0.82-0.91)]

Variable	AUC (CI)[1]	Cutoff value	Sensitivity	Specificity
B_{12}, pmol/l	0.69 (0.62-0.75)	≤ 148	0.20	0.94
Serum folate, ng/ml	0.87 (0.82-0.91)	≤ 7	0.74	0.94
Erythrocyte folate, ng/ml	0.78 (0.72-9.83)	≤ 175	0.17	1.00
Holo-TC, pmol/ml	0.75 (0.67-0.81)	≤ 35	0.44	0.94
MMA, nmol/l	0.77 (0.72-0.84)	≥ 300	0.53	1.00
tHcy, μmol/l	0.67 (0.61-0.74)	> 13	0.81	0.30

[1]Area under the curve and 95% confidence interval.

indicated that this variable presented the best diagnostic efficiency (table II). The cutoff value for serum folate (≤ 7 μg/l) had a sensitivity and specificity of 74% and 94%, respectively. The AUC (CI) for erythrocyte folate was the second larger area [0.78 (0.72-0.83)] and the cutoff value (≤ 175 μg/L) had a sensitivity (17%) much lower than the cutoff for serum folate, whereas a better specificity (100%). As both serum and erythrocyte folate presented the highest discrimination capacity we decide to start the algorithm A by determining folate status first (fig. 1). If the cutoff values for serum folate and erythrocyte folate were used together
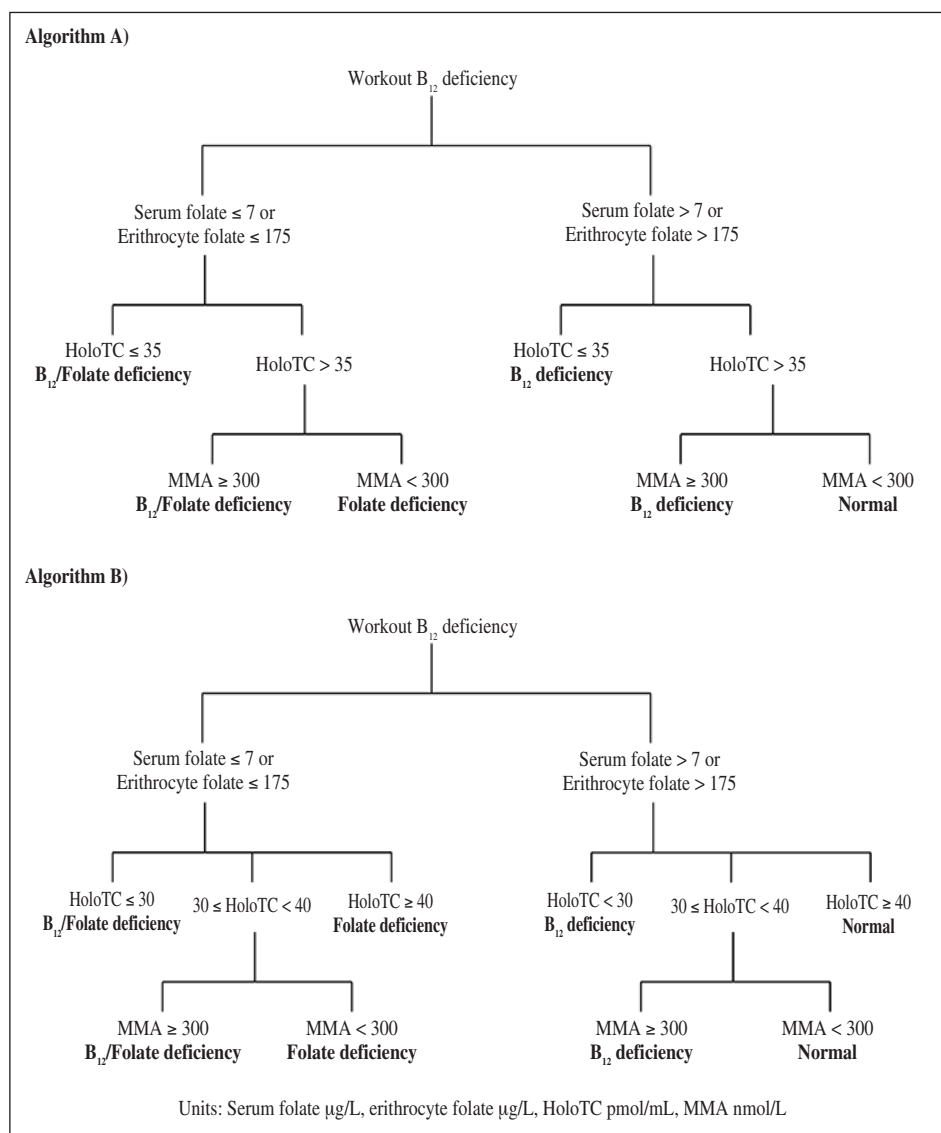

Fig. 1.—Algoritms A and B showing the different approaches for B_{12}/folate deficiency diagnosis.

Table III
Sensitivity and specificity for algorithm A

Measurement combination	Sensitivity	Specificity
Serum folata	0.74	0.94
Serum folate, erythrocyte folate	0.78	0.94
Serum folate, erythrocyte folate, Holo-TC	0.91	0.91
Serum folate, erythrocyte folate, Holo-TC, MMA	1.00	0.90

such that all individuals with low values for one or both of the measurement were combined, the sensitivity and specificity were 78% and 94%, respectively (table III).

AUCs for Holo-TC and MMA were similar (0.75 and 0.77, respectively) and they were higher than for the other two measurements not used yet, B_{12} and tHcy (0.69 and 0.67 respectively). Then we could use as a second measurement for the algorithm A both variables, holoTC or MMA. Because MMA analysis is expensive and MS-GC method is not available in most laboratories, whereas holoTC measurement can be carried out by using auto-mated immunoassays, this last variable was preferred for the second step of algorithm A. HoloTC was measured on all the individuals, having or not normal folate status. If we combined the three variables (serum folate, erythro-cyte folate and holoTC) the sensitivity reached 91%, while the specificity was slightly lower (91%) than measuring only serum and erythrocyte folate (Table 3). MMA was measured for the third and last step of algo-rithm A. The final sensitivity and specificity were 100% and 90%, respectively, and algorithm A included 6 possible outcomes: two diagnose an isolated B_{12} defi-ciency, two a combined deficiency, one an isolated folate deficiency and one normal status.

Algorithm B (fig. 1) represents a slight modification of algorithm A due to a change in the classification of individuals with or without holoTC deficiency. HoloTC measurements were divided in three groups: probable holoTC deficiency (< 30 pmol/l), possible holoTC deficiency (grey zone, between 30 and 40 pmol/l) and holoTC deficiency unlikely (> 40 pmol/l). Using algorithm B, MMA measurements would be carried out only for individuals with holoTC levels into the grey zone and this diagnostic strategy presented a sensitivity slightly lower than for algorithm A (91% and 100%) but a similar specificity (91% and 90%. Algorithm B included 8 possible outcomes: two diag-nose an isolated B_{12} deficiency, two a combined defi-ciency, two an isolated folate deficiency and two a normal status.

Significant differences were observed for all biomarkers between outcomes, except for B_{12}, in both algorithm, B_{12}. HoloTC levels were lowest and MMA levels highest in the combined deficiency group, serum folate was lowest in the isolated folate deficiency group, whereas erythrocyte folate was lowest in the combined deficiency group. tHcy was highest in the combined deficiency group. The only difference observed between algorithm A and B was for erythrocyte folate levels

which were higher in isolated B_{12} deficiency in algorithm A than in algorithm B. In the rest of the outcomes, levels and patterns were very similar.

Discussion

As has been stated before, especially in the elderly, there is a need to detect at the initial phase vitamin B_{12} deficiency, because they are at a greater risk for func-tional B_{12} deficiency. The clinical presentation of vitamin B_{12} deficiency varies considerably and rarely includes all the classic features, such as macrocytic anemia, peripheral neuropathy and sub-acute combined degeneration of the spinal cord[24]. More typically, vitamin B_{12} deficiency presents as non-specific symptoms fatigue, lassitude, malaise, vertigo, and cognitive impairment that could be attributed to old age and should be avoided by means of early detection. More-over, the clinical severity of vitamin B_{12} deficiency is often unrelated to B_{12} concentrations.[19,25] This has been demonstrated in our study by the small AUC (0.69) for B_{12} ROC curve and its low sensitivity (20%) when using a cutoff of 148 pmol/l as the only diagnostic criterion. This means, that functional deficiency occurs even in people whose B_{12} levels are in the reference range. Concerns about the limitations of standard vitamin B_{12} assays have been stated before.[17,24,26]

When using in single the rest of variables as the screen for vitamin B_{12} deficiency, the ROC curve for serum folate showed the larger AUC (0.87) with a good specificity (94%) but the sensitivity reached only 74%. The use of only erythrocyte folate measurement did not appear to be useful to discriminate individuals with inadequate vitamin B_{12} status due to its very low sensi-tivity (17%). When combining both folate measure-ments, specificity remained high (94%) but sensitivity (78%) increased only slightly compared with the sensi-tivity (74%) of serum folate used alone.

HoloTC has been proposed in the literature as a more sensitive marker.[25-27] However, measurement of HoloTC alone for vitamin B_{12} deficiency showed in our study a poor sensitivity (44%) that makes it unsuitable for screening. In order to increase the overall sensitivity detecting abnormal vitamin B_{12} status and preserving a good specificity, we combined both folate measure-ments with HoloTC values, obtaining a sensitivity and specificity of 91% for both (table III). Mean HoloTC levels are below 35 pmol/l in the isolated B_{12} and in the combined deficiency group in algorithm B. In fact, we found a strong correlation (r = 0.656; p < 0.001) between HoloTC and SCbl.

The clinical-economical advantage of algorithm B is that less MMA measurements have to be performed. MMA analysis is very expensive and the MS-GC method is complicated and not possible in the routine laboratory. There are several other physiological/pathological conditions where MMA is elevated, not only in B_{12} deficiency.[25] Furthermore, in the 93 subjects

with MMA ≥ 300 pmol/l, mean B_{12} levels were 234.05 ± 168 pmol/l and mean HoloTC levels were 54.63 ± 46.3 pmol/l. The advantage of HoloTC is that is specific for cobalamin metabolism and the analysis can be performed in the routine laboratory. Mean tHcy levels were 17.9 ± 6.66 µmol/l. MMA and HoloTC have a highly significant correlation (r = 0.500; p < 0.001).

The advantage of the diagnostic schemes proposed in this paper is that they allow not only to identify subclinical deficiencies, but also to distinguish between isolated B_{12}, isolated folate or combined B_{12}/folate deficiency. This diagnosis is important for the therapeutic treatment. The serum concentrations of the vitamins taken by their own can not give such specific information. On the other hand, as adequate cut-off points for blood parameters are still controversial, the decision tree seems to be a more adequate identification method.

Which of both algorithms is the most adequate must to be analysed further with more data. Perhaps it would be necessary to study a group of elderly in which B_{12} deficiency is not so highly prevalent. But in the meantime, using one of the algorithms seems to be more adequate than analysing data separately.

The AUCs obtained for Scbl and HoloTC in our study were somewhat different to those obtained by other research teams.[17,26,28] This difference may be attributable to different definitions of vitamin B_{12} used in these studies, where individuals are categorized as having likely vitamin B_{12} deficiency based on MMA and tHcy concentrations. The advantage of our study is that we used a full set of clinical diagnostic criteria (biochemical, haematological and morphological) to categorize individuals as presenting vitamin B_{12} deficiency.

Acknowledgements

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