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ORIGINAL ARTICLE

Association of vitamin B12, methylmalonic acid, and functional parameters

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ABSTRACT

Introduction: Diagnosis of vitamin B12 deficiency is difficult, as there is no conclusive single test for this disorder. We evaluated the association of serum B12 and methylmalonic acid (MMA) with haematologic parameters and physical and cognitive functioning in an effort to use such clinical parameters to improve the interpretation of serum values.

Methods: We used data of participants > 19 years of age from NHANES 2011-2012 and 2013-2014, a cross-sectional survey in the United States. Functional status was assessed with questionnaires on current health condition, disability, hospital utilisation, cognitive functioning, mental health and depression, and physical functioning. Muscle strength assessed with a handgrip dynamometer was used as a performance parameter. Results were evaluated both for the entire population and participants of Western European descent. Because renal function influences MMA concentrations and is a proxy for both frailty and comorbidity, all results were additionally stratified for individuals with normal vs impaired renal function (eGFR < 60 ml/min).

Results: In total, data of 9645 participants (mean age 49 (SD 17) years, 49.3% males) were included. Out of all participants with serum B12 < 140, 140-300, and 301-1000 pmol/l, 56.2%, 13.5%, and 4.1%, respectively had elevated MMA. MMA concentrations were more strongly associated with poor functional status and physical performance than serum B12. We identified a significant and independent association of MMA concentrations, as well as haemoglobin and co-morbidity with muscle strength.

Conclusions/interpretations: A large proportion of individuals with a decreased serum B12 concentration still has normal MMA concentrations. Elevated MMA concentrations were more strongly associated with poor functional performance than serum B12.

KEYWORDS

Epidemiology, methylmalonic acid, muscle strength, NHANES, vitamin B12

INTRODUCTION

The recognition of symptomatic cobalamin (vitamin B12) deficiency poses several challenges. First, the spectrum of complaints may be diverse, and symptoms such as paraesthesia in the hands and feet, muscle cramps, dizziness, cognitive disturbances, ataxia, fatigue, and depression may vary considerably between patients.[1-6] Second, the prevalence of anaemia in vitamin B12 deficiency is lower than anticipated[3,7], and neurological signs often occur in the absence of anaemia.[8] Third, serum B12 as a diagnostic test for tissue B12 deficiency may fail, as many people with such symptoms may have serum B12 concentrations above the lower population reference limit, which may cause individuals with relevant deficiency to be missed.[9,10] In some people, this may be caused by the recent use of oral supplementation with multivitamins, high-dose oral vitamin B12 preparations or even B12-fortified energy drinks.[11,12] Fourth, there is a poor correlation between serum B12 concentrations and complaints related to deficiency,[3,5,13] and some people with serum B12 concentrations below the lower reference limit may not have symptoms or may have normal active B12 concentrations.[14] Better information on the association between serum B12 concentrations and clinical symptoms is therefore warranted.

Yet, in daily practice, many general practitioners consider serum B12 concentrations within the reference interval for the population (i.e., 140-700 pmol/l) as proof of sufficiency, and possible complaints in this situation are determined to not be related to deficiency. Several authors have shown that many people with vitamin B12 deficiency

would be overlooked by incorrectly using only total serum B12 concentrations as status marker.[9,15,16] They therefore advocate measurement of one or more biomarkers, including methylmalonic acid (MMA), homocysteine (HCys), and/or holotranscobalamin in people with serum B12 concentrations in the grey zone of 140 to 300 pmol/l, in order to establish a possible diagnosis of deficiency.[9,17-19] Vitamin B12 is a pivotal cofactor in various enzymatic systems, and its deficiency will influence enzymes such as methylmalonyl-CoA mutase (MCM) and methionine synthase. As a consequence, vitamin B12 deficiency may result in high concentrations of MMA and HCys.[20] The sensitivity and specificity of elevated MMA concentrations as an indicator of symptomatic B12 deficiency are unknown. Earlier studies have even suggested that MMA concentrations are a poor predictor of symptom score or neurological complaints.[21] In addition, MMA concentrations may be elevated in people with severely impaired renal function.[22,23] Chronic kidney disease and impaired renal function are associated with more comorbidity and a higher risk of frailty, and there is evidence that chronic kidney disease is linked with impairments of physical and cognitive function and quality of life.[24,25] Similarly, elevated HCys concentrations may suggest symptomatic B12 deficiency, but HCys is also elevated in cases of folate deficiency or impaired renal function. Thus, although elevated MMA and HCys concentrations may be indicative of vitamin B12 deficiency, normal concentrations of these biomarkers do not rule out deficiency[26] or a favourable response to cobalamin therapy.[9] There are few large-scale studies that have reported on the association between serum B12, MMA, and functional status in the general population. A study in people > 70 years in Sweden showed that half of them had abnormal MMA or homocysteine concentrations, suggesting a latent or overt tissue deficiency of cobalamin or folate.[11] The 2001-2002 and 2003-2004 National Health and Nutrition Examination Survey (NHANES), a long-term epidemiologic survey in the USA,[27] showed that there is a large intermediate group of people whose B12 status is difficult to interpret.[20] In NHANES participants > 60 years, vitamin B12 deficiency was associated with an almost 10-fold increased risk in peripheral neuropathy and a 20-fold increased risk of total disability.[3] NHANES-based population reference values for MMA showed an age-related increase, due to both a gradual decline in kidney function with ageing, as well as vitamin B12 status.[28]

We combined data from two consecutive NHANES surveys of 2011-2012 and 2013-2014 in order to evaluate the association between serum B12 and MMA with haematological parameters and physical and cognitive functioning parameters. Furthermore, we also aimed to study the potential role of variation in serum B12 and MMA concentrations due to age and renal function on these associations.

MATERIALS AND METHODS

NHANES structure and inclusions

In short, NHANES is a cross-sectional survey in the U.S. that uses a complex, stratified, multistage probability sampling design.[20,27] NHANES has obtained written informed consent from all participants. The survey protocol was approved by the Research Ethics Review Board of the National Center for Health Statistics, Centers for Disease Control and Prevention. Interview questionnaires and examination response rates are publicly available.[29] Participants were first interviewed in their homes, during which demographic information and a variety of health-related information were collected. One to two weeks later, they underwent a standardised physical examination, as well as additional investigations such as exercise testing, 24-hour (h) dietary recall, and a blood draw in a mobile examination centre. Blood samples were taken with the participant fasting. Participants who visited the examination in the morning were requested to fast for nine hours; those visiting in the afternoon or evening were requested to fast for six hours. For this study, we created a dataset of NHANES 2011-2012 and 2013-2014 participants who were older than 19 years and had available serum B12 measurements. The NHANES survey included people from several ethnicities and the sample design for NHANES 2011-14 included an oversample of Asian Americans.

Outcomes

To estimate clinical complaints and functioning, we used data from the following NHANES questionnaires: current health condition, disability, hospital utilisation, medical conditions, cognitive functioning, mental health and depression, and physical functioning (supplementary table 1). Based on these questionnaires, we calculated symptom scores for current health status, mental health and depression, and physical functioning and disability, taking into account the most relevant questions/variables for each entity, as described in supplementary table 1. A higher symptom score conforms to a higher number of symptoms, complaints, and disturbance of functioning. We also evaluated the results of muscle strength which was measured through a grip test using a handgrip dynamometer as a separate parameter of physical functioning, and the results of cognitive functioning tests (the CERAD Word Learning sub-test, the Animal Fluency test, and the Digit Symbol Substitution Test (DSST), for details see supplementary table 1). The latter tests were only performed in participants aged 60 years and older.

Reference values were calculated for participants who could be considered 'healthy', i.e., defined as those participants with serum B12 between 301 and 1000 pmol/l, normal serum MMA, estimated glomerular filtration rate (eGFR) > 60, and no medication use.

Exposures

Haemoglobin and mean corpuscular volume (MCV) measurement were performed with a Beckman Coulter MAXM for 2011-2012 and the Beckman Coulter DxH 800 for 2013-2014 (Beckman Coulter Inc, Brea, CA, USA). No significant trend changes for haemoglobin and MCV were reported from NHANES 2011-2012 to NHANES 2013-2014. Serum B12 concentrations were measured with electrochemiluminescence immunoassay on a Modular Analytics E170® system (Roche Diagnostics, Indianapolis, IN). Serum MMA concentrations were analysed by LC-MS/MS as dibutylester after extraction from serum with tert-butylmethylether and derivatisation with butanol.[30] Serum creatinine was measured with the Jaffe rate method (kinetic alkaline picrate) on a Beckman Synchron DxC800 modular chemistry analyser. All information regarding these methods are publicly available on the NHANES website.[20,31]

On the basis of serum B12 concentrations, three groups were constructed: 1) probable vitamin B12 deficiency, defined as a serum B12 concentration < 140 pmol/l; 2) 'possible deficiency', serum B12 concentrations between 140 and 300 pmol/l; 3) normal concentrations, serum B12 > 300 pmol/l. Serum B12 concentrations greater than 1000 pmol/l were considered suggestive for supplementation with (parenteral) B12-containing preparations, and these participants were not included in the calculations. Serum MMA concentration ≥ 300 nmol/l was considered elevated. Anaemia was defined according to the World Health Organization criteria: haemoglobin concentration in men < 8.0 mmol/l and in women < 7.5 mmol/l, with MCV > 100 fl used as a definition for increased MCV.

Other variables

Medication use was scored in NHANES by the unique generic drug code from the Multum Lexicon Drug Database. The number of different medications reported by a participant was considered as a proxy for comorbidity.[32] Current smoking was defined as a positive answer to question SMQ690A/691A: Have you used tobacco/nicotine during the last 5 days? Renal function was calculated as eGFR with the formula developed by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI).[33] Impaired renal function influences MMA concentrations, and thereby the prognostic influence of elevated MMA concentrations may be different in people with impaired renal function. Also, impaired renal function itself is associated with more comorbidity and a higher risk of frailty.[24,25]

Statistics

Functional outcomes were assessed in relation to serum B12 and MMA concentrations. We calculated all variables for the entire population of participants > 19 years of age, and separately for people from Western European descent (described in NHANES as Non-Hispanic Whites, supplementary table 2) to evaluate for generalisability to Dutch individuals. Means were compared between groups with analysis of variance. When variables were not normally distributed, medians were compared with the nonparametric Mann-Whitney U or Kruskal Wallis test. Chi-square test was used to analyse categorical variables.

Univariable and multivariable linear regression analyses were performed to examine the association between relevant factors like serum B12 and MMA concentrations (both log transformed) and muscle strength. As age, sex, haemoglobin, serum creatinine (both log transformed), current smoking, and comorbidity are strong determinants of physical functioning and have been included in the multivariable model as co-factors.

NHANES has created specific sampling weights to account for its complex survey design (including oversampling), survey non-response, and post-stratification.[34] The incorporation of sampling weights into estimated regression coefficients helps protect against the potential existence of missing regressors. In addition, the linearisation variance estimator is suggested to be robust against the likelihood of correlated errors and the possibility of heteroscedasticity.[35,36] Although we did not intend to extrapolate our findings to the U.S. civilian non-institutionalised census population, we calculated our multivariable regression models with application of these weights. A p-value < 0.01 was used as a cut-off for statistical significance. Analyses were conducted using IBM SPSS Statistics (Version 24, IBM, Armonk, NY, USA) and Stata Statistical Software (version 16.0; Stata Corp).

RESULTS

NHANES 2011-2014 included 19931 participants. A total of 10286 participants were excluded because of age ≤ 18 years, unavailability of serum B12 measurements, serum B12 > 1000 pmol/l, pregnancy or breastfeeding (supplementary figure 1). The final study population included 9645 participants with a mean age of 49 (standard deviation (SD) 17) years; 50.1% were males. Mean haemoglobin was 8.7 (SD 1.0) mmol/l (in men: 9.2 ± 0.8 mmol/l, in women 8.2 ± 0.8 mmol/l), eGFR 96 (SD 24) ml/min, and median serum vitamin B12 concentrations and MMA concentrations were 377 pmol/l (IQR 280-509) and 140 nmol/l (IQR 108-189), respectively. In total, 159 (1.6%) participants had serum B12 concentrations < 140 pmol/l, while 2760 (28.6%) had concentrations between 140 and 300 pmol/l.

Table 1. *Association between serum B12 levels, methylmalonic acid, haematologic variables, and renal function*

	B12 < 140 pmol/l	B12 140-300 pmol/l	B12 301-1000 pmol/l	p-value
All	**n = 159**	**n = 2760**	**n = 6726**	
Males (%)	45	52	49	0.030
Age (years)	56 ± 17	49 ± 17	49 ± 18	< 0.001
MMA (nmol/l)	366 (69-5540)	161 (27-3020)	131 (37-1730)	< 0.001
MMA ≥ 300 (%)	56.2	13.5	4.1	< 0.001
Haemoglobin (mmol/l)	8.6 ± 0.9	8.8 ± 1.0	8.7 ± 1.0	0.038
Anaemia (%)	13.8	10.4	10.2	0.336
MCV (fl)	90 ± 7	89 ± 6	89 ± 6	0.176
MCV > 100 (%)	4.4	2.6	2.1	0.025
eGFR (ml/min)	87 ± 25	94 ± 23	94 ± 24	0.001
eGFR < 60 (%)	13.2	7.9	8.8	0.047
Western Europeans	**n = 65**	**n = 1249**	**n = 2635**	
Males (%)	47	49	51	0.390
Age (years)	58 ± 18	50 ± 18	51 ± 19	0.002
MMA (nmol/l)	309 (77-1890)	177 (59-1470)	151 (43-1240)	< 0.001
MMA ≥ 300 (%)	52.3	16.8	5.7	< 0.001
Haemoglobin (mmol/l)	8.8 ± 0.8	8.9 ± 0.9	8.9 ± 0.9	0.283
Anaemia (%)	7.6	6.2	5.9	0.805
MCV (fl)	91 ± 5	91 ± 5	91 ± 5	0.420
MCV > 100 (%)	4.5	2.5	3.0	0.472
eGFR (ml/min)	82 ± 23	89 ± 22	88 ± 23	0.032
eGFR < 60 (%)	12.1	11.4	12.3	0.689

Data are given as mean ± SD, median (range), or percentage.
eGFR = estimate glomerular filtration rate; MMA = methylmalonic acid; MCV = mean corpuscular volume; SD = standard deviation

Figure 1. *Relationship between levels of serum B12 and methylmalonic acid (MMA) in the entire population. The lower smoothed line shows the regression curve for participants with eGFR > 60 ml/min, the upper smoothed line is the regression curve for eGFR < 60 ml/min.*

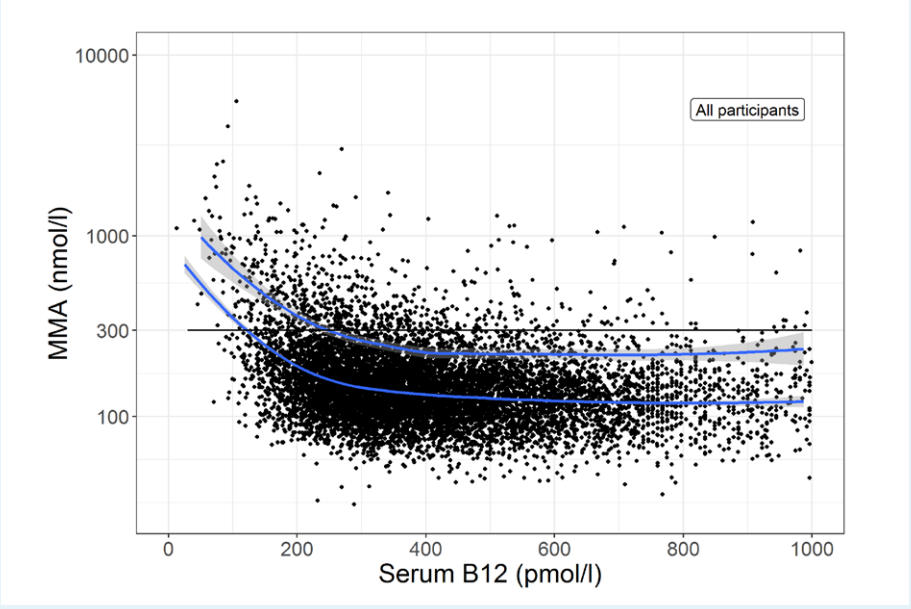

Table 2. *Association between methylmalonic acid, serum B12 levels, haematologic variables, and renal function*

	MMA < 300 nmol/l	MMA ≥ 300 nmol/l	p-value
All	**n = 8898**	**n = 742**	
Males (%)	50	51	0.581
Age (years)	48 ± 17	61 ± 16	< 0.001
Serum B12 (pmol/l)	385 (71-999)	260 (13-992)	< 0.001
Serum B12 < 140 (%)	0.8	12.1	< 0.001
Haemoglobin (mmol/l)	8.8 ± 0.9	8.5 ± 1.1	< 0.001
Anaemia (%)	9.7	18.1	< 0.001
MCV (fl)	89 ± 6	90 ± 6	0.008
MCV > 100 (%)	2.2	2.3	0.484
eGFR (ml/min)	96 ± 22	72 ± 29	< 0.001
eGFR < 60 (%)	6.6	32.5	< 0.001
Western Europeans	**n = 3553**	**n = 396**	
Males (%)	51	48	0.327
Age (years)	50 ± 18	64 ± 17	< 0.001
Serum B12 (pmol/l)	370 (87-999)	265 (70-992)	< 0.001
Serum B12 < 140 (%)	0.9	8.6	< 0.001
Haemoglobin (mmol/l)	9.0 ± 0.9	8.6 ± 1.0	< 0.001
Anaemia (%)	5.1	14.6	< 0.001
MCV (fl)	91 ± 5	91 ± 5	0.077
MCV > 100 (%)	2.9	2.5	0.410
eGFR (ml/min)	90 ± 21	70 ± 26	< 0.001
eGFR < 60 (%)	9.4	35.6	< 0.001

Data are given as mean ± SD, median (range), or percentage.
eGFR = estimate glomerular filtration rate; MMA = methylmalonic acid; MCV = mean corpuscular volume; SD = standard deviation

Of those participants with serum B12 < 140 pmol/l, 56.2% had elevated MMA concentrations, while only 4.4% had elevated MCV (table 1). In those with serum B12 < 100 pmol/l, only 32 of 42 (76.2%) had elevated MMA concentrations. In participants with serum B12 in the grey zone between 140 and 300 pmol/l, 13.5% had elevated MMA, while this was 4.1% in subjects considered to have normal serum B12 (301-1000 pmol/l). Similar results were obtained when only participants of Western European descent were studied. In these, only half (52.3%) had elevated MMA when serum B12 was < 140 pmol/l. Elevated MMA concentrations ≥ 300 nmol/l (table 2) were associated with significantly lower serum B12 and haemoglobin concentrations, and lower eGFR, but not with MCV or percentage of participants with elevated MCV.

There was a curvilinear association of serum B12 with MMA by which lower serum B12 was associated with higher MMA (figure 1). Elevated concentrations of MMA can be found across the entire spectrum of serum B12 concentrations (figure 1). Participants with an eGFR < 60 ml/min had a higher MMA for a given serum B12 concentration. In participants with serum B12 < 140 pmol/l, we identified that 43.8% had a normal MMA concentration (table 1). In participants with serum B12 in the grey zone and elevated MMA concentrations, 25.4% had an eGFR ≥ 60 ml/min, whereas in those with serum B12 between 301 and 1000 pmol/l, 46.8% of those with elevated MMA had an eGFR < 60 ml/min (supplementary table 3). Impaired renal function was found in 5.2-7.2% participants with normal serum MMA (supplementary table 3).

Table 3. *Health and functional parameters according to serum B12 levels*

	B12 < 140 pmol/l	B12 140-300 pmol/l	B12 301-1000 pmol/l	p-value
All	**n = 159**	**n = 2760**	**n = 6726**	
Current health status	186 (157-229)	180 (143-220)	180 (143-220)	0.052
Mental health & depression	14 (0-43)	17 (0-43)	17 (0-50)	0.115
Physical functioning	0 (0-40)	0 (0-20)	0 (0-18)	0.036
Any disability (%)[a]	28.4	20.3	20.5	0.108
Muscle strength (kg)	64 ± 19	72 ± 22	72 ± 23	0.001
Western Europeans	**n = 65**	**n = 1249**	**n = 2635**	
Current health status	200 (169-241)	180 (143-220)	180 (143-220)	0.037
Mental health & depression	14 (0-52)	17 (0-50)	17 (0-50)	0.889
Physical functioning	0 (0-40)	0 (0-26)	0 (0-30)	0.202
Any disability (%)[b]	23.5	22.9	23.4	0.955
Muscle strength (kg)	66 ± 20	71 ± 22	72 ± 23	0.192

Data are given as mean ± SD, median (IQR), or percentage.
Current health status, score 0-400, reference values 157 (129-180); mental health & depression, score 0-250, reference values 20 (0-40); physical functioning, score 0-190, reference values 0 (0-0);
for all three variables, higher score means worse performance.
Any disability: reference value 4%.
Muscle strength: the sum of the largest reading from each hand, reference values 78 ± 23 kg.
[a] only available in 116, 1863, and 4453 participants in the three different groups (B12 < 140 pmol/l, 140-300 pmol/l, and 301-1000 pmol/l, respectively)
[b] only available in 51, 892, and 1873 participants in the three different groups (B12 < 140 pmol/l, 140-300 pmol/l, and 301-1000 pmol/l, respectively)
n = number of patients

Table 4. *Health and functional parameters according to serum MMA levels and renal function*

	MMA < 300 nmol/l	MMA ≥ 300 nmol/l	p-value
All	**n = 8898**	**n = 742**	
Current health status	180 (143-220)	200 (160-240)	< 0.001
Mental health & depression	17 (0-50)	17 (0-57)	0.001
Physical functioning	0 (0-10)	12 (0-62)	< 0.001
Any disability (%)[a]	19.1	35.6	< 0.001
Muscle strength (kg)	72 ± 22	62 ± 21	< 0.001
Western Europeans with eGFR > 60 ml/min	**n = 3218**	**n = 255**	
Current health status	180 (143-220)	186 (157-240)	< 0.001
Mental health & depression	17 (0-50)	17 (0-50)	0.041
Physical functioning	0 (0-18)	9 (0-50)	< 0.001
Any disability (%)[b]	19.4	32.0	< 0.001
Muscle strength (kg)	74 ± 23	66 ± 21	< 0.001
Western-Europeans with eGFR < 60 ml/min	**n = 334**	**n = 141**	
Current health status	200 (169-240)	220 (183-260)	< 0.001
Mental health & depression	17 (0-52)	29 (0-62)	0.114
Physical functioning	27 (0-73)	50 (18-89)	< 0.001
Any disability (%)[c]	33.6	46.0	0.009
Muscle strength (kg)	59 ± 19	51 ± 19	0.001

Data are given as mean ± SD, median (IQR), or percentage.
For explanation: see legends for table 3.
[a] only available in 5831 and 596 participants of the two groups (< 300 vs ≥ 300 nmol/l MMA, respectively)
[b] only available in 2158 and 200 participants of the two groups (< 300 vs ≥ 300 nmol/l MMA, respectively)
[c] only available in 318 and 137 participants of the two groups (< 300 vs ≥ 300 nmol/l MMA, respectively)
eGFR = estimated glomerular filtration rate; IQR = interquartile range; kg = kilogram; MMA = methylmalonic acid
n = number of patients

Table 5. *Cognitive functioning in participants aged 60 years and older*

All participants	B12 < 140 pmol/l n = 69	B12 140-300 pmol/l n = 777	B12 301-1000 pmol/l n = 1896	p-value
CERAD total score	17.6 ± 5.2	18.6 ± 4.7	18.7 ± 4.9	0.248
CERAD score recall	5.3 ± 2.7	5.8 ± 2.3	5.8 ± 2.4	0.215
Animal Fluency Test	14.6 ± 5.2	16.7 ± 5.6	16.4 ± 5.6	0.009
Digit Symbol Substitution Test	40.2 ± 18.0	44.8 ± 17.1	46.4 ± 17.5	0.004

Reference values are:
CERAD total score 19.5 ± 4.6;
CERAD score recall 6.2 ± 2.3;
Animal fluency test 17.5 ± 5.8;
Digit symbol substitution test 50.0 ± 18.0

CERAD = Consortium to Establish a Registry for Alzheimer's Disease; MMA = methylmalonic acid
n = number of patients

Participants with serum B12 < 140 pmol/l reported significantly worse physical functioning and had lower muscle strength compared to those with serum B12 levels between 140-300 pmol/l and > 300 pmol/l (table 3). Both differences were lost when only people of Western European descent were evaluated. In the total population and in Western Europeans, we observed a significantly lower score in all functional outcomes, including muscle strength, for those with MMA concentrations > 300 nmol/l in comparison with people with normal MMA concentrations (table 4). In order to correct for impaired renal function, we re-calculated the composite outcomes and muscle strength for participants with an eGFR > 60 ml/min. Again, we observed lower performance with MMA ≥ 300 nmol/l, but the overall scores were slightly better for participants with an eGFR > 60 compared to those with an eGFR < 60 ml/min.

Table 5 shows the results of the cognitive function tests for all participants aged 60 years and older. The participants with serum B12 < 140 pmol/l had a lower score on the Animal Fluency Test and the Digit Symbol Substitution Test, compared to participants in the other groups, whereas the scores on the CERAD Word Learning and Recall test were similar. Participants with MMA ≥ 300 nmol/l had a lower score on all domains. Scores for the Intrusion Word Count were not different between the groups; 20.7% of participants with normal MMA compared with 19.5% with elevated MMA produced one or more intrusion words. Similar results were obtained for participants of Western European descent. Participants with normal renal function had significantly better score ($p < 0.001$ for all three domains) compared to those with impaired renal function. Comparison of participants with serum B12 < 140 pmol/l with normal or with elevated serum MMA concentrations showed that those with MMA ≥ 300 nmol/l were older, had slightly lower median serum B12 concentrations and more frequently impaired renal function; their haemoglobin, MCV concentrations and functional status were not different. Although the cognitive function tests showed lower scores for those with elevated MMA, these differences were not statistically significant due to low numbers (supplementary table 4).

Serum B12 concentration (log transformed) was not significantly associated with muscle strength (coefficient -0.279, SE 0.82, $p = 0.737$), while serum MMA (log transformed) was significantly associated with muscle strength (coefficient -6.90, SE 0.79, $p < 0.001$). The results of the multivariable regression analyses for muscle strength are depicted in table 6. For the entire population, we observed a significant and independent contribution for gender, age, and MMA concentrations, as well as haemoglobin concentrations and co-morbidity. Serum B12 was not independently associated with muscle strength. Similarly, age, serum B12, MMA concentrations, and co-morbidity were independently associated with the physical functioning symptom score, whereas only age and co-morbidity were significantly associated with current health status and mental health.

DISCUSSION

In this study, we have shown that a large proportion of individuals with a decreased serum B12 concentration still has normal MMA concentrations. In addition, in people with serum B12 concentrations in the grey zone between 140 and 300 pmol/l, 13.5% had elevated MMA concentrations. Only a very small proportion of participants with low serum B12 and elevated MMA had anaemia, with or without elevated MCV. Participants with serum B12 concentrations < 140 pmol/l had lower physical functioning and muscle strength in the entire

Table 6. *Multivariable regression analysis for muscle strength/combined grip test, corrected for NHANES survey weights*

A. Entire population (n = 8134)				
Variable	Coefficient	Linearized SE	t	p-value
Male	-28.700	0.644	-44.56	< 0.001
Age (years)	-0.324	0.021	-15.77	< 0.001
Log serum B12 (pmol/l)	-1.275	0.615	-2.07	0.046
Log MMA (nmol/l)	-4.414	0.607	-7.27	< 0.001
Log haemoglobin (mmol/l)	17.276	2.567	6.73	< 0.001
Log creatinine (mcmol/l)	8.989	1.191	7.55	< 0.001
Log N prescription meds	-1.746	0.278	-6.27	< 0.001
Current smoking	-0.353	0.459	-0.77	0.447
R-squared 0.648				
B. Western Europeans (n = 3482)				
Variable	Coefficient	Linearised SE	t	p-value
Male	-29.929	0.834	-35.89	< 0.001
Age (years)	-0.341	0.027	-12.73	< 0.001
Log serum B12 (pmol/l)	-1.463	0.825	-1.77	0.086
Log MMA (nmol/l)	-4.335	0.791	-5.48	< 0.001
Log haemoglobin (mmol/l)	21.012	3.724	5.64	< 0.001
Log Creatinine (mcmol/l)	6.035	1.486	4.06	< 0.001
Log N prescription meds	-2.321	0.372	-6.23	< 0.001
Current smoking	-0.762	0.610	-1.25	0.220
R-squared 0.678				

MMA = methylmalonic acid; N = number; SE = standard error of the mean; t = result of t-statistic test

population, but not in the subgroup of participants of Western European descent. Serum MMA concentrations were strongly associated with all clinical outcomes and with muscle strength in both the total population as well as participants of Western European descent.

When evaluating the functional status or performance of the participants, serum MMA concentrations proved to be a better indicator of poor functional status than serum B12 concentrations. In all domains, participants with elevated MMA ≥ 300 nmol/l had worse scores compared to those with MMA < 300 nmol/l. This is confirmed in our evaluation of muscle strength as a functional marker. Regression analysis showed that MMA, but not serum B12, was a significant and independent predictor of muscle strength in all subpopulations evaluated; i.e., the entire population, participants of Western European descent, and those with normal renal function. In an earlier study in NHANES participants > 60 years, it was demonstrated that vitamin B12 deficiency was associated with an almost 10-fold increased risk in peripheral neuropathy for participants with serum B12 < 200 pmol/l and homocysteine > 20 µmol/l, but only a 1.4 fold increase for participants with B12 (< 258 pmol/l) or MMA (> 210 nmol/l).[3]

We aimed to investigate cognitive domains in relation to serum B12 and MMA concentrations. As shown in tables 3, 4, and 6, elevated MMA concentrations were more strongly associated with poor functional performance than serum B12. There is limited literature on the relationship between serum B12, its biomarkers, and cognitive performance. Hooshmand et al. reported that higher serum homocysteine concentrations were associated with poorer performance in global cognition, memory, executive functions, and verbal expression, while higher baseline holotranscobalamin (holoTC) was significantly associated with better performance in global cognition,

executive functioning, and psychomotor speed.[37] In the Maine-Syracuse study, serum B12 concentrations and total homocysteine concentrations were positively and negatively associated, respectively, with cognitive performance.[38] Lewis et al. reported that elevated MMA concentrations appeared to be more reflective of cognitive impairment than serum B12, even when corrected for serum creatinine concentreations,[39] and similarly, high plasma homocysteine and serum MMA concentrations correlated inversely with movement and cognitive performance.[40] Taken together, these studies support our observation that biomarkers show a stronger association with functional outcome than serum B12 measurements. A study in people aged 75 and above showed that serum folate concentration was a more important determinant of cognitive performance than serum B12.[41]

The results of this observational study are of clinical importance for our approach towards patients with presumed or possible vitamin B12 deficiency. Some clinicians and clinical chemists consider elevated MMA as the single proof of existing B12 deficiency, and they base their diagnostic algorithms on this. However, almost 25% of people with serum B12 concentrations < 100 pmol/l had normal MMA concentrations. This has been previously observed: approximately 63% of people with low holo-transcobalamin (holoTc) levels < 20 pmol/l, indicative of true deficiency, had normal serum MMA concentrations.[26] This supports observations that serum MMA is not a very sensitive indicator of tissue B12 deficiency. Indeed, Schrempf W. et al. reported that both serum B12 and holoTC levels were weak predictors of abnormal MMA levels.[15] Other studies have confirmed that normal levels of MMA may be measured even in situations of very low B12 levels.[42] In addition, there are isolated reports showing that serum B12, homocysteine, and MMA levels are unreliable predictors of B12-responsive neurological disorders.[43]

The curvilinear association between serum B12 and MMA has been shown by Bailey et al. [20] in an earlier subset of NHANES participants, combining all available data from three consecutive NHANES screenings (1999-2000, 2001-2002, 2003-2004). These authors also showed that for each level of serum B12, MMA concentrations were higher in groups of participants with higher age.[20] Possibly, reductions in eGFR may mediate some of these differences, and they also raise the question whether age-specific reference values for MMA should be used.[23] This is supported by the observation that eGFR is independently associated with MMA in multivariable analysis. In the current paper, we confirmed that a serum B12 within the normal reference range, i.e., > 140 pmol/l, does not definitively reflect normal tissue B12 activity as estimated by serum MMA concentrations. This is also supported by our regression analysis, as depicted in figure 1, where the curvilinear course has an inflection point between 300 and 400 pmol/l of vitamin B12. This clearly supports the assumption that the area of vitamin B12 insufficiency extends above the lower reference value of serum vitamin B12.

Clinicians must consider that an impairment in renal function may increase serum MMA concentrations. As shown, NHANES participants with impaired renal function (eGFR < 60 ml/min) had poorer functional outcomes compared to those with an eGFR above 60 ml/min. A recent publication has provided information on how to adjust serum MMA concentrations for a reduction in eGFR in people with serum B12 levels between 90 and 300 pmol/l, and these calculations were intended to reduce the number of patients classified as vitamin B12 deficient.[23] However, based on the current data (table 1 and supplementary table 4), as well as earlier observations, serum MMA below 300 nmol/l does not exclude vitamin B12 deficiency.[15,26]

Classically, vitamin B12 deficiency has been associated with macrocytic anaemia. However, neurological signs of vitamin B12 deficiency are often present in the absence of anaemia.[8] The prevalence of anaemia in vitamin B12 deficiency appears to be lower than anticipated.[7] In NHANES participants, fewer than 10% of people considered to be vitamin B12 deficient had macrocytosis.[3] In the current study, the number of people with serum B12 < 140 pmol/l and anaemia and/or elevated MCV is small: anaemia was observed in 13.8% of these participants and elevated MCV in 4.4%. Prevalence of anaemia in those with MMA ≥ 300 nmol/l was 18.1%. Causes of anaemia may be complex and concomitant iron deficiency may mask macrocytosis.[44] It should be noted however, that we currently report data from an epidemiological survey and not data regarding patients referred for suspected vitamin B12 deficiency. In the latter group, prevalence of anaemia may be higher. Nevertheless, as anaemia is only seen in a minority of patients with vitamin B12 deficiency, its absence should not be considered as proof that vitamin B12 status is normal.

The results of the current study may help clinicians to identify pitfalls in diagnosing vitamin B12 deficiency. First, since 13.5% of these people with serum B12 concentrations > 140 and < 300 pmol/l have elevated MMA provides evidence that such serum B12 concentrations should not always be interpreted as normal, which is in accordance with several earlier reports.[6,9,15,19] Second, serum MMA is not a sensitive marker; the high percentage of people with low serum B12 but normal MMA suggests that the prevalence of tissue B12 deficiency may even be higher than can be estimated based on abnormal serum MMA concentrations. As the natural course of vitamin B12 deficiency is not well-known, it cannot be excluded that participants with low serum B12 but normal MMA may be in the early, still asymptomatic phase of their

deficiency.[45] Earlier studies have shown the importance of treatment response. In one of his papers, Solomon concluded that if cobalamin therapy had been restricted to symptomatic patients with both low or intermediate serum B12 concentrations and increased MMA or homocysteine concentrations, 63% of responders would not have been treated.[9] Functional vitamin B12 deficiency can be present in patients with apparently normal serum B12 concentrations, either related to defects in intracellular transport of B12,[46] due to interference of serum B12 assays by intrinsic factor antibodies,[47-51] or by masking due to the use of oral vitamin B12-containing supplements.[11,12] The high prevalence of elevated MMA in people with serum B12 > 140 pmol/l refutes the proposed algorithm for cost minimisation which was reported in this journal in 2013.[52] Applying the proposed algorithm will therefore, leave several people undiagnosed, who do have a high probability of vitamin B12 deficiency. Taken together, there is a great need of a generally accepted definition of vitamin B12 deficiency, which takes into account complaints, baseline biochemical results, and response to treatment.[9] Most studies have until now focus on normalisation of serum B12 or MMA, and do not specifically address the clinical syndrome, complaints, or quality of life.[6,53]

Strengths and limitations

This study was adequately powered to study the associations of interest, because we used a large NHANES dataset that reflects the general U.S. population. In addition, we considered potential effects of renal function and age, and have been able to provide sub-analyses in participants of Western European descent to evaluate for generalisability to Dutch individuals. Earlier studies have suggested that ethnicity may influence this association.[20] Because of its cross-sectional nature, we cannot be sure that the same results will apply to patients who are evaluated because of specific (neurological) complaints and are found to have low serum B12 concentrations. In NHANES 2011-2012 and 2013-2014, no direct assessment of peripheral neuropathy was available. The observational nature of this study does not allow for conclusions regarding causality. Also, the use of the NHANES dataset could limit the generalisability to the Dutch population.

CONCLUSIONS

MMA concentrations are elevated in only 56% of people with serum B12 concentrations < 140 pmol/l, and in 13.5% of people with serum B12 in the grey zone of 140-300 pmol/l. MMA concentrations proved to be a more reliable predictor of complaints, functional status, and physical performance than serum B12. Measuring serum MMA may also assist with diagnosing tissue B12 deficiency in cases of doubt when serum B12 concentrations are higher than 140 pmol/l, but this biomarker may also be elevated in people with (severely) impaired renal function.

DISCLOSURES

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Availability of data and material

We used publicly available and de-identified NHANES data collected by the National Center for Health Statistics, Centers for Disease Control and Prevention for the present study. https://www.cdc.gov/nchs/nhanes/index.htm

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Conflicts of interest

All authors declare no conflicts of interest.

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APPENDIX

Supplementary table 1. ***Questionnaire data*** *For each item, the total sum of score was calculated and divided by the number of answers given, to correct for missing answers/data. For calculation purposes, the resulting sum was multiplied by 100. If needed, scores were recoded, with the highest score indicating a worse performance or a higher level of complaints or dysfunctioning.*

Domain 1. Current health status **HSQ = current health status; maximum score 400**	
2011-2012 Questions	**Scoring**
HSD010 - General health condition	1 = Excellent; 2 = Very Good; 3 = Good; 4 = Fair; 5 = Poor
HSQ493 - Pain makes it hard for usual activities (number of days)	Scores recoded: 0-5 days = 1; 6-12 days = 2; > 12 days = 3
HSQ496 - How many days feel anxious (number of days)	Scores recoded: 0-5 days = 1; 6-12 days = 2; > 12 days = 3
2013-2014* Question	**Scoring**
HSD010 - General health condition	1 = Excellent; 2 = Very good; 3 = Good; 4 = Fair; 5 = Poor
* In the years 2013-2014, only question HSD010 was available, no t the other questions HSQ493 and HSQ496, In 2014, these questions were dropped from the survey.	
Domain 1. Current health status **HUQ = hospital utilisation and access to care**	
2011-2012 Questions	**Scoring**
HUQ010 - General health condition	1 = Excellent; 2 = Very Good; 3 = Good; 4 = Fair; 5 = Poor
HUQ020 - Health now compared with 1 year ago	1 = better; 2 = worse; 3 = about the same Scores recoded: 1 = 1; 2 = 4; 3 = 2
HUQ050 - # times receive healthcare over past year	0 = None; 1 = 1; 2 = 2 to 3; 3 = 4 to 9; 4 = 10 to 12; 5 = 13 or more
HUQ090 - Seen mental health professional/past year	Recoded: 0 = no; 1 = yes
2013-2014* Questions	**Scoring**
Same as 2011-2012, except HUQ050 and HUQ051	
HUQ051 - # times receive healthcare over past year	Scores recoded to match the scores of question HUQ050 which was used in 2011-2012.
Domain 2. Mental health & depression **DPQ = mental health and depression**	
Questions	**Scoring**
DPQ010 - Have little interest in doing things	Scores recoded: 0 = 0; 1 = 1; 2 = 2; 3 = 3; 7, 9 = missing
DPQ020 - Feeling down, depressed, or hopeless	Scores recoded: 0 = 0; 1 = 1; 2 = 2; 3 = 3; 7, 9 = missing
DPQ040 - Feeling tired or having little energy	Scores recoded: 0 = 0; 1 = 1; 2 = 2; 3 = 3; 7, 9 = missing
DPQ070 - Trouble concentrating on things	Scores recoded: 0 = 0; 1 = 1; 2 = 2; 3 = 3; 7, 9 = missing
DPQ100 - Difficulty these problems have caused	Scores recoded: 0 = 0; 1 = 1; 2 = 2; 3 = 3; 7, 9 = missing
HUQ090 - Seen mental health professional/past year	0 = no; 1 = yes
MCQ084 - Difficulties in thinking or remembering	0 = no; 1 = yes

Domain 3. Physical functioning **PFQ = physical functioning**	
Questions	**Scoring**
PFQ049 - Limitations keeping you from working	Scores recoded: 1 = 1; 2 = 0; 7, 9 = missing
PFQ051 - Limited in amount of work you can do	Scores recoded: 1 = 1; 2 = 0; 7, 9 = missing
PFQ057 - Experience confusion/memory problems	Scores recoded: 1 = 1; 2 = 0; 7, 9 = missing
PFQ059 - Physical, mental, emotional limitations	Scores recoded: 1 = 1; 2 = 0; 7, 9 = missing
PFQ061B - Walking for a quarter of a mile difficulty	Scores recoded: 1 = 0; 2 = 1; 3 = 2; 4 = 3; 5, 7, 9 = missing
PFQ061C - Walking up ten steps difficulty	Scores recoded: 1 = 0; 2 = 1; 3 = 2; 4 = 3; 5, 7, 9 = missing
PFQ061D - Stooping, crouching, kneeling difficulty	Scores recoded: 1 = 0; 2 = 1; 3 = 2; 4 = 3; 5, 7, 9 = missing
PFQ061I - Standing up from armless chair difficulty	Scores recoded: 1 = 0; 2 = 1; 3 = 2; 4 = 3; 5, 7, 9 = missing
PFQ061M - Standing for long periods difficulty	Scores recoded: 1 = 0; 2 = 1; 3 = 2; 4 = 3; 5, 7, 9 = missing
PFQ061P - Grasp/holding small objects difficulty	Scores recoded: 1 = 0; 2 = 1; 3 = 2; 4 = 3; 5, 7, 9 = missing
Original scores for PFQ061B through P 1 = no difficulty; 2 = some difficulty; 3 = much difficulty; 4 = unable to do	
Domain 4. Any disability **DLQ = disability (only for 2013-2014 survey). Maximum score 100**	
Questions	**Scoring**
MCQ084 - Difficulties in thinking or remembering	0 = no; 1 = yes.
DLQ040 - Have serious difficulty concentrating?	0 = no; 1 = yes.
DLQ050 - Have serious difficulty walking?	0 = no; 1 = yes.
DLQ060 - Have difficulty dressing or bathing?	0 = no; 1 = yes.
DLQ080 - Have difficulty doing errands alone?	0 = no; 1 = yes.
Domain 5. **MGX = muscle strength**	
The muscle strength/grip test component measured the isometric grip strength using a handgrip dynamometer. We used the combined grip strength (kg), i.e., the sum of the largest reading from each hand.	
Domain 6.	
CFQ = cognitive functioning, only available in participants aged 60 years and older **CFDCSR - CERAD: Score delayed recall, maximum score 10.** **CFDCIR - CERAD: Intrusion word count recall, maximum score 8.**	
The word learning and recall modules from the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) assesses immediate and delayed learning ability for new verbal information (memory sub-domain). The test consists of three consecutive learning trials and a delayed recall. Participants are instructed to read aloud 10 unrelated words, one at a time, and immediately following the presentation of the words, they recall as many words as possible. In each of the three learning trials, the order of the 10 words is changed. The delayed word recall occurred after the other two cognitive exercises (Animal Fluency and DSST) were completed (approximately 8-10 minutes from the start of the word learning trials). In addition to scores for each word learning trial and the delayed word recall, a score for the number of intrusions (incorrect words that were not on the list) is included in the data file.	
CFDAST - Animal Fluency Test, maximum score 39	
The Animal Fluency test examines categorical verbal fluency, a component of executive function. Participants are asked to name as many animals as possible in one minute. A point is given for each named animal.	
CFDDS - Digit Symbol Substitution Test, maximum score 105	
Digit Symbol Substitution test (DSST), a performance module from the Wechsler Adult Intelligence Scale, relies on processing speed, sustained attention, and working memory. The exercise is conducted using a paper form that has a key at the top containing nine numbers paired with symbols. Participants have two minutes to copy the corresponding symbols in the 133 boxes that adjoin the numbers.	

Supplementary table 2. *Distribution of ethnicity in the current dataset of 9645 NHANES 2011-2014 participants*

		Number	Percent
1	Mexican American	1140	11.8
2	Other Hispanic	918	9.5
3	Non-Hispanic White	3951	41.0
4	Non-Hispanic Black	2141	22.2
6	Non-Hispanic Asian	1213	12.6
7	Other Race, Including Multi-Racial	282	2.9

Supplementary table 3. *Distribution of renal function (eGFR) according to groups of serum B12 and MMA concentrations*

		MMA < 300 nmol/l		MMA ≥ 300 nmol/l	
Serum B12 (pmol/l)	n	eGFR > 60 ml/min	eGFR ≤ 60 ml/min	eGFR > 60 ml/min	eGFR ≤ 60 ml/min
< 140	157	63 (94.0%)	4 (6.0%)	74 (8.2%)	16 (17.8%)
140-300	2757	2259 (94.8%)	124 (5.2%)	279 (74.6%)	95 (25.4%)
301-1000	6722	5983 (92.8%)	461 (7.2%)	148 (53.2%)	130 (46.8%)

eGFR = estimate glomerular filtration rate; MMA = methylmalonic acid

Supplementary table 4. *Comparison of participants with serum B12 < 140 pmol/l with normal vs. elevated serum MMA concentrations*

	B12 < 140 and MMA < 300 nmol/l	B12 < 140 and MMA ≥ 300 nmol/l	p-value
All	**n = 67**	**n = 90**	
% males	43.3	46.7	0.746
Age (years)	49 ± 17	61 ± 16	< 0.001
Serum B12 (pmol/l)	121 (71-140)	113 (13-140)	0.005
Serum MMA (nmol/l)	200 (77-298)	698 (301-5540)	N.A.
Haemoglobin (mmol/l)	8.7 ± 1.0	8.6 ± 0.9	0.553
Anaemia (%)	14.9	12.2	0.396
MCV (fl)	90 ± 6	89 ± 7	0.343
MCV > 100 (%)	6.0	3.3	0.340
eGFR (ml/min)	98 ± 21	79 ± 25	< 0.001
eGFR < 60 (%)	6.0	17.8	0.023
Current health status	186 (157-220)	186 (143-241)	0.589
Mental health & depression	20 (0-60)	17 (9-50)	0.526
Physical functioning	0 (0-30)	0 (0-52)	0.127
Grip strength	69 ± 18	62 ± 20	0.033
CERAD total score *	18.9 ± 4.9	17.2 ± 5.3	0.213
CERAD score recall *	6.0 ± 2.4	5.0 ± 2.7	0.154
Animal Fluency Test *	16.1 ± 4.9	14.0 ± 5.3	0.136
Digit Symbol Substitution Test *	45.7 ± 12.7	37.8 ± 19.5	0.109

Data are given as mean ± SD, median (range), or percentage.
CERAD = Consortium to Establish a Registry for Alzheimer's disease; eGFR = estimate glomerular filtration rate; MMA = methylmalonic acid; MCV = mean corpuscular volume.
* only available in 20 and 49 participants aged 60 years and older, for groups MMA < 300 nmol/l and MMA ≥ 300 nmol/l, respectively

Supplementary figure 1.