Mild cobalamin deficiency and cognitive function in elderly people

Efficacy of oral supplements

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Milde vitamine B12 deficiëntie en het cognitief functioneren van ouderen

De effectiviteit van orale supplementen

Simone Josephina Petra Maria Eussen

The truth is rarely pure and never simple
(Oscar Wilde)

ABSTRACT

Cobalamin deficiency is common in older people and has been recognised as a possible cause for several clinical manifestations such as anaemia and cognitive impairment. Markers for cobalamin deficiency include increased concentrations of plasma total homocysteine (tHcy) and methylmalonic acid (MMA), and decreased concentrations of holotranscobalamin (holoTC). Cross sectional analysis in this thesis confirmed that impaired cognitive performance was associated with relatively unfavourable concentrations of markers for cobalamin status. These results are in line with findings from previous cross-sectional and prospective studies and suggest a role for cobalamin status in cognitive function, in particular because cobalamin deficiency is highly prevalent in old age. According to our recruitment activities it appeared that 26.6% of the older people had mild cobalamin deficiency, which we defined as low to low-normal cobalamin concentrations in combination with increased MMA concentrations. Normalizing mild cobalamin deficiency, defined as a decrease of respectively 80% to 90% of the estimated maximum reduction in plasma MMA concentrations, could be achieved by supplementing daily oral doses of 647 μg to 1032 μg crystalline cobalamin. The main purpose of our research was to investigate whether daily supplementation with such a high dose of oral cobalamin alone or in combination with folic acid has beneficial effects on cognitive function in people aged 70 years or older with mild cobalamin deficiency. We did this in a double-blind, placebo-controlled trial with a relatively large number of carefully selected participants, and an extensive assessment of cognitive function. In total, 195 individuals were randomized to receive either 1,000 μg cobalamin, or 1,000 μg cobalamin + 400 μg folic acid, or placebo for 24 weeks. Markers for cobalamin status and cognitive function were assessed before and after 24 weeks of treatment. Assessment of cognitive function included the domains of attention, construction, sensomotor speed, memory and executive function. Cobalamin status did not change in the placebo group, whereas oral cobalamin supplementation corrected mild cobalamin deficiency. Improvement in one domain (memory function) was observed in all treatment groups, and was greater in the placebo group than in the group who received cobalamin alone ($P = 0.0036$). Oral supplementation with cobalamin alone or in combination with folic acid for 24 weeks was not associated with improvements in other cognitive functions. Blood collection after cessation of oral cobalamin supplementation showed that adequate cobalamin status may maintain for a period of up to 5 months after cessation. Despite the null finding of this trial, recent studies provide clues for future research in improving cognitive function.

CONTENTS

Introduction

With increasing life expectancy across the world, the number of elderly who suffer from cognitive impairment and dementia also increase.[1] Knowledge of how to get older in good mental health will benefit quality of life of elderly people. The evidence to date suggests that, even in old age, improvements in nutritional status may improve cognitive functioning.[2, 3] Cobalamin deficiency is a particular problem in the aging population given its high prevalence.[4] It is associated with anemia, cerebrovascular diseases, and several neurological disorders, such as neuropathy, myelopathy, depression, and cognitive impairment.[5, 6] This thesis focuses on the effects of cobalamin supplementation on cobalamin status and subsequently on cognitive performance of elderly people.

COBALAMIN

Terminology

Cobalamin, or vitamin B12, was first isolated in 1948,[7, 8] and its molecular structure was described in 1956.[9] The term cobalamin refers to a family of substances composed of a central cobalt nucleus surrounded by a corrin ring with a complex side chain consisting of benzimidasole. The molecule is completed by linkage with one of several different radicals to the cobalt nucleus. Many forms of cobalamin can be formed through the replacement of different radicals or by oxidation and reduction of the cobalt nucleus. The corrin ring has a variable ligand that can contain a methyl-, adenosyl-, hydroxo-, or cyanogroup, resulting in methylcobalamin, adenosylcobalamin, hydroxocobalamin, and cyaonocobalamin, respectively.[10]

Cobalamin intake

The usual dietary sources of cobalamin are meat and meat products, and to a lesser extent dairy products. Although bacteria in the large bowel of humans produce cobalamin, it cannot be taken up in the body from this site. Therefore, humans fully depend on animal food products or on cobalamin-fortified products for their daily needs of the vitamin. A normal diet contains approximately 5-15 µg cobalamin from which a maximum of 3 µg is absorbed.[11] The Dutch Recommended Dietary Allowance (RDA) for cobalamin is set to 2.8 µg/day by the Health Council of the Netherlands. However, due to insufficient knowledge of the effect of cobalamin intake on serum cobalamin status, the Health Council took the estimated average requirement for adults to be the amount of cobalamin that is required to compensate for daily losses of 0.2% of the minimum required bodily reserve of 5000 µg. Hereby, an absorption rate from food of 50% and a coefficient of variation of 20% was taken into account.[12] Recently, one trial examined the relation between cobalamin intake and plasma markers for cobalamin status and showed that an intake of 6 µg cobalamin /day was accompanied with normal cobalamin status, whereas a lower intake was associated with a mildly impaired cobalamin status.[13]

Absorption and function

For the transport from food into body cells, cobalamin is absorbed by either active absorption (intrinsic factor mediated) or passive diffusion. The intrinsic factor transports cobalamin in the digestive system. Once absorbed in the distal ileum, it is transported in the plasma by transcobalamin and haptocorrin. The intrinsic factor related absorption has a limited capacity of approximately 3 µg per meal. However, when large amounts are ingested, e.g. in the form of supplements, approximately 1% of cobalamin can be absorbed by passive diffusion. For efficient absorption and retention of cobalamin, several tissues, receptors, and transport systems are involved. These include the gastric mucosa, pancreas, distal ileum, liver and biliary system, and the kidneys. Once present in the metabolism, cobalamin serves as a co-factor for methionine synthase, an enzyme that remethylates homocysteine (Hcy) to methionine, and for methylmalonyl-CoA mutase, an enzyme that converts methylmalonyl-CoA to succinyl-CoA (Figure 1[14]). Cobalamin plays an important role in DNA synthesis, methylation reactions and energy metabolism.[15]

COBALAMIN DEFICIENCY

Definition

The definition of cobalamin deficiency has not been clearly established as there is no gold standard available. Consequently, there is no consensus on criteria to diagnose the deficiency. A low serum cobalamin concentration does not always indicate cobalamin deficiency and a normal serum cobalamin concentration does not always exclude it.[16-18] Increased concentrations of methylmalonic acid (MMA)[19] and total homocysteine (tHcy) in plasma or serum[6] are established as useful diagnostic indicators of cobalamin deficiency. Homocysteine is regarded as a less specific marker for cobalamin deficiency because concentrations are also elevated with folate deficiency and many other life-style factors.[20] Recently, reduced holo-transcobalamin (holoTC) concentration has been studied as a new marker for cobalamin deficiency, because it is assumed to represent the biological active fraction of cobalamin in blood.[21-23] In addition to the different biochemical markers to diagnose cobalamin deficiency, also different cut off values for these markers are used to identify individuals with cobalamin deficiency. The research described in this thesis includes mildly cobalamin deficient elderly people and were selected on the basis of low to low-normal cobalamin concentrations in combination with elevated MMA concentrations.

Causes of cobalamin deficiency

Cobalamin deficiency is a slowly progressive process that may take many years to develop.[24] Risk factors for development of cobalamin deficiency mainly include age[25], inadequate intake by vegans and vegetarians or malnutrition[26], use of medications such as proton pump inhibitors[27], and malabsorption of the vitamin from food or from the intestine due to gastro-intestinal diseases including atrophic gastritis, gastric surgery, and bacterial overgrowth.[11, 14, 28, 29] Furthermore, less frequent causes include heavy smoking, chronic alcoholism, autoimmune diseases such as Sjögren's syndrome and polyglandular autoimmune syndrome, and genetic factors such as juvenile pernicious anemia, polymorphisms in cobalamin metabolism and the Imerslund Gräsbeck syndrome.[14]
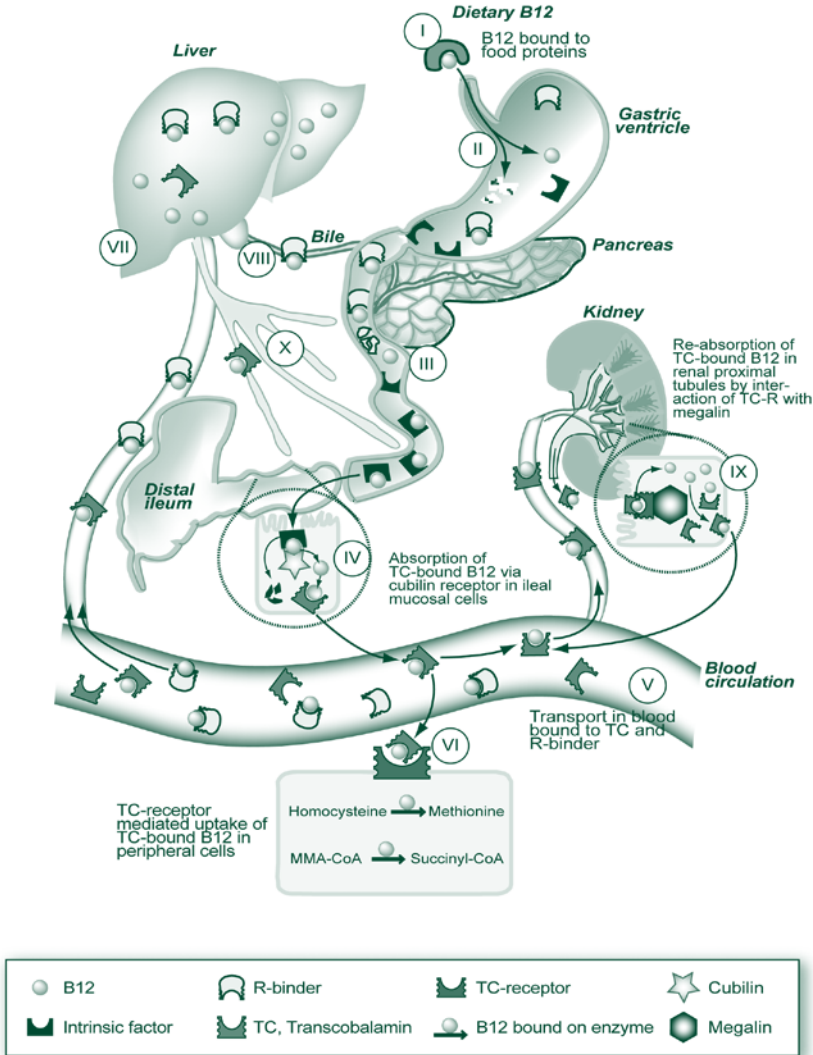
Figure 1. Cobalamin absorption and metabolism.Dietary cobalamin (vitamin B12) is normally protein-bound (**I**) and provided by food products of animal origin. Pepsin and low pH in the gastric ventricle degrade food proteins, resulting in release of cobalamin (**II**). Free cobalamin is then bound to R-binder, which is produced by the salivary glands and parietal cells. R-binders are degraded by pancreas proteases, and cobalamin (both newly ingested and cobalamin bound to R-binder in the bile) is released again and binds with high affinity to intrinsic factor produced in the stomach (**III**). Intrinsic factor has high affinity to cobalamin at neutral or alkaline pH of the pancreatic juice. In the mucosal cells of the distal 80 cm of the ileum, the cobalamin-intrinsic factor complex is recognized by cubilin receptors (in a functional complex with the amnionless molecule) (**IV**). Cobalamin enters the blood circulation bound to TCII. There, the majority of cobalamin (70-80%) is bound to R-binder and only a minor portion (20-30%) is bound to transcobalamin II (TC-II) (**V**). TC-II-bound cobalamin (holoTC) is the biologically active fraction of total cobalamin in serum, as only this fraction is taken up by the majority of cells in the body. Cellular up-take of holoTC is mediated by transcobalamin (TC)-receptors (**VI**). Cobalamin absorbed in the intestine subsequently enters the liver (**VII**) via the portal system (X). Within the cells, holoTC-molecules are degraded and cobalamin enzymatically converted into its two coenzyme forms, methylcobalamin (co-factor for the methionine-synthase enzyme) and adenosylcobalamin (co-factor for the methylmalonyl-CoA mutase in mitochondria) (**VI**). There is extensive enterohepatic circulation transporting 3-5 times more cobalamin than is newly absorbed from food. Cobalamin and cobalamin analogues are bound to R-binders in the bile (**VIII**). The kidneys seem to have a more important role for cobalamin homeostasis than earlier recognized. HoloTC is filtered in the glomeruli and quantitatively reabsorbed in the proximal tubuli in a process involving the TC-receptor and megalin (IX). Cobalamin leaves tubulus cells at the basal membrane bound to TC-II.

Treatment of cobalamin deficiency

There is no consensus on how to treat cobalamin deficiency with respect to dosage, and route of administration. In general practice, intramuscular cobalamin injections are used to treat cobalamin deficiency.[30] However, intramuscular injections may be inconvenient and painful for patients, and the frequent need for assistance of health professionals are expensive for the health care system and makes individuals dependent.[31, 32] More convenient and cost-effective alternatives would benefit the health care system in general and individuals in particular. Intranasal administration[33, 34] and fortification of milk[35] with cobalamin can serve as alternatives to treat cobalamin deficiency. Moreover, supplementation by daily oral doses with 1,000 to 2000 μg of cyanocobalamin administered orally has been shown to be as effective[36] or even more effective[30] than cobalamin administered by intramuscular injections to correct biochemical markers of cobalamin deficiency. A major knowledge gap concerns the minimum effective dose of oral cobalamin supplementation that would normalise cobalamin deficiency. This gap has lead to one of the two intervention studies described in this thesis. A dose-finding study, as described in chapter 2, aimed to determine the minimum effective dose of oral crystalline cobalamin that is required for a maximal reduction in MMA concentrations.

Monitoring

Very little is known about the persistence of the effects of oral cobalamin treatment in elderly people with mild cobalamin deficiency. In general practice, cobalamin status is not monitored in patients who are treated for cobalamin deficiency. Consequently, there is no consensus on when and how to monitor effects of treatment. Chapter 3 evaluates changes in markers for cobalamin status after cessation of oral cobalamin supplementation in participants with mild cobalamin deficiency prior to supplementation.

CONSEQUENCES OF COBALAMIN DEFICIENCY

Cobalamin deficiency has been recognised as a possible cause for several clinical manifestations. The haematological and gastrointestinal symptoms include anemia and glossitis, respectively. In addition, the neuropathological and neuropsychological signs include subacute combined degeneration of the spinal cord[37], paresthesia in feet and fingers, disturbances in vibratory sense, psychomotor slowing, delirium, depression, behavioural disorders, and cognitive impairment.[38, 39] Historically, the clinical definition of cobalamin deficiency was based on presence of severe megaloblastic anemia combined with neuropsychological symptoms.[40] However, this believe was refuted by Lindenbaum et al, who indicated that neuropsychological symptoms such as cognitive impairment, may occur in the absence of haematological signs.[5] During the last 25 years it has been proposed that the neuropsychological symptoms are often the first clinical manifestation of cobalamin deficiency, preceding the hematological and neuropathological symptoms.[5, 41] This thesis focuses on mild cobalamin deficiency in relation to the neuropsychological symptoms of cognitive impairment.

Cognitive impairment

Cognitive functions are related to a variety of different brain-mediated functions and processes. Information from internal sources such as experience, memory, concepts and thoughts, and information from external sources such as the environment are perceived, evaluated, stored, and manipulated. Humans constitute the response to this information. Impairment in one or more cognitive functions may result in mild cognitive impairment or dementia. It is estimated that worldwide 24.3 million people have dementia today, with 4.6 million new cases of dementia every year. Consequently, the social, medical, and economical impact of cognitive impairment is large.[1] Early stages of cobalamin deficiency, as indicated by increased concentrations of plasma tHcy and MMA6, and decreased concentrations of holoTC21, may result in milder forms of cognitive impairment in the absence of anemia.[42, 43]

The pathogenesis of neurological damage and relevance to cognitive impairment is still uncertain.[44, 45] Therefore, a better understanding of the risk factors for cognitive impairment is needed in order to prevent and possibly reverse cognitive impairment in elderly people. In this respect, B-vitamins and homocysteine are of interest because of their link with cardiovascular disorders and cognitive impairment.[46, 47] This thesis focuses on the association of cognitive function with mild cobalamin deficiency, a condition in which concentrations of MMA and homocysteine are elevated. In most tissues, homocysteine is remethylated to methionine by the cobalamin-dependent enzyme methionine synthase (MS), and 5-methyltetrahydrofolate provides a methyl group. The classical signs of cobalamin deficiency have been related to the hypomethylation theory because aberrations in the cobalamin dependent methylation reactions are thought to cause myelin damage and disturbed neurotransmitter metabolism.[48-50] In addition, elevated concentrations of MMA may induce neuronal damage in vitro.[51]

Studies to address the role of cobalamin in neuropsychological performance

Existing cross-sectional studies[52-65] show associations between cobalamin status with neuropsychological functions in elderly people with and without severe cognitive impairment. However, these associations are inconsistent and can be ascribed to various markers that are used to evaluate cobalamin status, and concurrently to a large variety of neuropsychological test batteries. Therefore, chapter 4 evaluates whether there were any associations between cobalamin and folate status and specific cognitive domains by using sensitive markers for cobalamin and folate status and an extensive neuropsychological test battery. Studies on the prediction of cognitive performance by B-vitamin status[55, 66-68] and intake[69] also show associations between markers for cobalamin and folic acid status with cognitive function, but these results are inconclusive with respect to the markers which have been measured.

Although results of cross-sectional and prospective studies suggest a role of cobalamin and folate status in neuropsychological function, confirmation is needed from randomised controlled intervention trials. Small non-randomized and placebo controlled trials[70-74] showed beneficial effects of cobalamin treatment on cognitive function. However, results from placebo controlled intervention studies would provide the most compelling evidence for the effects of B-vitamins on cognitive function, since extraneous variables which might affect cognitive function will be controlled for. Existing evidence for the effects of
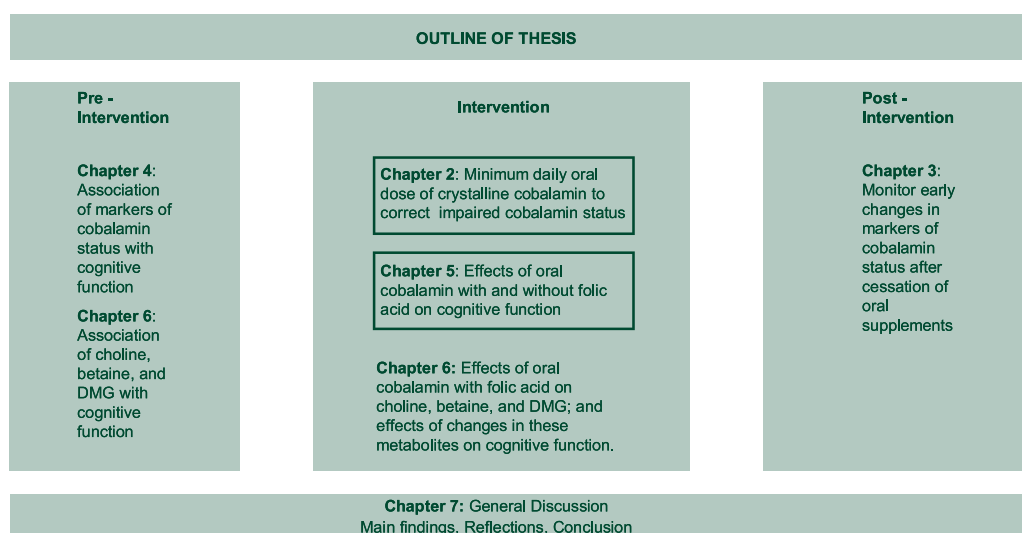
cobalamin supplementation on cognitive function from randomized trials is limited and inconclusive.[75-77] This can possibly be explained by variations in study duration, sample size, characteristics of study population, diagnosis and treatment of cobalamin deficiency, and assessment of cognitive function. Based on results of existing cross-sectional, prospective and intervention studies, we tested the hypothesis that oral cobalamin supplementation improves cognitive performance or prevents cognitive decline. The study of van Asselt et al[74] served as a pilot study and enabled us to design a randomized placebo controlled trial in which we aimed to overcome the methodological shortcomings of previous trials. Chapter 5 describes the efficacy of oral cobalamin supplementation on cognitive function.

Homocysteine is not only remethylated by the cobalamin-dependent enzyme methionine synthase (MS), but also by the enzyme betaine-homocysteine methyltransferase (BHMT). The latter reaction predominantly takes place in the liver and kidneys, and methionine and dimethylglycine (DMG) are the products of this reaction. The methyl donor, betaine, is formed from choline, which also is a precursor for the neurotransmitter acetylcholine.[78] Previous studies have focused on the associations of homocysteine, cobalamin and folate with cognitive performance.[50, 79-83] However, the possible relation of choline, betaine and DMG with cognitive performance has not been explored previously, and is addressed in Chapter 6.

OUTLINE OF THESIS

The main objectives of this thesis were to study the lowest oral dose of cobalamin to normalise mild cobalamin deficiency, and to study its efficacy on cognitive function. The data collection in order to answer these two main research questions enabled us to shed more light on other cobalamin related research questions. The chapters of this thesis are placed into perspective in Figure 2.

Figure 2: Outline of this thesis. The chapters within the frames indicate the two main research questions.

OUTLINE OF THESIS

Pre - Intervention

Chapter 4: Association of markers of cobalamin status with cognitive function
Chapter 6: Association of choline, betaine, and DMG with cognitive function

Intervention

Chapter 2: Minimum daily oral dose of crystalline cobalamin to correct impaired cobalamin status

Chapter 5: Effects of oral cobalamin with and without folic acid on cognitive function

Chapter 6: Effects of oral cobalamin with folic acid on choline, betaine, and DMG; and effects of changes in these metabolites on cognitive function.

Post - Intervention

Chapter 3: Monitor early changes in markers of cobalamin status after cessation of oral supplements

**Chapter 7:** General Discussion
Main findings, Reflections, Conclusion

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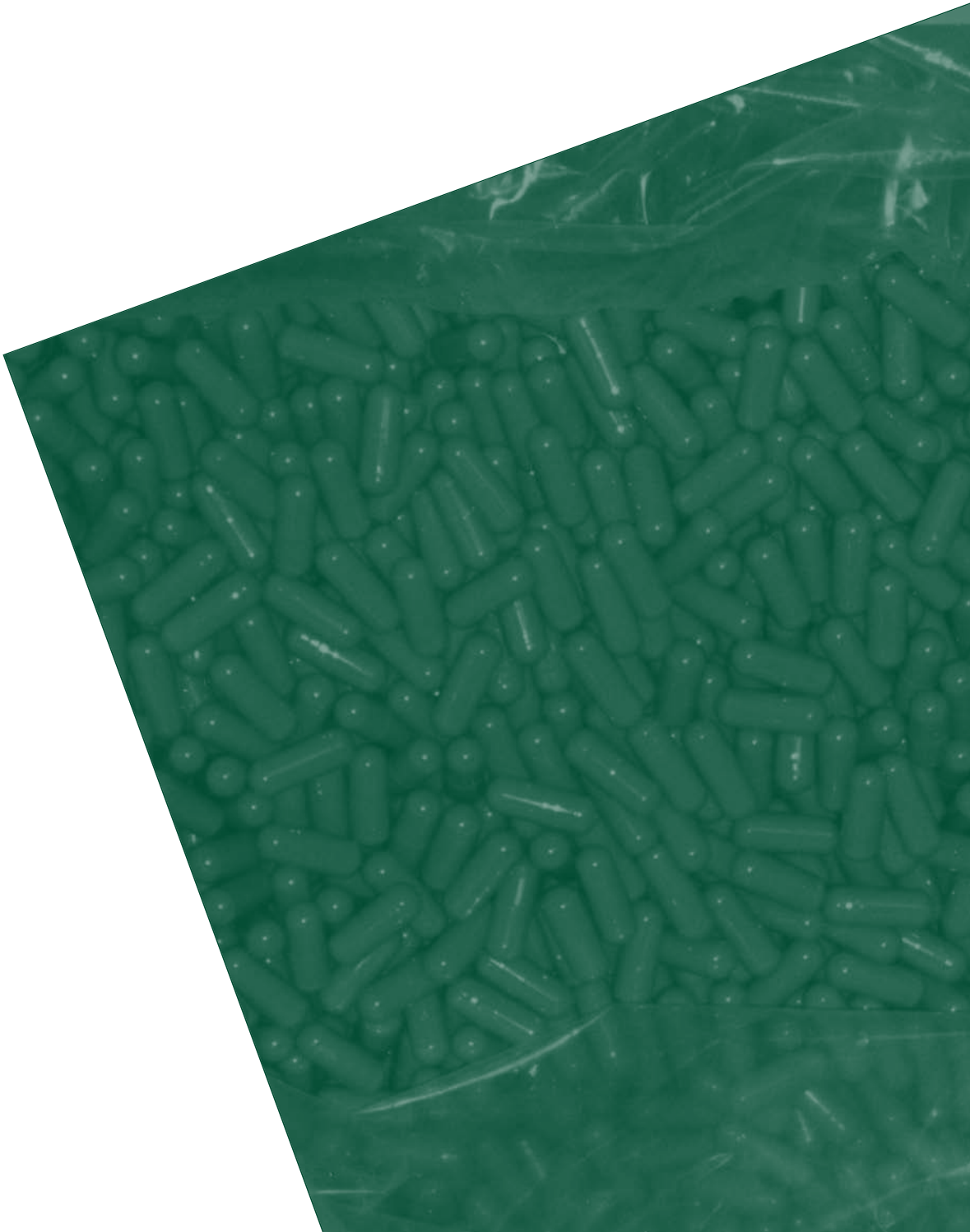
Oral cyanocobalamin supplementation in elderly people with cobalamin deficiency: a dose-finding trial

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ABSTRACT

Background: Supplementation with high doses of oral cobalamin are as effective as cobalamin administered by intra-muscular injection to correct plasma markers of cobalamin deficiency, but the effects of lower oral doses of cobalamin on such markers are uncertain.

Purpose: To determine the lowest oral dose of cobalamin required to normalise biochemical markers of cobalamin deficiency in older people with mild cobalamin deficiency, defined as serum cobalamin between 100 and 300 pmol/L and metthylmalonic acid (MMA) of 0.26 µmol/L or greater.

Design: A randomized, parallel group, double-blind dose-finding trial assessed the effects on biochemical markers for cobalamin deficiency of daily oral doses of 2.5, 100, 250, 500 and 1,000 µg of cobalamin administered for 16 weeks in 120 people.

Main outcome measure: The dose of oral cobalamin that produces 80% to 90% of the estimated maximal reduction in plasma MMA concentration.

Main findings: Supplementation with cobalamin in daily oral doses of 2.5, 100, 250, 500 and 1,000 µg were associated with mean reductions in plasma MMA concentrations of 16%, 16%, 23%, 33% and 33%, respectively. Daily doses of 647 µg to 1032 µg of cobalamin were associated with 80% to 90% of the estimated maximum reduction in plasma MMA concentration.

Conclusions: The lowest dose of oral cobalamin required to normalise mild cobalamin deficiency is over 200 hundred times greater than the recommended dietary allowance, which is about 3 µg daily.

Key words: cobalamin deficiency, methylmalonic acid, oral supplementation, dose finding, elderly

INTRODUCTION

Cobalamin deficiency, due to intrinsic factor deficiency, hypochlorhydria or food bound malabsorption, mainly affects older people.[1-3] Symptoms of cobalamin deficiency include anaemia, neuropathy or neuropsychiatric disorders, but more commonly lead to non-specific tiredness or malaise in older people.[3-5] Approximately 20% of the circulating plasma cobalamin is transported as holotranscobalamin (holoTC), which can be taken up by all cells, and the remaining 80% is transported as haptocorrin which is not believed to be metabolically active.[6, 7] In the cell, cobalamin acts as a cofactor for methionine synthase, an enzyme that remethylates homocysteine (Hcy) to methionine, and for methylmalonyl-CoA mutase, an enzyme that converts methylmalonyl-CoA to succinyl-CoA. In the setting of cobalamin deficiency, methylmalonyl-CoA is hydrolysed to methylmalonic acid (MMA). Thus, elevated plasma concentrations of MMA and total homocysteine (tHcy) can be used as biochemical markers to aid in the diagnosis of cobalamin deficiency and to monitor the response to cobalamin supplementation.[8, 9]

Active absorption of protein bound cobalamin in food is impaired in individuals with cobalamin deficiency, but approximately 1% of orally administered crystalline cobalamin is absorbed by passive diffusion.[3, 10] Consequently, cobalamin deficiency is usually treated by monthly intra-muscular injections of 1,000 μg hydroxy- or cyanocobalamin. However, daily dietary supplementation with 1,000 to 2,000 μg of cyanocobalamin administered orally has been shown to be as effective[11] or even more effective[12] as cobalamin administered by intramuscular injections to correct biochemical markers of cobalamin deficiency.[11, 12] Previous trials that examined the effects on biochemical markers of cobalamin status of daily dietary oral supplements ranging from 10 to 100 μg of cyanocobalamin were unable to determine the lowest effective dose required to correct cobalamin deficiency.[13, 14] A major knowledge gap concerns the lowest dose of oral cobalamin supplementation that would normalise elevated MMA concentrations. The aim of the present trial is to determine the lowest dose of cobalamin that is required for a maximal reduction in MMA concentrations in a randomised, parallel, double blind controlled dose-finding study in older people with mild cobalamin deficiency. The doses used cover the total spectrum from the RDA to the commonly used dose in cobalamin injections.

METHODS

Participants

Free-living older people aged 70 years or older were recruited in the Wageningen area of the Netherlands, and through a database of individuals who had previously indicated interest in participation in such a trial. Individuals with self-reported anaemia, surgery or diseases of the stomach or small intestine, or any life-threatening diseases were excluded, as were individuals who reported current use of multivitamin supplements containing folic acid, cobalamin, or pyridoxine hydrochloride and those currently receiving cobalamin injections. The concomitant medication known to affect cobalamin absorption (e.g. proton pump inhibitors, H2-antagonists, and metformin) was permitted if the medication had been provided at least 3 months prior to enrolment and was scheduled to be continued for the duration of the trial. Individuals who fulfilled the above criteria were invited to give a blood sample at a screening

visit. People were eligible for the trial if their serum cobalamin concentration was between 100 and 300 pmol/L, their plasma MMA concentration ≥ 0.26 µmol/L and their serum creatinine concentration ≤ 120 µmol/L, the latter reflecting normal kidney function.[3] Figure 1 shows the recruitment procedure and the flow of participants through the phases of the study. The study protocol was approved by the Medical Ethical Committee of Wageningen University and written informed consent from all participants was obtained before the screening visit.

Protocol

Eligible people who agreed to be enrolled in a 3 to 4 week placebo run-in period prior to randomisation and who had proven compliant (> 90% intake of capsules) during the run-in period were randomised to receive 16 weeks of treatment in a parallel group design with daily oral doses of 2.5, 100, 250, 500 or 1,000 µg cyanocobalamin (Figure 1).

Figure 1: Recruitment procedure and flow of participants during the study
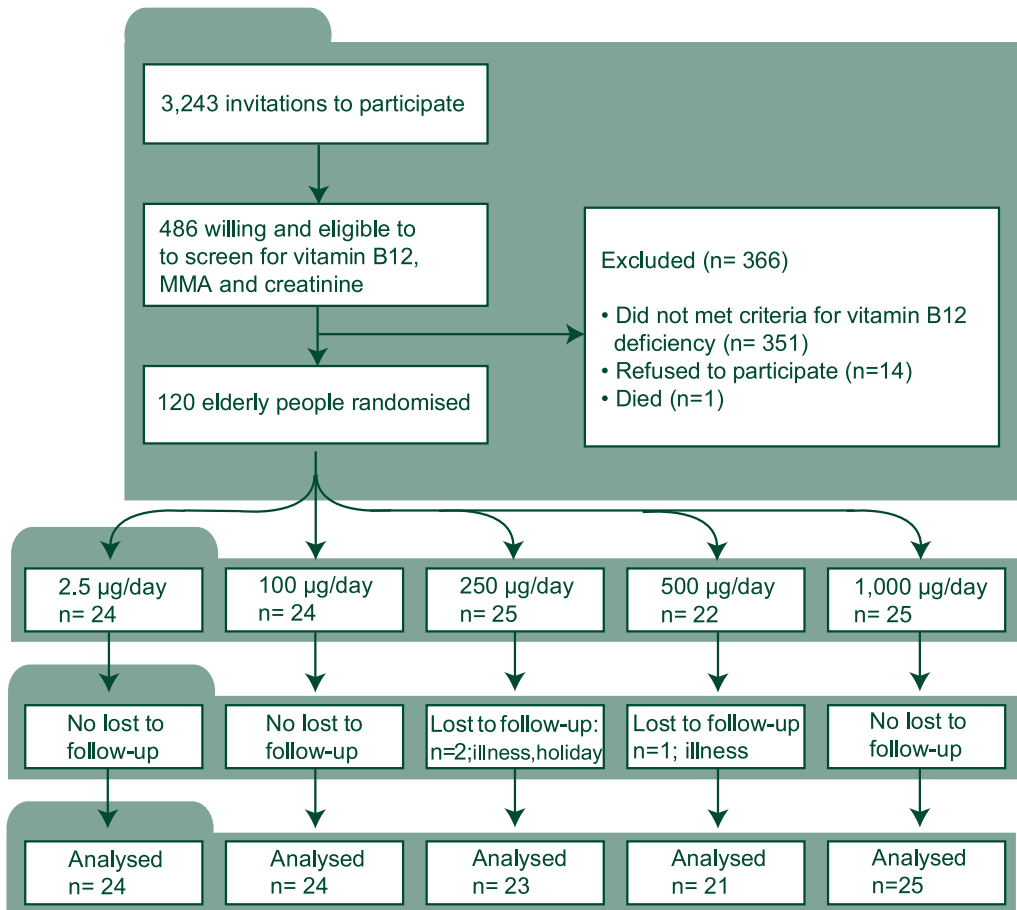
The doses selected for this study were based on the RDA of The Netherlands, which was 2.5 µg daily at the start of the trial, and 1,000 µg which served as a positive control and is administered in the form of intramuscular injections to treat cobalamin deficiency. The 100-, 250- and 500- µg doses were chosen to provide an optimum dose-response curve. We did not include a placebo in our study design because of ethical reasons.

Randomisation was based on plasma MMA concentration at the screening visit, age and sex. We used strata to ensure a balanced distribution of participants with respect to MMA ($0.26 \leq$ MMA ≤ 0.309, $0.31 \leq$ MMA ≤ 0.359, and MMA ≥ 0.36), age (≤ 75 and > 75 years) and sex. All investigators and participants were masked for study treatment.

Assuming a within-person SD for MMA of 0.25 µmol/L for changes in plasma MMA concentrations induced by cobalamin supplementation[15], sample size calculations indicated that 17 participants per group provided 80% power to detect an absolute difference of 0.22 µmol/L in MMA concentrations between the treated groups. In order to control for an estimated drop out rate of 23%[16], at least 20 participants were to be enrolled in each group.

Cobalamin was to be administered as cyanocobalamin in capsules that were identical in appearance, smell and taste among all treatment groups. The mean (SD) measured dose of cobalamin for the capsules intended to contain 2.5 µg, 100 µg, 250 µg, 500 µg and 1,000 µg were 3 (single pooled assessment), 112 (4.7), 270 (3.4), 553 (1.7) and 860 (9.7) µg, respectively.

Participants were asked to maintain their regular diet and to avoid use of supplements containing B-vitamins during the trial. All participants were asked to complete a diary to record their daily intake of capsules, their use of non-study medication, and the occurrence of any new illnesses during the trial. No adverse events were reported. Compliance was checked by counting unused capsules remaining in capsule dispensers and by verifying pill count in the participants' diaries. Mean compliance was 98% and since the compliance for each participant was greater than 90%, data for all participants were included in the analyses.

Data collection and analytical methods

A blood sample was collected at the screening and randomisation visits and after 8 and 16 weeks of active treatment. Height and weight were also measured at the randomisation visit. Participants were asked to be fasting at the randomisation visit, but were allowed to eat a light breakfast (without fruit, fruit juices, meat or eggs) at least one hour before attending for the screening and follow-up visits. The study was carried out between February 27, 2002 and February 28, 2003. A sample of blood for subsequent measurement of MMA (primary outcome measure), tHcy and holoTC (both secondary outcome measures), respectively, was collected in a 10-ml vacutainer containing EDTA. This blood sample was placed in ice water and centrifuged at 2600 rpm for 10 min at a temperature of 4 °C within 30 minutes of collection. All plasma samples were stored at -80 ºC prior to laboratory analyses. Plasma concentrations of MMA and tHcy were determined by gas chromatography – mass spectroscopy (GC-MS) after derivatization with methylchloroformate.[17] The plasma concentration of holoTC was measured by the

AXIS-Shield radioimmunoassay method.[18] A blood sample was collected in a 5 ml gel tube for measurement of serum cobalamin (secondary outcome measure) and creatinine concentrations. The serum samples for cobalamin determination were stored at room temperature in the dark for measurement later that day using the IMMULITEr 2000 cobalamin method.[19] In addition, at the randomisation visit, a sample of blood was collected in 5 ml evacuated tubes containing EDTA, stored at room temperature for measurement later that day of haematological parameters (haemoglobin, haematocrit, mean cell volume, hypersegmentation of neutrophils), and plasma folate concentrations.

Statistical analysis

Baseline concentrations of the biochemical parameters were calculated as the average of the measurements recorded at the screening and randomisation visits for each individual. The proportional changes in plasma concentrations of MMA, tHcy and holoTC and serum cobalamin were calculated by dividing each participant's absolute change in concentration after 16 weeks of treatment by their concentration at baseline. The lowest dose of oral cobalamin required to achieve a maximum reduction in MMA concentrations was determined using a 'closed test procedure'.[20] The Kruskall Wallis test was used to investigate whether differences in median proportional changes were present between dose groups, while the Mann Whitney U test was used to investigate between which two dose groups differences in the median changes occurred. In addition, curve fitting that plots the proportional reductions in MMA concentrations against the incremental doses of cobalamin was used to assess the dose-response relationship. The best fit dose-response curves showed a one phase exponential decay estimated by the following nonlinear regression equation: change (%) = (top – bottom) * exp (-k*cobalamin dose) + bottom.

This regression equation was used to identify the lowest oral dose of cyanocobalamin required to achieve a maximal reduction in MMA concentrations. This dose was defined as the dose that produces 80% to 90% of the maximum estimated reduction in plasma MMA concentrations. Statistical analyses were conducted using SAS statistical software (SAS Institute Inc., Cary, USA), and curve fitting was performed by GraphPad Prism (GraphPad Software Inc., San Diego, USA).

RESULTS

Characteristics of participants

Selected characteristics of the study participants are given in Table 1.

At baseline, the study population was on average not undernourished since the median body mass index (BMI) was 25.3 kg/m[2,21] There were no significant differences in the mean concentrations of MMA, tHcy, holoTC and cobalamin between the screening and the randomisation visits. The median baseline concentrations of serum cobalamin and of plasma MMA were well matched by treatment groups, indicating that the randomisation procedure had been successful. At baseline, serum cobalamin concentrations were correlated with plasma holoTC (ρ=0.53, p<0.0001), plasma MMA (ρ= -0.34, p=0.0002), and tHcy concentrations (ρ= -0.25, p=0.0056). Plasma holoTC concentrations were correlated with MMA (ρ= -0.41, p<0.0001) and plasma tHcy concentrations (ρ= -0.38 p<0.0001), while plasma tHcy concen-

trations were correlated with MMA concentrations (ρ= 0.85, p<0.0001), but not with folate concentrations (ρ= –0.01, p=0.91).

Table 1: Characteristics of the study population at baseline

	Prevalence	Median (IQR)*	Absolute range
Descriptive characteristics:			
Age (y)		80 (7)	64 - 94
Sex, % male	44%		
Use of medication†	53%		
Anemia‡	12%		
Macrocytosis §	6%		
Hypersegmentation ‖	57%		
Hypersegmentation ¶	7%		
BMI (kg/ m^2)		25.3 (4.6)	19.7 – 35.3
Folate (nmol/L)		6.4 (4.8)	1.1 – 18.8
Creatinine (µmol/L)		88 (20)	53 – 122
Biochemical characteristics:			
MMA (µmol/L)		0.33 (0.16)	0.23 – 5.16
tHcy (µmol/L)		14.5 (5.7)	7.8 – 114.0
holoTC (pmol/L)		47 (35)	8 – 121
Cobalamin (pmol/L)		208 (87)	113 – 362

*IQR=Q3–Q1. † Use of proton pump inhibitors, H2 antagonists or metformin. ‡ defined as Hb<8.1 mmol/L in males and <7.4 mmol/L in females. § defined as MCV>100 fl. ‖ defined as 5-lobed neutrophils/100 neutrophils. ¶ difined as 6 lobed neutrophils/100 neutrophils.

Absolute effects of different doses of cobalamin

On average, the absolute decreases in plasma MMA and tHcy concentrations and absolute increases in plasma cobalamin and holoTC concentrations increased with increasing doses of cyanocobalamin (Table 2). The reductions in MMA concentrations in all cobalamin-treated groups were significant during the first 8 weeks of treatment and remained stable during the second 8 weeks of treatment. The absolute reduction in MMA concentrations of at least 0.22 µmol/L observed after 8 and 16 weeks of supplementation with 500 and 1,000 µg of cobalamin supplementation indicated that the study had sufficient power to detect differences between the randomly allocated doses of cobalamin. In addition, the absolute effects of cobalamin supplementation on MMA concentrations were assessed using the proportion of the trial population that achieved an MMA concentration below the laboratory reference interval for MMA of 0.26 µmol/L (Personal communication, J Schneede). Daily supplementation of 2.5, 100, 250, 500 or 1,000 µg cobalamin resulted in reductions in MMA concentrations to below the reference interval of 0.26 µmol/L, in 21%, 38%, 52%, 62% and 76% of the participants, respectively.

Table 2: Concentrations of MMA, tHcy, HoloTC and cobalamin at 8 and 16 weeks, and absolute effects after 8 and 16 weeks of cyanocobalamin supplementation by intervention group

|	Dose*		8 weeks			16 weeks	
	(μg/day)	n	Median(IQR)†	Response (95% CI)	n	Median (IQR)†	Response (95% CI)
MMA	2.5	24	0.29 (0.13)	-0.04 (-0.06 to -0.02)	24	0.28 (0.07)	0.07 (-0.09 to -0.04)
(μmol/L)	100	24	0.29 (0.06)	-0.09 (-0.14 to -0.04)	24	0.30 (0.07)	0.08 (-0.13 to -0.03)
	250	25	0.25 (0.11)	-0.13 (-0.21 to -0.05)	23	0.28 (0.11)	0.14 (-0.22 to -0.05)
	500	22	0.25 (0.09)	-0.22 (-0.37 to -0.07)	21	0.26 (0.03)	0.23 (-0.37 to -0.08)
	1,000	25	0.26 (0.04)	-0.31 (-0.69 to 0.07)	25	0.25 (0.04)	0.34 (-0.74 to 0.06)
tHcy	2.5	24	14.4 (8.8)	-0.7 (-1.4 to -0.1)	24	14.0 (6.5)	-0.9 (-1.7 to -0.1)
(μmol/L)	100	24	13.7 (4.0)	-0.5 (-1.1 to -0.1)	24	13.6 (4.5)	-0.7 (-1.5 to 0.1)
	250	25	14.1 (5.3)	-1.0 (-1.7 to -0.3)	23	13.8 (6.1)	-1.4 (-2.4 to -0.4)
	500	22	13.8 (6.0)	-1.9 (-2.9 to -1.0)	21	13.1 (5.4)	-2.4 (-3.4 to -1.5)
	1,000	25	11.1 (4.2)	-5.1 (-11.3 to 1.1)	25	10.4 (5.1)	-5.7 (-13.1 to 1.7)
HoloTC	2.5	24	67 (43)	8 (2 to 15)	24	63 (40)	8 (3 to 14)
(pmol/L)	100	24	70 (43)	26 (16 to 35)	24	77 (38)	28 (19 to 38)
	250	25	85 (40)	40 (29 to 50)	23	94 (67)	48 (36 to 60)
	500	22	97 (39)	49 (39 to 59)	21	106 (48)	60 (49 to 71)
	1,000	25	127 (60)	67 (55 to 79)	25	132 (43)	73 (61 to 85)
Cobalamin	2.5	24	269 (81)	70 (29 to 88)	24	290 (119)	64 (20 to 74)
(pmol/L)	100	24	300 (98)	108 (72 to 145)	24	279 (184)	129 (74 to 183)
	250	25	327 (158)	128 (100 to 157)	23	347 (188)	153 (111 to 195)
	500	22	372 (47)	182 (141 to 224)	21	404 (293)	212 (149 to 274)
	1,000	25	449 (334)	248 (185 to 311)	25	574 (418)	328 (247 to 109)

*The treatment groups of 2, 5, 100, 250, 500 and 1.000 µg cobalamin contained on average 3, 112, 270, 553 and 860 µg cobalamin respectively. † IQR=Q3-Q1

Proportional effects of different doses of cobalamin

The determination of the lowest dose of cobalamin associated with the maximum reductions in MMA or maximum increases in holoTC using the closed test procedure[20] (which defined the optimum dose as that dose that differed significantly from the lower doses, but did not differ significantly from the higher doses) concluded that the intended dose of 500 μg/day of cobalamin was the lowest oral dose associated with a maximum reduction in MMA concentrations and a maximum increase in holoTC concentrations, respectively. The proportional reductions in MMA concentrations after daily supplementation with 2.5 μg, 100 μg, 250 μg and 500 μg cobalamin differed significantly from each other, whereas the proportional reductions in MMA concentrations did not differ significantly after supplementation with 500 μg and 1000 μg cobalamin (P=0.2).

The proportional decreases in MMA and tHcy, and proportional increases in cobalamin and holoTC concentrations observed with incremental doses of cobalamin after 16 weeks of supplementation are presented in Figure 2. The mean reduction in plasma MMA concentration after 16 weeks of supplementation compared with baseline varied from 16% to 33% in the groups receiving 2.5 μg/day to 1000 μg/day of cobalamin. The proportional reduction in MMA after 16 weeks supplementation was calculated by means of the following formula: "25.82*exp (-0.0018626* cobalamin dose) – 39.6". The lowest daily oral doses of cobalamin that resulted in an 80% to 90% of the maximum reduction in MMA varied between 647 to 1032 μg of cobalamin. On average, such doses of cobalamin reduced plasma MMA concentrations by approximately 33%.

Figure 2: Proportional effects of different doses of cobalamin on MMA, tHcy, holoTC and cobalamin concentrations after 16 weeks of supplementation. Error bars represent SD.
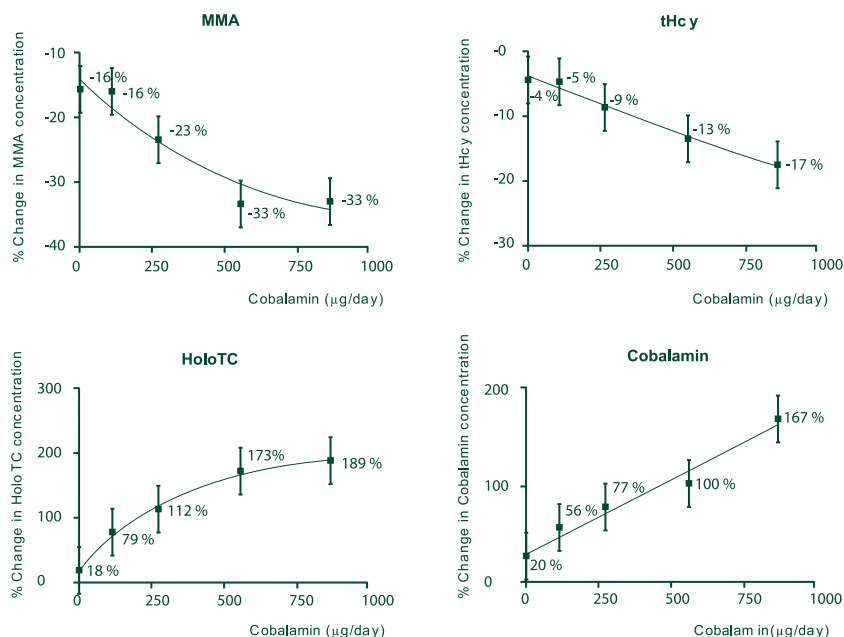
DISCUSSION

The results of this dose-finding trial demonstrate that the lowest oral dose of cyanocobalamin associated with 80% to 90% of the estimated maximum reduction in plasma MMA concentration in an elderly population with mild cobalamin deficiency varied from 647 to 1032 µg daily, and such doses reduce plasma MMA concentrations by approximately 33%. However, daily doses of 2.5 to 250 µg cyanocobalamin produce statistically significant reductions in MMA concentrations of 16% to 23% in this population. The conclusions of this trial are based primarily on reductions in plasma MMA concentrations because MMA reflects tissue levels of cobalamin.[3, 9, 12]

Comparable proportional increases in concentrations of serum cobalamin and plasma holoTC were observed in response to the different doses of cyanocobalamin. The dose-finding curve for holoTC demonstrated that daily oral doses of 527 to 759 µg of cobalamin resulted in an 80% to 90% increase of the estimated maximum increase in holoTC concentrations.

In contrast to the dose-finding curves for MMA and holoTC, the dose-finding curve for Hcy does not show a plateau. This finding may be related to the selection criteria, which did not include tHcy, since tHcy is not a specific marker of cobalamin status, but is also affected by folate status and a variety of life-style factors.[22] Most likely, tHcy concentrations in these participants are less responsive to cobalamin supplementation. Therefore, we cannot assume that, based on our data, a full dose-response curve can be fitted for tHcy.

The conclusions of this trial may reflect the definition of cobalamin deficiency and the variable absorption of cobalamin in older people. The diagnosis of cobalamin deficiency is complicated by the limitations of current assay techniques because serum cobalamin concentrations alone may misclassify a significant proportion of individuals with cobalamin deficiency. [3, 9, 23] Moreover, there is no consensus about the cut-off points for cobalamin deficiency or metabolites to define cobalamin deficiency. The present trial enrolled healthy older people with mild cobalamin deficiency defined as serum cobalamin between 100 and 300 pmol/L in combination with plasma MMA of 0.26 µmol/L or greater in individuals without renal dysfunction. Analysis of a sub-group of participants with more severe cobalamin deficiency (using MMA concentrations of 0.32 µmol/L or greater at baseline, present in 67 participants) resulted in more pronounced changes in MMA, tHcy, HoloTC and cobalamin concentrations, and confirmed the results of closed test procedure (Data not shown). According to the corresponding dose-finding curves for MMA and HoloTC, 830 µg/d would provide 80% of the maximal reduction in MMA, and 449 µg/d would provide 80% of the maximal increase of HoloTC.

Cobalamin can be absorbed actively with a limited capacity of about 3 µg per meal in the presence of intrinsic factor, and a normal function of the stomach, pancreas and terminal ileum. However, the bioavailability of crystalline cobalamin is unaffected by the underlying causes of cobalamin deficiency and about 1% of crystalline cobalamin (typically used in oral cobalamin supplements) is absorbed by passive absorption.[3] This study was unable to distinguish the extent to which differences in individual responses were due to active as opposed to passive absorption of cobalamin.

The results of this trial differ from the results of Seal et al who compared the effects on serum cobala-

min and tHcy concentrations of oral cyanocobalamin using daily oral doses of 10 to 50 μg or placebo for 4 weeks in 31 older people who had a pre-treatment cobalamin concentration between 100 and 150 pmol/L. Seal et al showed that supplementation with 50 μg/day increased serum cobalamin, but it had no significant effects on tHcy concentrations.[13] Rajan et al compared the effects of sequential daily treatment with 25, 100, and 1,000 μg of cyanocobalamin for six weeks on serum cobalamin and plasma MMA concentrations in 23 elderly people who had a pre-treatment cobalamin concentration less than 221 pmol/L in combination with MMA concentration greater than 0.27 μmol/L. Rajan et al reported that daily treatment with 25 μg or 100 μg lowered, but did not normalize, MMA concentrations and a daily dose of 1,000 μg of cobalamin was required to normalise MMA concentrations.[14]

The results of this trial indicate that the lowest dose of oral cobalamin required to normalise biochemical markers of mild cobalamin deficiency in older people with a mild cobalamin deficiency is more than 200 times greater than the Recommended Dietary Allowance for cobalamin of approximately 3 μg/day. Clinical trials are currently assessing the effects of high doses of oral cobalamin on markers of cognitive function and depression. If such trials can demonstrate that the reported associations of cobalamin deficiency with cognitive impairment or depression are causal and reversible by treatment[24], the relevance of correction of cobalamin deficiency in older people could be substantial. However, the present trial demonstrates that much higher doses of cobalamin are required to normalise cobalamin deficiency than were previously believed.

ACKNOWLEDGEMENTS

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Changes in markers of cobalamin status after cessation of oral B-vitamin supplements in elderly with mild cobalamin deficiency

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ABSTRACT

Objective: To monitor early changes in markers of cobalamin status and to compare the sensitivity of different markers for cobalamin status after cessation of oral supplementation.

Design, subjects, and intervention: Participants aged 70 years or older with mild cobalamin deficiency were treated daily for 6 months with a capsule containing either 1,000 µg cobalamin (group C, n=34), a combination of 1,000 µg cobalamin with 400 µg folic acid (group CF, n=31) or placebo (n=30). Participants provided one single blood sample at 3, 5 or 7 months after cessation of study supplements to determine concentrations of cobalamin, holotranscobalamin (holoTC), and methylmalonic acid (MMA) after cessation.

Results: Cobalamin status was assumed to be replete at the end of the 6 month supplementation period. The pooled intervention groups (group C + CF) indicate that serum cobalamin declined by 43% and holoTC by 55% within the first 3 months after cessation, with no significant further decline thereafter. Within the same period, mean MMA increased by 15% (P = 0.07) within the first 5 months, and by 50% (P = 0.002) after 7 months, thereby approaching the baseline concentration of 0.40 µmol/L.

Conclusions: After cessation of a 6 month daily oral cobalamin supplementation period, there is a parallel decrease of serum cobalamin and holoTC concentrations. These decreases precede the attainment of tissue cobalamin depletion, as measured by increase in MMA concentrations. Oral supplementation may afford adequate cobalamin status for a period of up to 5 months after cessation.

Key words: cobalamin deficiency, cessation of oral supplementation, elderly people

INTRODUCTION

Cobalamin deficiency is common in elderly people and results from either the inability to release cobalamin from food proteins (food malabsorption), intestinal malabsorption, or inadequate intake.[1-4] Cobalamin deficiency causes anemia, as well as a variety of neuropsychiatric symptoms, including myelopathy, which may become irreversible within 12 months after onset.[5, 6] Therefore, early diagnosis and treatment of cobalamin deficiency are of major importance.

Increased concentrations of methylmalonic acid (MMA) and total homocysteine (tHcy) in plasma or serum[6] and decreased concentrations of cobalamin are established as useful diagnostic indicators of cobalamin deficiency. Recently, reduced holo-transcobalamin (holoTC) concentration has been proposed as a new test for cobalamin deficiency, since it is believed to represent the biologically active fraction of cobalamin in blood.[7, 8]
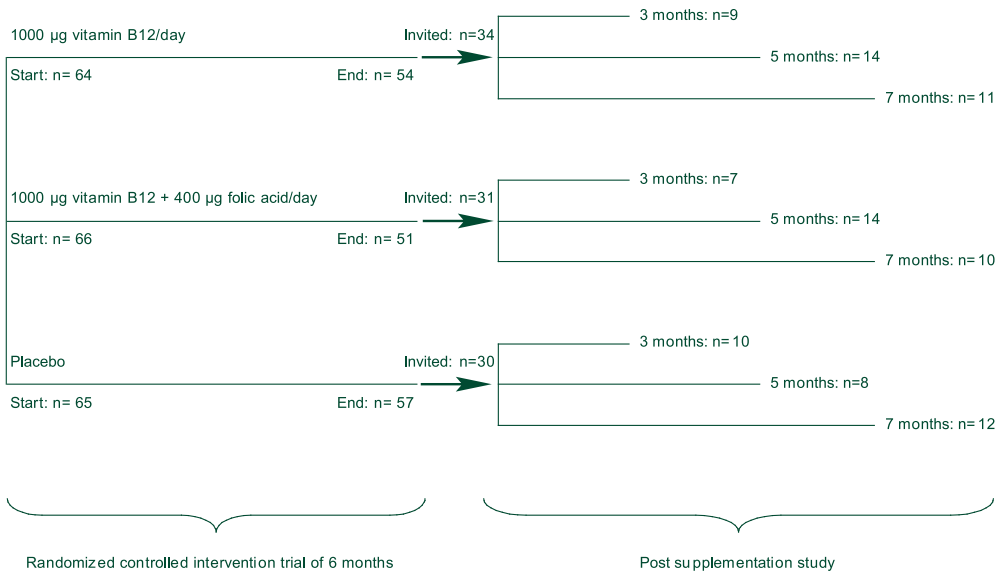
We recently conducted a dose-finding trial, investigating the normalization of markers of cobalamin status during 4 months oral cobalamin treatment in elderly subjects with biochemical evidence of mild deficiency. A daily dose of 650 to 1,000 µg/day crystalline cyanocobalamin was required to correct biochemical signs of mild cobalamin deficiency.[9] However, little is known about the duration of the effects of oral treatment with cobalamin in elderly people with mild cobalamin deficiency. Monitoring cobalamin markers after cessation may provide this valuable information. In the present study, we therefore investigated the changes in markers for cobalamin status after cessation of oral cobalamin supplementation in participants treated with 1000 µg/day. This gives a unique opportunity to compare the sensitivity of different markers for cobalamin status by monitoring early changes in markers after cessation of supplementation, during a period in which participants gradually attain a negative cobalamin balance.

MATERIALS AND METHODS

Protocol

The present study is a follow up study that measures markers for cobalamin status after completion of a randomized placebo controlled intervention trial. This intervention trial investigated the effects of cobalamin supplementation of cognitive performance.[10] During the intervention study, participants were treated daily for 6 months with a capsule containing either 1,000 µg cobalamin (group C), a combination of 1,000 µg cobalamin and 400 µg folic acid (group CF) or placebo (group P). A number of participants were invited to provide one single blood sample after either 3, 5 or 7 months after they stopped taking the study supplements (follow-up). Figure 1 presents the flow of participants during the intervention trial and the present study. Both these studies were approved by the Medical Ethics Committee of Wageningen University, and written informed consent was obtained from all participants.

Figure 1: Flow of participants during the intervention trial[10] and the present trial

1000 µg vitamin B12/day   Invited: n=34   3 months: n=9

Start: n= 64   End: n= 54   5 months: n= 14

7 months: n= 11

1000 µg vitamin B12 + 400 µg folic acid/day   Invited: n=31   3 months: n=7

Start: n= 66   End: n= 51   5 months: n= 14

7 months: n= 10

Placebo   Invited: n=30   3 months: n= 10

Start: n= 65   End: n= 57   5 months: n=8

7 months: n= 12

Randomized controlled intervention trial of 6 months   Post supplementation study

Participants

Free-living older people and older people living in care facility homes who were 70 years or older and fulfilled criteria for mild cobalamin deficiency were enrolled in the intervention trial. Mild cobalamin deficiency was defined as either serum cobalamin concentrations between 100 and 200 pmol/L, or as serum cobalamin concentrations between 200 and 300 pmol/L in combination with plasma MMA concentrations ≥ 0.32 µmol/L.[11] Participants had serum creatinine concentration ≤ 120 µmol/L to exclude severe impairment of renal function.[3]

Blood collection

Blood samples were collected at the start of the intervention (baseline), after 6 months of intervention, and during follow-up. Blood samples for measurement of holoTC, MMA and tHcy were collected into a 10 ml Vacutainer® tube containing EDTA. This blood sample was placed in ice water and centrifuged at 2600 rpm for 10 min at a temperature of 4 °C within 30 minutes of collection. All plasma samples were stored at –80 ºC prior to laboratory analyses. Plasma concentrations of MMA were determined by a Liquid Chromatography Electro Spray Ionisation Tandem Mass Spectrometry (LC-ESI-MS/MS) system (H.J.B., oral communication, July 28, 2005). Plasma tHcy concentrations were determined by a method based on methylchloroformate derivatization and gas chromatography-mass spectrometry[12], and plasma holoTC was measured using the AXIS-Shield radioimmunoassay method.[13] A second blood sample was

taken into a 5 ml gel tube for measurement of cobalamin and creatinine. The serum samples for cobalamin determination were stored at room temperature in the dark for measurement later that day using the IMMULITE 2000 cobalamin method.[14] A third blood sample was collected into a 5 ml Vacutainer® tube containing EDTA, and stored between 4 and 8 °C to determine red blood cell (RBC) folate at the same day of blood collection. Analytical coefficients of variation (CV) of the assays for cobalamin, MMA, holoTC, and RBC folate were 6.3%, < 2.2%, < 2.2%, 12% and 5.9%, respectively.

Statistical methods

Changes in concentrations during supplementation, and during the follow-up period of 3-7 months, were calculated as proportional changes relative to concentrations before or relative to concentrations at the end of supplement use. Differences between absolute concentrations of vitamins or markers at the start and end of supplement use, and differences between concentrations at follow-up vs. start or end of supplement use were analyzed with a paired Student's t-test. The intervention groups (C, CF) given cobalamin did not differ with respect to cobalamin, holoTC and MMA at either the start or end of supplement use (by unpaired t-tests). Therefore, these data were pooled, and proportional changes at follow-up relative to the end of supplement use were compared using one-way analysis of variance (ANOVA) with Tukey post hoc tests. These changes were considered to be significant at the 0.05 level. Statistical analyses were conducted using SAS statistical software (version 9.1; SAS Institute Inc., Cary, USA), and the graph was prepared using GraphPad Prism (version 4; GraphPad Software Inc., San Diego, USA).

RESULTS

The mean (SD) age of the study population was 82 (6) years, 22% were males and 52% lived in a care facility home. Compliance of capsule intake during supplementation period was 99%. No adverse effects were reported.

In the intervention groups (C, CF) at the end of supplementation, mean serum cobalamin had increased by 195% (C) and 226%, whereas holoTC had increased by 286% (C) and 369%. MMA was reduced by 39% and 36% in group C and group CF, respectively. Concentrations of serum cobalamin and holoTC were still elevated by 35% and MMA still reduced by 30% after 7 months of follow up (Table). These proportional changes are relative to baseline concentrations.

As expected, only participants treated with cobalamin plus folic acid, showed substantially increased concentrations RBC folate and reduced plasma tHcy during supplementation, and these changes were partly maintained during follow-up. In the placebo group, mean concentrations of markers for cobalamin status (cobalamin, holoTC, MMA, and tHcy) remained stable throughout the supplementation period and during follow-up (Table).

Table: Concentrations of vitamins and metabolites during and after supplementation in older people with mild cobalamin deficiency[a]

Marker	6 month supplementation period		After cessation of supplementation		
	Start [b]	End	3 months	5 months	7 months
Supplement group C:	cobalamin				
	n=34	n=34	n=9	n=14	n=11
Cobalamin	190 ± 61	527 ± 214[c]	311 ± 99[c,d]	235 ± 48[c,d]	275 ± 107[c,d]
pmol/L (%)		(195 ± 109)	(49 ± 23)	(47 ± 39)	(35 ± 20)
HoloTC	60 ± 21	221 ± 124[c]	97 ± 32[c,d]	74 ± 28[c,d]	88 ± 36[c,d]
pmol/L		(286 ± 176)	(76 ± 60)	(41 ± 56)	(35 ± 26)
MMA	0.39 ± 0.15	0.22 ± 0.06[c]	0.24 ± 0.06[c]	0.22 ± 0.05[c]	0.33 ± 0.12[c,d]
µmol/L (%)		(-39 ± 16)	(-33 ± 13)	(-28 ± 20)	(-29 ± 20)
tHcy	15.0 ± 4.3	12.6 ± 3.5[c]	14.5 ± 3.9[d]	14.1 ± 4.0[d]	13.9 ± 3.6
µmol/L (%)		(-15 ± 17)	(-9 ± 13)	(3 ± 21)	(-7 ± 15)
RBC Folate	557 ± 159	749 ± 326[c]	532 ± 130[d]	622 ± 289[d]	435 ± 217[c,d]
nmol/L (%)		(35 ± 30)	(-5 ± 13)	(10 ± 26)	(-21 ± 28)
Supplement group CF: cobalamin plus folic acid					
	n=31	n=31	n=7	n=14	n=10
Cobalamin	199 ± 40	622 ± 200[c]	374 ± 136[c,d]	294 ± 25[c,d]	259 ±41[c,d]
pmol/L (%)		(226 ± 110)	(86 ± 47)	(48 ± 28)	(35 ± 19)
HoloTC	65 ± 26	285 ± 192[c]	107 ± 34[c,d]	99 ± 33[c,d]	72 ± 27[c]
pmol/L		(369 ± 255)	(45 ± 50)	(60 ± 44)	(37 ± 45)
MMA	0.41 ± 0.22	0.24 ± 0.1[c]	0.25 ± 0.08[c]	0.27 ± 0.12[c]	0.33 ± 0.17[c,d]
µmol/L (%)		(-36 ± 21)	(-34 ± 7)	(-23 ± 15)	(-31 ± 15)
tHcy	14.9 ± 5.0	9.1 ± 2.5[c]	11.8 ± 4.2[c,d]	13.4 ± 3.9[c,d]	12.0 ± 2.0[c,d]
µmol/L (%)		(-38 ± 12)	(-26 ± 10)	(-10 ± 15)	(-4 ± 22)
RBC Folate	570 ± 210	1448 ± 431[c]	788 ± 194[c,d]	771 ± 97[c,d]	533 ± 251[d]
nmol/L (%)		(173 ± 81)	(57 ± 28)	(43 ± 25)	(-11 ± 40)

Table continued

Supplement group P: placebo					
	n=30	n=30	n=10	n=8	n=12
Cobalamin	176 ± 47	160 ± 45	171 ± 45	162 ± 49	175 ± 59
pmol/L (%)		(-9 ± 11)	(-9 ± 26)	(-18 ± 31)	(-4 ± 27)
HoloTC	66 ± 28	56 ± 25	56 ± 24	57 ± 28	60 ± 27
pmol/L		(-6 ± 26)	(-5 ± 21)	(3 ± 36)	(-8 ± 13)
MMA	0.45 ± 0.23	0.48 ± 0.25	0.46 ± 0.24	0.32 ± 0.05	0.55 ± 0.41
µmol/L (%)		(7 ± 1 8)	(2 ± 26)	(0 ± 20)	(-3 ± 34)
tHcy	17.3 ± 5.6	18.1 ± 8.5	19.7 ± 7.6	18.1 ± 8.1	17.4 ± 5.6
µmol/L (%)		(6 ± 2 0)	(-2 ± 14)	(17 ± 23)	(9 ± 17)
RBC Folate	609 ± 277	629 ± 261	451 ± 192 [c]	553 ± 170 [d]	477 ± 320 [c,d]
nmol/L (%)		(5 ± 2 5)	(-17 ± 18)	(-8 ± 27)	(-30 ± 25)

[a] Concentrations, mean ± SD, are given in units or as proportions (%) relative the concentrations at start of supplementation.

[b] No significant differences between participants who provided a blood sample at 3, 5 or 7 months after cessation, except for MMA in group C, Tukey's post-hoc test (P<0.05).

[c] Significantly different from start of supplement use, paired Student's t test (P<0.05)

[d] Significantly different from end of supplement use, paired Student's t test (P<0.05)

Data of the pooled intervention groups (C, CF) are presented as proportional changes during follow up relative to the concentrations at the end of intervention (Figure 2). Serum cobalamin declined by 43% and holoTC by 55% within the first 3 months of follow- up with no significant further decline thereafter. Notably, in the same period, mean MMA increased marginally by 15% (paired t-test, P = 0.07) within the first 5 months, and markedly by 50% (P=0.002), i.e. to 0.33 µmol/L, after 7 months (Figure 2), thereby approaching the baseline concentration of 0.40 µmol/L (Table).

Figure 2: Changes in markers of cobalamin status after cessation of oral vitamin B12 supplementation [a,b]
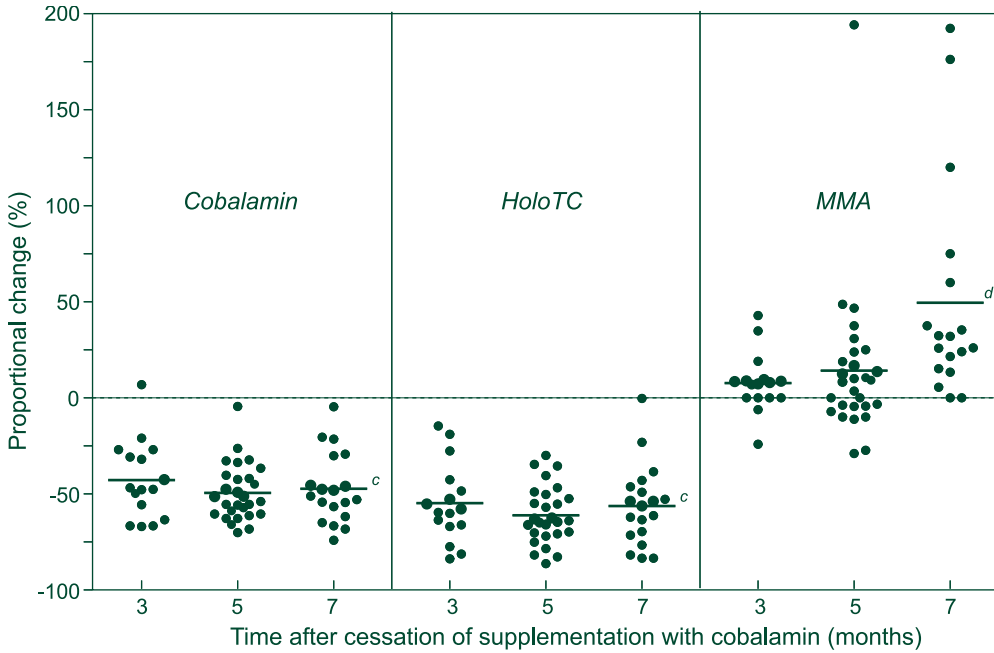
[a] Data are given as proportional changes relative to concentrations at the end of 6 months of supplement use

[b] Significant changes, except for MMA at 3 and 5 months after cessation, paired Student's t test (P<0.05)

[c] No significant differences between 3, 5 and 7 months after cessation, Tukey's post-hoc test (P=0.48 for cobalamin and P=0.50 for holoTC).

[d] Change at 7 months significantly different from changes at 3 and 5 months after cessation, Tukey's post-hoc test (P<0.01).

DISCUSSION

The present study was designed "a posteriori", and conducted after completion of an efficacy trial investigating the effects of oral cobalamin supplementation on cognitive performance in participants with mild cobalamin deficiency.[10] Unfortunately, we were not able to monitor longitudinal changes in individual cases after cessation of supplementation, and some subgroups were rather small for logistics reasons. Despite these weaknesses of our study, it is possible to evaluate the relative sensitivity of the different cobalamin markers by monitoring their early changes after cessation of supplementation when participants gradually attain a negative cobalamin balance. Moreover, this is the first study so far that allows assessment of the duration of treatment effects after oral cobalamin supplementation in patients with signs of mild cobalamin deficiency.

The most important findings from the present follow-up study were the relatively rapid fall of serum cobalamin and holoTC concentrations after cessation of cobalamin supplementation within the first 3 months after cessation, whereas MMA showed a marked increase between month 5 and 7 (Table and Figure 2). Moreover, the parallel time courses for cobalamin and holoTC during follow-up (Figure 2) suggest that these cobalamin fractions are equally sensitive to detect the recurrence of a negative cobalamin balance. The significant increase in MMA to concentrations above normal already 7 months after cessation indicates that a tissue deficiency may be re-established within this time frame.

Concentrations of MMA and tHcy decreased within the first 3 months of supplementation and then remained stable between the 4th and 6th month of supplementation (data not shown). We therefore assume that cobalamin stores were replete at the end of the supplementation period. The duration of treatment effects may depend on the severity of the deficiency prior to supplementation. One published investigation in elderly people without cobalamin deficiency demonstrated that cobalamin returned to pre-treatment concentrations and MMA and tHcy were still slightly reduced 9 months after injection with high doses of cobalamin, folic acid and vitamin B6.[15] Another study indicated that after treatment serum cobalamin clearance in patients with pernicious anaemia was higher than clearance in vegans and people with an adequate cobalamin status. This can be explained by impaired re-absorption of biliary cobalamin due to absence of intrinsic factor in pernicious anaemia.[16]

In conclusion, after cessation of daily oral supplementation with 1,000 µg cobalamin with or without additional 400 µg folic acid during 6 months, there is a parallel decrease of serum cobalamin and holoTC concentrations. The decline precedes the attainment of tissue cobalamin depletion, as measured by increase in MMA concentrations. Oral supplementation may afford an adequate cobalamin status for a period of up to 5 months after cessation. After that, a negative cobalamin balance may occur. We therefore suggest that biannual monitoring of cobalamin status is warranted in cobalamin deficient elderly who stop taking regular cobalamin supplementation.
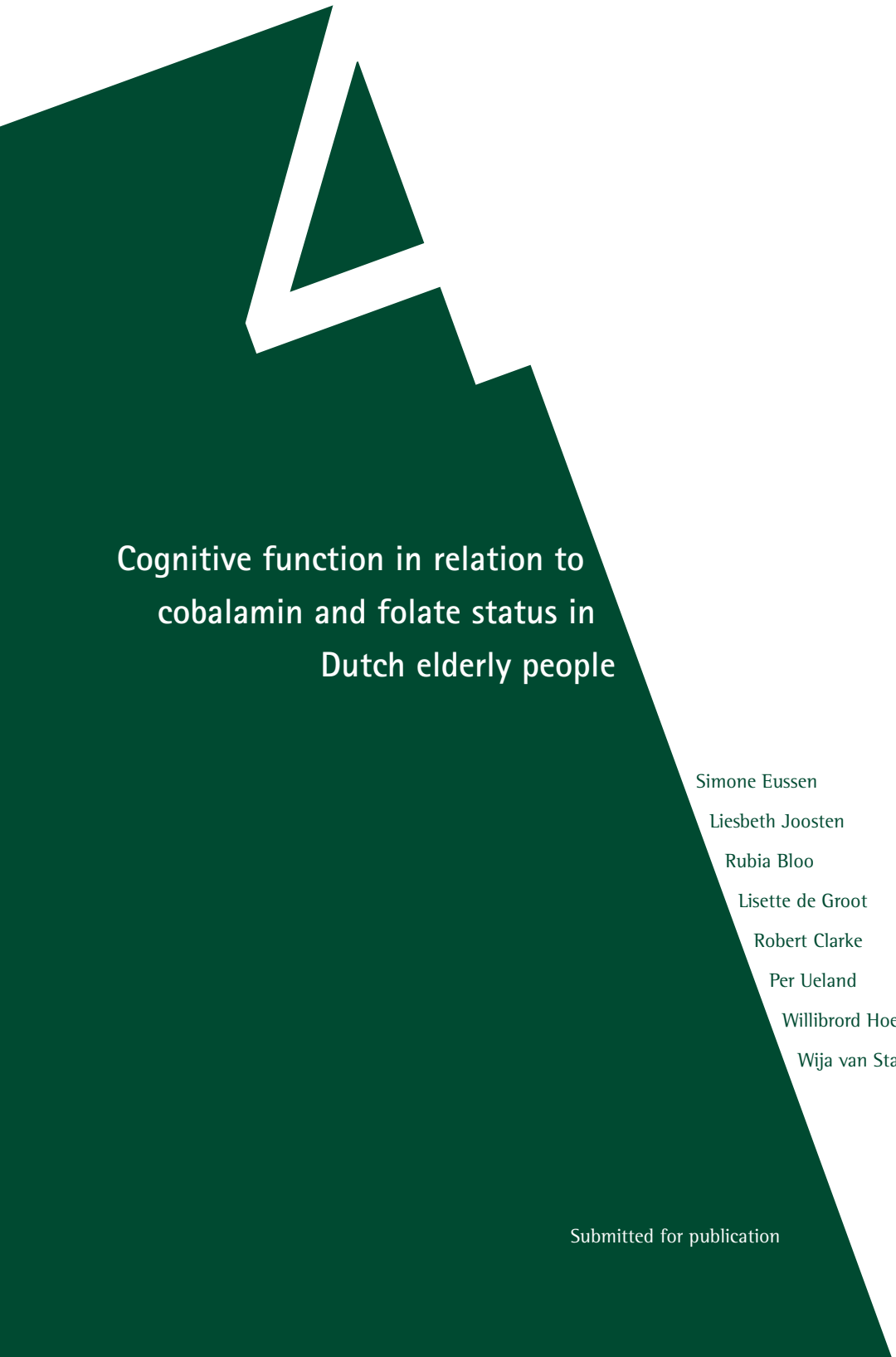
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Cognitive function in relation to cobalamin and folate status in Dutch elderly people

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ABSTRACT

The associations of different markers of cobalamin status, as measured by cobalamin, holotranscobalamin (HoloTC), methylmalonic acid (MMA), and total homocysteine (tHcy); and of folate status, as measured by tHcy and red blood cell (RBC) folate, were assessed in relation to different cognitive domains. Cross-sectional analysis, adjusted for age, education and interviewers in 242 people aged > 70 years revealed significant tests for trend (P for trend < 0.05) for cobalamin with sensomotor speed and executive function, holoTC with sensomotor speed, MMA with attention, tHcy with construction, sensomotor speed and executive function, and RBC folate with attention, sensomotor speed, memory and executive function. These results suggest that impaired folate and cobalamin status were associated with impairments of some cognitive domains, but there was no obvious pattern to distinguish these from each other.

Key words: elderly, cobalamin and folate status, cognitive performance

INTRODUCTION

Cognitive performance declines with increasing age, resulting in an exponential increase in dementia with age. Evidence from observational and experimental studies suggests an association between markers of cobalamin and folate status with cognitive impairment.[1-3] The association between cobalamin status and cognitive performance was first recognized in 1855 when Addison described pernicious anaemia and noted that "the mind occasionally wandered".[4] Later on, in addition to causing anemia, cobalamin deficiency has been linked with a variety of neuropsychiatric symptoms, such as neuropathy, myelopathy, dementia, depression, memory impairment, and cerebrovascular diseases.[5-7]

While prolonged cobalamin deficiency may eventually cause irreversible neurological damage and cognitive impairment[8, 9,] early stages of cobalamin deficiency may result in milder forms of cognitive impairment in the absence of anemia.[10, 11] Milder forms of cobalamin deficiency can be diagnosed by increased concentrations of plasma total homocysteine (tHcy) and methylmalonic acid (MMA)[6] and decreased concentrations of holotranscobalamin (holoTC).[12] Elevated concentrations of MMA and tHcy have been linked to mechanisms by which cobalamin deficiency may lead to neurological damage and cognitive dysfunction.[13, 14] Furthermore, cobalamin and folate play a role in the maintenance of the blood brain barrier (BBB)[15] and one carbon metabolism.[16]

The published data[17-29] on the associations between different markers for cobalamin and folate status and cognitive performance are inconsistent. However, various B-vitamin markers and neuropsychological test batteries have been used in these studies, which may explain the discrepancies. We therefore investigated whether associations between cobalamin and folate status were confined to some cognitive domains by using sensitive markers for cobalamin and folate status and an extensive neuropsychological test battery.

METHODS

Participants

Data from 242 Dutch men and women aged 70 years and older were included in the present cross sectional analyses. The study investigates the associations between markers for cobalamin and folate status in elderly people with no to moderate cognitive impairment and a cobalamin status ranging from an adequate status to moderate deficiency. Among the 242 elderly included in the present trial, 202 elderly started with a run-in period of a randomized controlled trial to investigate the efficacy of oral cobalamin with or without folic acid supplements on cognitive performance.[30] Main inclusion criterion for the intervention trial was mild cobalamin deficiency, which was defined as either serum cobalamin concentrations between 100 and 200 pmol/L, or as serum cobalamin concentrations between 200 and 300 pmol/L in combination with plasma MMA concentrations ≥ 0.32 µmol/L. Participants had serum creatinine concentration ≤ 120 µmol/L to exclude severe impairment of renal function.[31] Another 40 elderly people with an adequate cobalamin status performed the cognitive test battery and were included in the present study. Exclusion criteria for all individuals were history of cobalamin deficiency, use of

cobalamin (> 50 μg/day) or folic acid (> 200 μg/day) supplementation or injections, surgery or diseases of the stomach or small intestine, anemia, life-threatening diseases, severe hearing or visual problems, and severe cognitive impairment, defined as a score < 19 points on the Mini-Mental State Examination (MMSE). The Medical Ethical Committee of Wageningen University approved the study protocol. Daily boards and client councils of those individuals living in an institution gave their consent and written informed consent from all participants was obtained before blood collection.

Data collection

Blood collection

Blood samples were collected during a screening visit, which took place between April 2003 and March 2004. Cobalamin status was assessed by serum cobalamin concentrations, and plasma concentrations of MMA, tHcy and holoTC. Folate status was assessed by plasma concentrations of tHcy and red blood cell (RBC) folate concentrations. Participants were allowed to eat a light breakfast (without fruit, fruit juices, meat or eggs) at least one hour before blood collection. A sample of blood for subsequent measurement of MMA, tHcy and holoTC was collected into a 10 ml Vacutainer® tube containing EDTA. This blood sample was placed in ice water and centrifuged at 2600 rpm for 10 min at a temperature of 4 °C within 30 minutes of collection. All plasma samples were stored at –80 ºC prior to laboratory analyses. Plasma concentrations of MMA were determined by a Liquid Chromatography Electro Spray Ionisation Tandem Mass Spectrometry (LC-ESI-MS/MS) system (H.J.B., oral communication, July 28, 2005). Plasma tHcy concentrations were determined by a method based on methylchloroformate derivatization and gas chromatography-mass spectrometry[32], and plasma holoTC was measured using the AXIS-Shield radioimmunoassay method.[33] A second blood sample was taken into a 5 ml gel tube for measurement of serum cobalamin and creatinine. The serum samples for cobalamin determination were stored at room temperature in the dark for measurement later that day, using the IMMULITE 2000 cobalamin method[34]. A third blood sample was collected into a 5 ml Vacutainer® tube containing EDTA, and stored between 4 and 8 °C to determine red blood cell folate at the same day of blood collection. The interassay coefficient of variation (CV) for serum cobalamin, RBC folate, and plasma concentrations of MMA, tHcy and holoTC were 6.3%, 5.9%, 5%, < 2.2% and 12% respectively.

Medical history, life style and anthropometry

The questionnaire provided self-reported information on medical history and issues related to cobalamin status and cognitive function.[35] Individuals were asked to indicate "yes" or "no" to questions about history or presence of myocardial infarction, coronary bypass, stroke, transient ischemic attack, angina pectoris, diabetes mellitus, and hypertension, use of medication, subjective memory and depressive complaints, in addition to questions on smoking, alcohol consumption and diet (vegetarian or vegan). Education was classified as ''low'' (i.e. less than primary school or primary school), "intermediate" (i.e. less than low vocational training, low vocational training or mean vocational training) or "high" (i.e. high vocational training or university level). Body height and weight were measured in a standing position.

Cognitive performance

Cognitive performance was assessed during a 1.5 to 2 -hour session by six trained and registered neuropsychologists. A Mini-Mental State Examination (MMSE)[36] score < 19 points was used to exclude individuals with severe cognitive impairment. To describe the study population, the MMSE, Clinical Dementia Rating (CDR) Scale[37] and Geriatric Depression Scale (GDS)[38] were used. The CDR classified the study population into participants with no cognitive impairment (CDR=0), mild cognitive impairment (MCI; CDR=0.5), moderate cognitive impairment (CDR=1), or severe cognitive impairment (CDR=2).[39] The test battery consisted of tests that have been indicated to be sensitive to the effects of B-vitamin treatment and aging in previous studies[2, 40] The Finger Tapping Test[41] and Motor Planning Test[41] are computerised tasks in which measurements are obtained form a six-button panel, containing one red button and five white target buttons, laid out in a 180 ° arc, all at 6 cm distance from the red button. The participant is requested to press the red button as quickly and often as possible during 30 seconds (Finger Tapping), and afterwards to press the white button immediately adjacent (clockwise) to a white lit button, instead of the lid button itself (Motor Planning). Participants are asked to copy, draw immediate after copying (immediate recall, maximum score 36 points), and draw 30 minutes after copying (delayed recall, maximum score 36 points) the Complex Figure of Rey.[42] With the 15 Word Learning Task[43], a list of 15 words is read 5 times to the participant, and in between the participant is asked to recall as many words as possible (immediate recall, maximum score 75 points). After 30 minutes, the participant is asked to recall as many words as possible (delayed recall, maximum score 15 points). This was followed by reading a list of 30 words to the participant who has to indicate the 15 words read out originally (recognition, maximum score 30 points). In the Trailmaking Test[44], pseudo randomly placed circles with numbers (Trailmaking A), and with both letters and numbers (Trailmaking B) have to be connected with a line as fast as possible in a fixed order (score, seconds needed to complete the task). In the Digit Span, a subtest of the Wechsler Adult Intelligence Scale[45], participant are asked to repeat a string of digits in the original order (digit span forward, maximum score 8 points) and in reverse order (digit span backward, maximum score 7 points). Ravens' Coloured Progressive Matrices46 consist of 24 figures. The principle on which a figurative matrix is constructed can be deduced from the design of the figure that is presented to the participant (maximum score 24 points). In the Stroop[47], participants are asked to read the name of colours (red, green, yellow, blue) (Stroop 1; score, seconds needed to complete the task), naming colour blocks (Stroop 2; score, seconds needed to complete the task), and naming the colour of the ink rather than the word (Stroop 3; score, seconds needed to complete the task). Within the WAIS[48], participants are asked to mention similarities between 5 pairs of nouns. Finally, the Verbal Fluency test[49] requests to list as many animals (Fluency Animal) and nouns (Fluency Letter) as possible within 2 minutes (score, number of items mentioned). All these single tests were clustered into domains of attention, construction, sensomotor speed, memory and executive function, as indicated in the statistical methods. Since cognitive status can be influenced by depression[50], the presence of depression (defined as a ≥ 5 out of 15 points) was assessed by the Geriatric Depression Scale (GDS).

Statistical methods

Generally, higher individual neuropsychological tests scores indicate a better cognitive performance, except for tests that include measures of speed and some tests of executive function. Higher scores on speed related tasks reflect more time needed to complete a task and thus lower performance. In addition, higher scores on stroop test (part 3/part 2) and the trail making tests (part C/part A) reflect lower concept shifting and interference abilities. In order to achieve consistency in interpretation of results, we multiplied crude test scores with -1 of those tests of which higher scores indicated lower performance. The association between individual neuropsychological tests and the vitamin markers were assessed by partial spearman rank correlation coefficients, which were adjusted for age and education. Individual neuropsychological tests were clustered into the neuropsychological domains of attention, construction, sensomotor speed, memory, and executive function to reduce chance findings and to facilitate the interpretation of the cognitive data. The domains of attention and construction were assessed by one cognitive test, while the other domains were assessed by multiple tests. All crude test scores were transformed to z-scores, i.e. z-score = (individual result – mean result at baseline)/SD at baseline) and the multiple tests for the domains of sensomotor speed, memory, and executive function were clustered to compound z-scores: Sensomotor speed = $(-Z_{(Motor\ Planning(2))} + -Z_{(finger\ tapping)} + -Z_{(trail\ making,\ part\ A)}/3$; Memory = $(Z_{15WordLearning,\ immediate} + Z_{15WordLearning,\ delayed} + Z_{15WordLearning,\ recognition} + Z_{Rey,\ immediate} + Z_{Rey,\ delayed} + Z_{DigitSpan\ backward})/6$; Executive function = $(-Z_{(Motor\ Planning(3))} + -Z_{(Trail\ Making\ (partC/partA)} + -Z_{(Stroop(part3/part2)} + Z_{Similarities\ (WAIS)} + Z_{Raven} + Z_{WordFluency(Animals)} + Z_{WordFluency(Letter))}/7$. The Digit Span Forward test, a measure for attention, and the copy of the Complex Figure of Rey, a measure for construction, were not included in the compound measures. However, for comparison we transformed these crude scores into z-scores as well: Attention = $Z_{Digit\ Span\ Forward}$; Construction = $Z_{Rey,\ copy}$. Some participants were unable to complete all tests because of performance difficulties, e.g. tiredness. Compound z-scores were calculated when data for at least 2, 4, and 5 tests for the domains of sensomotor speed, memory and executive function respectively were available.

To examine differences in cognitive performance between individuals with relatively unfavorable, intermediate, and favorable concentrations of biochemical markers, we calculated tertile categories for biochemical markers. These analyses were conducted by using the general linear models procedure in which each tertile variable was defined as a class variable. For those with a significant test for trend (across median concentrations for each tertile), least squares means were compared across tertile categories with Tukey's adjustment for multiple comparisons. In addition to crude analysis, models were adjusted for age, education, and random effects of neuropsychologists, and then for other possible confounders. Possible confounders were depression, smoking (current, past or never), alcohol consumption (social drinking yes or no), living situation (free-living or institutionalized) and co-morbidity factors including stroke, transient ischemic attack, angina pectoris, diabetes mellitus, and hypertension. All analyses were conducted using SAS statistical software (version 9.1; SAS Institute Inc., Cary, USA) and the graph was made using GraphPad Prism (version 4; GraphPad Software Inc., San Diego, USA).

RESULTS

Characteristics of participants

Table 1 shows demographic, lifestyle, co-morbidity data, and neuropsychological characteristics of all participants. The mean (SD) age was 81 (6) years, 26% were males and 40% lived in a care facility home. The proportion of self perceived memory impairment (53%) and self perceived depression (26%) was higher than the proportion as measured by the MMSE, CDR and GDS. Cognitive impairment was present in 16% of the study population according to the MMSE, and in 35% according to the CDR.

Table 1: Characteristics of Dutch elderly people who participated in neuropsychological assessment (n=242)

Demography	Age, mean (SD) years	81 (6)
	Sex, Male, n (%)	63 (26)
	Living, institutionalised, n (%)	96 (40)
	Education; low/ intermediate/ high, n (%)	84 (35)/ 109 (45)/ 49 (20)
Lifestyle	Ex-smokers/ Smokers, n (%)	66 (27)/ 20 (8)
	Social drinking, n (%)	93 (38)
	Vegetarian, n (%)	10 (4)
	Multivitamin use, n (%)	42 (17)
Medical history	Myocardial infarction, n (%)	30 (12)
	Stroke, n (%)	10 (4)
	Transient ischemic attack, n (%)	40 (17)
	Angina Pectoris, n (%)	38 (16)
	Diabetes Mellitus, n (%)	22 (9)
	Hypertension, n (%)	60 (25)
	H_2 antagonist/proton pump inhibitors, n (%)	50 (21)
Neuropsychological symptoms	MMSE, mean (SD)	27.0 ± 3.1
	19 - 24 points (cognitive impairment), n (%)	38 (16)
	GDS, mean (SD)	3.0 ± 2.9
	> 5 points (depression), n (%)	47 (19)

Cognitive test battery

As expected, cognitive tests that have been clustered within each domain correlated well with each other. Spearman rank correlation coefficients varied from 0.43 to 0.50 (all P values <0.0001) within the domain of sensomotor speed, from 0.37 to 0.82 (all P values <0.0001) within the domain of memory, and from 0.28 to 0.57 (all P values <0.0001) in the domain of executive function. Moreover, the compound z-scores for the five domains also correlated well with each other (all P-values < 0.0002).

Associations of cobalamin and folate status with individual neuropsychological tests

Table 2 shows strong associations between age and education level with cognitive performance and concentrations of vitamin markers. Therefore, we adjusted partial spearman rank correlation coefficients for age and education level. Table 2 shows that all vitamin markers, except RBC folate, were associated with the mini-mental state examination (MMSE), a measurement of global cognitive performance. More specifically, partial rank correlation coefficients revealed positive correlations of cobalamin, holoTC and RBC folate with individual specific neuropsychological tests. In line with this, MMA and tHcy were negatively correlated with the tests. MMA, tHcy and RBC folate were correlated with more neuropsychological tests than cobalamin and holoTC were. The strongest correlations were observed for tHcy and RBC folate in the domains of sensomotor speed, memory, and executive function.

Associations of cobalamin and folate status with global and specific compound cognitive domains

All vitamin markers revealed a significant test for trend with MMSE through tertile categories of vitamin markers (all P for trends < 0.001). However, after adjustment for age, education, and neuropsychologists, tests for trend remained significant for cobalamin (P for trend 0.002) and holoTC (P for trend 0.039), and borderline with MMA (P for trend 0.057).

From the 242 participants, data of 222, 237, and 234 participants were included for analysis on the domains of respectively sensomotor speed, memory, and executive function because some participants were unable to complete all tests. Table 3 presents crude cognitive performance for tertile categories of vitamin markers, and shows significant tests for trend for all vitamin markers with cognitive domains, except for cobalamin and holoTC with attention. However, after adjustment for age, education, and random effects of neuropsychologists, tests for trend were attenuated, as shown in Figure 1. After adjustment for these variables, elderly with cobalamin concentrations in the 1st tertile (< 171 pmol/L) had lower scores on the domains of sensomotor speed and executive function compared to elderly with concentrations in the 3rd tertile (> 218 pmol/L). The mean differences in z-scores between these tertile categories were 0.33 (95% CI: 0.09 to 0.58) for sensomotor speed and 0.21 (95% CI: 0.03 to 0.38) for executive function. Although a significant test for trend was observed for holoTC with sensomotor speed, there was no significant difference between the lowest and highest tertile. Those individuals with tHcy concentrations in the 3rd tertile (> 15.1 μmol/L) had lower scores on the domains of construction and sensomotor speed compared to those with concentrations in the 1st tertile (< 11.3 μmol/L). The mean differences in z-scores between these tertiles were 0.39 (95% CI: 0.09 to 0.69) for construction,

and 0.32 (95% CI: 0.07 to 0.57) for sensomotor speed.

Elderly with RBC folate concentrations in the 1st tertile (< 521 nmol/L) showed lower scores on the domains of attention, sensomotor speed, memory and executive function compared to those with concentrations in the 3rd tertile (> 707 nmol/L), although the difference between these tertile categories was not significant for the domain of sensomotor speed. The mean differences in z-scores between 1st and 3rd tertile were 0.57 (95% CI: 0.26 to 0.89) for attention; 0.28 (95% CI: 0.06 to 0.50) for memory; and 0.32 (95% CI: 0.15 to 0.50) for executive function. Only the associations of tHcy with construction, and

Table2: Spearman correlation coefficients of age and education with vitamin markers and cognitive tests, and partial spearman rank correlation coefficients (adjusted for age and education) between cognitive tests and vitamin markers

Domain	Neuropsychological test	N	Age	Education	Cobalamin	HoloTC	MMA	tHcy	folate
			Demography		Vitamin Markers				
Global	Mini Mental State Examination	242	-0.37**	0.39**	0.16**	0.12*	-0.11*	-0.12*	0.09
Attention	Digit Span Forward	238	-0.20**	0.25**	-0.05	0.00	-0.12*	-0.12*	0.19**
Construction	Figure of Rey – copy	236	-0.27**	0.30**	0.09	0.07	-0.08	-0.18**	0.08
Sensomotor	Finger Tapping	222	-0.35**	0.33**	0.13*	0.08	0.00	-0.25**	0.24**
speed	Motor Planning_2	221	-0.41**	0.17**	0.11*	0.07	-0.05	-0.18**	0.12*
	Trail Making A	236	-0.33**	0.17**	0.11*	0.14**	0.00	-0.15**	0.11*
	Compound speed	222	-0.46**	0.30**	0.15*	0.12*	-0.04	-0.26**	0.21**
Memory	Figure of Rey - immediate recall	235	-0.34**	0.25**	0.02	0.08	-0.11*	-0.04	0.10
	15 Word Learning –immediate	237	-0.40**	0.30**	0.09	0.08	-0.11*	-0.03	0.10
	Digit Span Backward	239	-0.23**	0.38**	0.12*	0.06	-0.11*	-0.13**	0.12*
	Figure of Rey - delayed recall	233	-0.35**	0.24**	0.03	0.06	-0.13**	-0.07	0.13**
	15 Word Learning - delayed recall	235	-0.35**	0.20**	0.07	0.10	-0.15**	-0.04	0.16**
	15 Word Learning – recognition	235	-0.35**	0.22**	0.07	0.07	-0.09	-0.03	0.07
	Compound memory	237	-0.41**	0.32**	0.08	0.08	-0.14**	-0.07	0.15**
Executive	Motor Planning_3	213	-0.41**	0.17**	0.10	0.07	-0.12*	-0.12*	0.15**
function	Trail Making (A/B)	227	-0.26**	0.26**	0.05	0.00	-0.02	0.04	0.05
	Raven	234	-0.33**	0.43**	0.16**	0.12*	-0.08	-0.09	0.14**
	Stroop (part3/part2)	209	-0.18**	0.28**	0.12*	0.12*	0.03	-0.07	0.28**
	Similarities (WAIS)	236	-0.25**	0.53**	0.18**	0.18**	-0.13**	-0.05	0.15**
	Verbal Fluency, letter	235	-0.20**	0.37**	0.10*	0.08	-0.09	-0.22**	0.14**
	Verbal Fluency, animal	236	-0.30**	0.26**	0.10	0.05	-0.12*	-0.18**	0.17**
	Compound executive function	233	-0.39**	0.48**	0.16**	0.13*	-0.11**	-0.14**	0.24**
Age		242			-0.22**	-0.16**	0.27**	0.35**	-0.22**
Education		242			0.15**	0.17**	-0.21**	-0.13**	0.18**

* P < 0.10, ** P < 0.05

RBC folate with attention, memory and executive function remained statistically significant after further adjustment for depression, smoking, alcohol consumption, living situation, and co-morbidity factors such as stroke, transient ischemic attack, angina pectoris, diabetes mellitus, and hypertension.

The cut-off points for the tertiles of cobalamin were 171 and 218 pmol/L; for HoloTC they were 52 and 76 pmol/L; for MMA they were 0.31 and 0.41 µmol/L; for tHcy they were 11.3 and 15.1 µmol/L; and for RBC folate they were 521 and 707 nmol/L. P values indicate significant tests for trend across median concentrations for each tertile, corrected for age, education and neuropsychologist.

Table 3: Crude cognitive performance (mean z-score ± se) by tertile categories for markers for cobalamin and folate status in Dutch elderly people.

		Cobalamin	HoloTC	MMA	tHcy	RBC folate
Attention	Tertile 1	-0.046 ± 0.114	-0.159 ± 0.110	0.203 ± 0.111	0.159 ± 0.111	-0.318 ± 0.107
	Tertile 2	0.044 ± 0.112	0.194 ± 0.114	0.061 ± 0.111	0.086 ± 0.111	-0.061 ± 0.109
	Tertile 3	0.000 ± 0.112	-0.023 ± 0.109	-0.254 ± 0.109	-0.244 ± 0.111	0.377 ± 0.107
	P for trend	0.830	0.566	0.003	0.008	< 0.001
Construction	Tertile 1	-0.348 ± 0.112	-0.220 ± 0.111	0.186 ± 0.112	0.284 ± 0.108	-0.281 ± 0.109
	Tertile 2	0.160 ± 0.108	0.047 ± 0.114	-0.006 ± 0.112	0.052 ± 0.109	0.059 ± 0.112
	Tertile 3	0.166 ± 0.108	0.167 ± 0.109	-0.178 ± 0.111	-0.339 ± 0.109	0.227 ± 0.110
	P for trend	0.004	0.018	0.023	< 0.001	0.002
Sensomotor speed	Tertile 1	-0.276 ± 0.089	-0.164 ± 0.089	0.121 ± 0.091	0.335 ± 0.085	-0.257 ± 0.086
	Tertile 2	0.036 ± 0.088	-0.028 ± 0.092	0.041 ± 0.093	-0.040 ± 0.088	0.052 ± 0.092
	Tertile 3	0.261 ± 0.088	0.216 ± 0.089	0.129 ± 0.089	-0.288 ± 0.087	0.245 ± 0.088
	P for trend	< 0.001	0.002	0.045	< 0.001	< 0.001
Memory	Tertile 1	-0.253 ± 0.088	-0.217 ± 0.087	0.128 ± 0.086	0.210 ± 0.087	-0.299 ± 0.085
	Tertile 2	0.096 ± 0.086	0.122 ± 0.089	0.209 ± 0.086	-0.046 ± 0.088	0.059 ± 0.087
	Tertile 3	0.158 ± 0.087	0.110 ± 0.086	-0.315 ± 0.084	-0.154 ± 0.088	0.252 ± 0.085
	P for trend	0.002	0.017	< 0.001	0.005	< 0.001
Executive function	Tertile 1	-0.304 ± 0.078	-0.162 ± 0.079	0.166 ± 0.077	0.208 ± 0.076	-0.344 ± 0.073
	Tertile 2	0.062 ± 0.074	-0.011 ± 0.080	0.054 ± 0.078	0.003 ± 0.077	0.049 ± 0.075
	Tertile 3	0.207 ± 0.075	0.147 ± 0.076	0.232 ± 0.077	0.231 ± 0.077	0.280 ± 0.073
	P for trend	< 0.001	0.005	< 0.001	< 0.001	< 0.001

The cut-off points for the tertiles of cobalamin were 171 and 218 pmol/L; for HoloTC they were 52 and 76 pmol/L; for MMA they were 0.31 and 0.41 µmol/L; for tHcy they were 11.3 and 15.1 µmol/L; and for RBC folate they were 521 and 707 nmol/L. P values indicate significant tests for trend across median concentrations for each tertile.

Figure 1: Cognitive performance (mean z-score) in Dutch elderly people by tertile categories for markers for cobalamin and folate status, adjusted for age, education, and neuropsychologists.
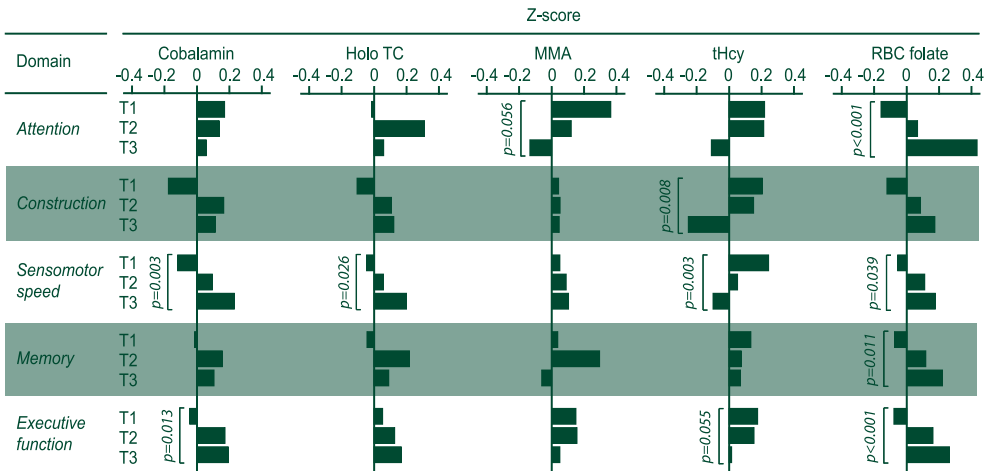

The cut-off points for the tertiles of cobalamin were 171 and 218 pmol/L; for HoloTC they were 52 and 76 pmol/L; for MMA they were 0.31 and 0.41 µmol/L; for tHcy they were 11.3 and 15.1 µmol/L; and for RBC folate they were 521 and 707 nmol/L. P values indicate significant tests for trend across median concentrations for each tertile, corrected for age, education and neuropsychologist.

DISCUSSION

The present cross-sectional study reveals associations for cobalamin with sensomotor speed and executive function, holoTC with sensomotor speed, MMA with attention, tHcy with construction, sensomotor speed and executive function, and RBC folate with attention, sensomotor speed, memory and executive function after adjustment for age, education and neuropsychologist.

These cross sectional associations were found in a population without severe cognitive impairment. Other cross sectional studies in elderly without severe cognitive impairment, using a limited range of neuropsychological tests, found comparable associations.[17-22] Although the present study did not reveal associations with low cobalamin concentrations in the domains of construction and memory, other studies did show associations with low cobalamin status and lower abstract thinking[17], lower episodic memory[18], and lower spatial copying skills.[19, 20] Other studies showed that the effects of low folate concentrations on episodic memory skills[18] and high tHcy concentrations on spatial copying skills[19, 20] were stronger than the effects of low cobalamin concentration on these neuropsychological functions. These findings are in line with results of the present study. Elevated concentrations of MMA previously have been associated with lower information processing speed, memory, verbal fluency and nonverbal reasoning[21], whereas the present study did not reveal associations of MMA with attention and memory

after adjustment for age, education and neuropychologists. Furthermore, elevated concentrations of tHcy predicted lower performance on tasks of simple motor and psychomotor speed, verbal memory and verbal learning.[22] These results are partially in line with the present study which indicated associations with construction and sensomotor speed.

Also in severely cognitively impaired elderly[23-28], impaired cobalamin status has been related to dementia. Low cobalamin concentrations have been associated with lower scores on the MMSE[23, 24] and behavioral and psychological symptoms of dementia.[24-26] In contrast, other studies indicate no associations of dementia with B-vitamins and tHcy[27], or only associations with low RBC folate, but not with cobalamin or tHcy.[28]

Results of the present study and previous cross-sectional studies suggest a role of cobalamin and folate status in neurpsychological performance. Both low cobalamin and folate concentrations conferred a double risk of developing Alzheimer's disease, particularly in persons with baseline MMSE scores > 26 points[51], whereas other prospective studies indicate that only low folate concentrations[20, 52], or neither of the vitamins nor tHcy[53] predict the rate of cognitive decline. Furthermore, predictive effects of tHcy on cognitive decline have mainly been observed in the age category of 60 to 80 years.[54-56] Another prospective study revealed that high cobalamin intake was associated with slower cognitive decline among the oldest participants, whereas high folate intake from food and from vitamin supplementation (> 400 µg/day) unexpectedly predicted a faster rate of cognitive decline.[57] Thus, results from studies on the prediction of cognitive performance by B-vitamin status and intake are inconclusive. Small non-randomized and placebo controlled trials[9, 40, 58-60] showed beneficial effects of cobalamin treatment on cognitive performance, but evidence for the effects of cobalamin supplementation on cognitive function from randomized trials is limited and inconclusive.[61-63]

Comparison of study results is hampered by differences in study methodology, including study population, biochemical markers of cobalamin and folate status, and neuropsychological tests utilized. The test battery of the present study varied not only with the abilities assessed, but also with relative task difficulties. Attention and sensomotor speed are relatively simple cognitive abilities, whereas construction, memory and executive function involve complex cognitive abilities. To our knowledge, only one study investigated the profile of cognitive impairment associated with low vitamin concentrations.[29] Their study indicated that cobalamin and folate concentrations were only associated with more complex neuropsychological tests[29], in contrast to the present study which also observed associations with the relatively simple cognitive domains of attention and sensomotor speed.

In summary, this study shows that unfavorable concentrations of cobalamin, holoTC, MMA, tHcy and RBC folate are associated with lower performance in some of the cognitive domains of attention, construction, sensomotor speed, memory, and executive function. These results suggest that impaired folate and cobalamin status were associated with impairments of some cognitive domains, but there was no obvious pattern to distinguish these from each other.

ACKNOWLEDGEMENTS

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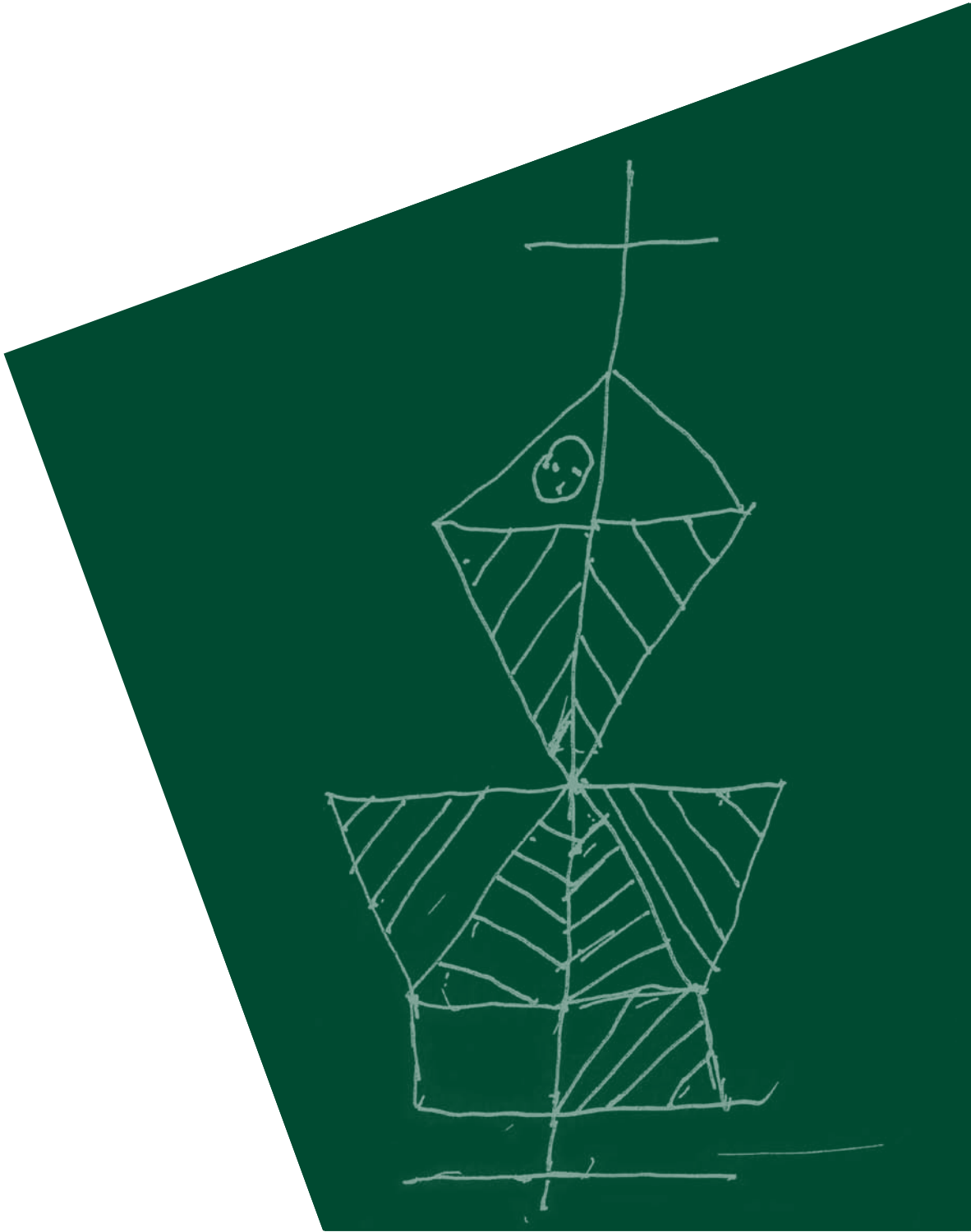
Effect of oral cobalamin with or without folic acid on cognitive function in older people with mild cobalamin deficiency: a randomized, placebo-controlled trial

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ABSTRACT:

Background: Cobalamin deficiency is associated with cognitive impairment in older people. However, randomized evidence for the effects of cobalamin supplementation on cognitive function is limited and inconclusive.

Objective: To investigate whether daily supplementation with high doses of oral cobalamin alone or in combination with folic acid has any beneficial effects on cognitive function in people aged 70 years or older with mild cobalamin deficiency.

Design: In a double-blind, placebo-controlled trial, 195 individuals were randomized to receive either 1,000 µg cobalamin, or 1,000 µg cobalamin plus 400 µg folic acid, or placebo for 24 weeks. Cobalamin status was assessed using methylmalonic acid, total homocysteine (tHcy) and holotranscobalamin (holoTC) before and after 12 and 24 weeks of treatment. Cognitive function was assessed before and after 24 weeks of treatment using an extensive neuropsychological test battery that included the domains of attention, construction, sensomotor speed, memory and executive function.

Results: Cobalamin status was unchanged after treatment in the placebo group, and oral cobalamin supplementation corrected mild cobalamin deficiency. Cobalamin plus folic acid supplementation increased red blood cell folate concentrations and reduced tHcy concentrations by 36%. Improvement in memory function was greater in the placebo group than in the group who received cobalamin alone (P = 0.0036). Neither supplementation with cobalamin alone or in combination with folic acid was accompanied by any improvement in other cognitive domains.

Conclusions: Oral supplementation with cobalamin alone or in combination with folic acid for 24 weeks was not associated with any improvement in cognitive function.

Key words: elderly, cobalamin deficiency, oral supplementation, cognitive function

INTRODUCTION

Cobalamin deficiency is common in older people and results from either the inability to release cobalamin from food proteins (food malabsorption) or intestinal malabsorption, or inadequate intake.[1-3] Cobalamin is involved in the one-carbon metabolism where it plays a role in the transfer of methyl groups and methylation reactions that are important for the synthesis and metabolism of neurotransmitters and phospholipids in the central nervous system.[4] Moreover, cobalamin is also required for nucleic acid synthesis and hematopoiesis[3], and the metabolism of fatty acids and amino acids in the mitochondrial citric acid cycle [5]. In addition to causing anemia, cobalamin deficiency has been linked with several neurological disorders, such as neuropathy, myelopathy, dementia, depression, memory impairment, and cerebrovascular disease.[6, 7] Although prolonged cobalamin deficiency may eventually result in irreversible neurological damage and cognitive impairment[8, 9], early stages of cobalamin deficiency -detected by increased concentrations of plasma total homocysteine (tHcy) and methylmalonic acid [7] and decreased concentrations of holotranscobalamin (holoTC)[10] - may result in milder forms of cognitive impairment in the absence of anemia.[11, 12]

Several cross-sectional and prospective studies in both healthy and cognitively impaired older people have reported associations between impaired cobalamin status and cognitive function.[13-15] Intervention trials of cobalamin supplementation and cognitive function have been performed[9, 16-26], of which only three were randomized placebo controlled.[23-25] The results of these trials were inconclusive, possibly because of variations in study duration, sample size, characteristics of study population, diagnosis and treatment of cobalamin deficiency, and assessment of cognitive function. It is possible that beneficial effects of cobalamin supplementation on cognition may be related to the duration and severity of cognitive impairment.[8, 27] For example, Martin et al postulated that there is limited time frame to reverse milder forms of cognitive impairment by cobalamin treatment in older people.[9]

In a recent dose-finding study in older people to determine the minimum effective dosage for oral cobalamin supplementation to correct mild cobalamin deficiency, we found that a daily dose of 650 to 1,000 µg/day was required to correct biochemical signs of impaired cobalamin status.[28] The aim of the present trial was to investigate the effects of oral cobalamin supplementation alone or in combination with folic acid for 24 weeks on cognitive function in older people with mild cobalamin deficiency with none to moderate cognitive impairment.

SUBJECTS AND METHODS

Recruitment and eligibility of participants

Free-living older people and older people living in care facility homes aged 70 years or older were recruited from different parts of The Netherlands via mailed health questionnaires. Individuals were excluded if they reported a history of cobalamin deficiency, use of cobalamin (> 50 µg/day) or folic acid (> 200 µg/day) supplementation or injections, surgery or diseases of the stomach or small intestine,

anemia, dementia, life-threatening diseases, or severe hearing or visual problems. Medication interfering with cobalamin absorption29 was permitted if it had been provided at least 3 months prior to the screening of cobalamin status and was intended to be continued for the duration of the trial. Screening for cobalamin status was carried out between April 2003 and March 2004. Individuals who fulfilled the criteria for mild cobalamin deficiency were eligible to enter the run-in period. Mild cobalamin deficiency was defined as 1) serum cobalamin concentration between 100 and 200 pmol/L, or 2) serum cobalamin concentrations between 200 and 300 pmol/L with plasma MMA concentrations ≥ 0.32 µM and serum creatinine concentration ≤ 120 µmol/L, the latter intended to exclude severe impairment of renal function.[3] A summary of the recruitment procedure and the flow of participants included in the study is shown in Figure 1. The Medical Ethics Committee of Wageningen University approved the study protocol. The management of care-facility homes provided informed consent, and written informed consent was obtained from all individuals before the screening for impaired cobalamin status began.

Study design and protocol

Individuals with mild cobalamin deficiency took a placebo capsule for 2 weeks before the randomization (run-in period). The mean (SD) time elapsed between the screening and run-in period was 6 (3) weeks. Within the run-in period, individuals were excluded from further participation if they ingested < 90% of the capsules, or if they scored < 19 points (maximum 30 points) on the Mini-Mental State Examination (MMSE). Eligible participants were randomly assigned to receive 24 weeks of treatment in a parallel group design with daily oral doses of 1) 1,000 µg cobalamin, 2) a combination of 1,000 µg cobalamin and 400 µg folic acid, or 3) a placebo capsule (Figure 1). The doses selected for this study were based on previous dose-finding studies for oral cobalamin[28] and folic acid.[30] Cobalamin was administered as cyanocobalamin. The capsules given to the separate treatment groups were identical in appearance, smell and taste. The placebo capsules contained AVICEL PH102 (Medipulp GmbH, Aschaffenburg, Germany) as a filler. The mean (SD) measured doses of cobalamin for the capsules containing cobalamin or cobalamin + folic acid were 986 (3.4) µg and 987 (3.8) µg, respectively. The mean (SD) measured dose of folic acid for the cobalamin + folic acid capsules was 357 (6.0) µg.

Sample size calculations indicated that 45 participants per group had 80% power to detect an absolute difference of 3 points between the intervention groups in Verbal Fluency scores induced after cobalamin injections, assuming a within-person SD of 4.4 points in Verbal Fluency.[21] In order to control for an estimated drop out rate of 23%[31], at least 55 participants were to be enrolled in each group.

Randomization was stratified according to MMA concentration at screening visit (below and above 0.45 µmol/L), age (below and above 80 years), sex, and MMSE (below and above 24 points). The study had a double blind design.

The participants were asked to maintain their regular diet and to record in a diary their daily intake of capsules, use of medication, and occurrence of any new illnesses during the trial. Compliance was checked by counting unused capsules remaining in capsule dispensers and by verifying pill count in the participants' diaries. Nurses were asked to monitor the daily capsule intake of the institutionalized participants.

Figure 1: Recruitment procedure and flow of participants during the study. MMA, methylmalonic acid; MMSE, Mini–Mental State Examination
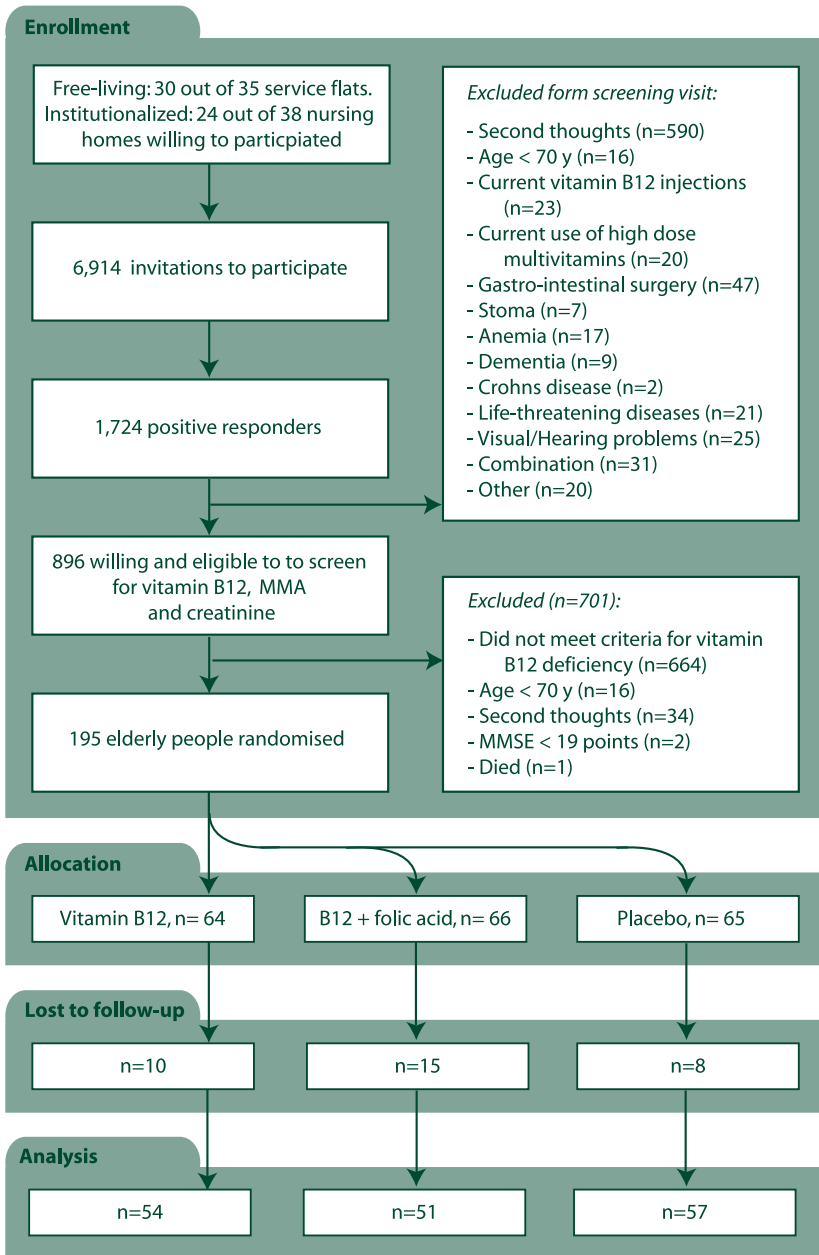
Data collection

Medical history, life style and anthropometry

The questionnaire collected information on medical history and issues related to cobalamin status and cognitive function.[32] The participants were asked to indicate "yes" or "no" to questions about history or presence of myocardial infarction, coronary bypass, stroke, transient ischemic attack, angina pectoris, diabetes mellitus, and hypertension, use of medication, subjective memory and depressive complaints, in addition to questions on smoking, alcohol consumption and diet (vegetarian or vegan). Education was classified as ''low'' (i.e. less than primary school or primary school), "intermediate" (i.e. less than low vocational training, low vocational training or mean vocational training) or "high" (i.e. high vocational training or university level). Body height and weight were measured in a standing position at the baseline visit and with participants dressed in light clothing and without shoes. Body weight (kg) was measured to the nearest 0.5 kg with a calibrated mechanical balance (Seca, Hamburg, Germany), and body height (cm) to the nearest 0.1 cm.

Blood

A blood sample was collected at both screening and randomization visits and after 12 and 24 weeks of active treatment. The participants were allowed to eat a light breakfast (without fruit, fruit juices, meat or eggs) at least one hour before blood collection. A sample of blood for subsequent measurement of MMA, tHcy and holoTC was collected into a 10 ml Vacutainer® tube containing EDTA. This blood sample was placed in ice water and centrifuged at 2600 rpm for 10 min at a temperature of 4 °C within 30 minutes of collection. All plasma samples were stored at –80 °C prior to laboratory analyses. Plasma concentrations of MMA were determined by a liquid chromatography electro spray ionisation tandem mass spectrometry (LC-ESI-MS/MS) system (H.J.B., oral communication, July 28, 2005). Plasma tHcy concentrations were determined by a method based on methylchloroformate derivatization and gas chromatography-mass spectrometry[33], and plasma holoTC was measured using the AXIS-Shield radioimmunoassay method[34]. A second blood sample was taken into a 5 ml gel tube for measurement of serum cobalamin and creatinine. The serum samples for cobalamin determination were stored at room temperature in the dark for measurement later that day using the IMMULITE 2000 cobalamin method.[35] A third blood sample was collected into a 5 ml Vacutainer® tube containing EDTA, and stored between 4 and 8 °C to determine red blood cell folate at the same day of blood collection. From this Vacutainer® tube, also hematological parameters (hemoglobin, hematocrit, mean cell volume, hypersegmentation of neutrophils) were determined at the randomization visit.

Cognitive function

Cognitive function was assessed by 6 trained and registered neuropsychologists during the run-in period (referred to as "baseline") and at week 24 of intervention during a 1.5 to 2 -hour session. The MMSE[36], Clinical Dementia Rating (CDR) Scale[37] and Geriatric Depression Scale (GDS)[38] were used to describe the study population. Individuals with an MMSE score < 19 points (maximum 30 points) were excluded.

Table 1: Description of neuropsychological test battery with corresponding domain and neuropsychological focus

Task[1]	Domain	Neuropsychological focus	Description
MMSE[36]	All	Global cognitive function	Screening tool. Exclusion from further participation if score < 19 points at first visit
Finger Tapping, computerized[41]	Sensomotoric speed	Simple sensomotor speed	Press a single button as often as possible within 30 seconds
Motor Planning_2, computerized[41]	Sensomotoric speed	Simple visuomotor reaction	Press a lit button out of 3 buttons as quickly as possible
Motor Planning_3, computerized[41]	Executive function	Complex visuomotor reaction	Inhibit automatic reaction in pressing a button immediately adjacent to a lit button as quickly as possible
Figure of Rey – copy[42]	Construction	Visuoconstruction	Copy the complex figure of Rey from an example
Figure of Rey – immediate recall[42]	Memory	Visual immediate memory	Draw the complex figure of Rey without the example immediately after the copy
15 Word Learning – immediate recall[43]	Memory	Verbal immediate memory	Read 15 words 5 times and recall words in between reading
Trail Making B[44]	Executive function	Concept shifting	Connect randomly placed numbers and letters alternated with a line as fast as possible
Digit Span Forward[45]	Attention	Attention	Repeat a string of digits in original order
Digit Span Backward[45]	Memory	Working memory	Repeat a string of digits in reverse order
Raven[46]	Executive function	Visual reasoning	Choose a design that fits into a matrix
Stroop[47, 48]	Executive function	Interference	Name color of the ink while inhibiting the automatic response of reading rather than the word (part 3). Part 1: reading names of colors red, green, yellow, blue; part 2) naming colored blocks red, green, yellow, blue
Figure of Rey - delayed recall[42]	Memory	Visual delayed memory	Draw the complex figure of Rey without the example 30 minutes after seeing the copy
15 Word Learning - delayed recall[43]	Memory	Verbal delayed memory	Recall the words of the 15 word learning test
Verbal Fluency, letter[50]	Executive function	Word generation	List as many nouns beginning with letter P (0 w) of G (24 w) as possible in 2 minutes
Verbal Fluency, animal[50]	Executive function	Word generation	List as many animals as possible in 1 minute
GDS[38]	Emotional status	Depression	Self-rating scale for depression

[1] Ordered by assessment

The CDR classified the study population into participants with no cognitive impairment (CDR=0), mild cognitive impairment (MCI; CDR=0.5), moderate cognitive impairment (CDR=1), or severe cognitive impairment (CDR=2). The neuropsychologists ascribed a score to the CDR according to results of the cognitive test battery described in Table 1, and an interview based on the criteria as composed by Petersen.[39] Tests that have been shown sensitive to the effects of B vitamin treatment and aging in previous studies[14, 21] were used to measure the potential effects of cobalamin supplementation on cognitive function. Since the cognitive status can be influenced by depression[40], the presence of depression (defined as a ≥ 5 out of 15 points) was assessed by the Geriatric Depression Scale (GDS). The order of assessment and description of the tests, including their corresponding cognitive domain and neuropsychological focus, are listed in Table 1.

Statistical methods

All analyses were carried out, on a per protocol basis, including the 162 participants (84%) who completed the trial. Baseline characteristics between treatment groups were compared by one-way analysis of variance (ANOVA) for continuous variables and chi-square analysis for categorical variables. The average concentrations of the biochemical parameters at the screening and randomization visits were calculated for each participant, and defined as "baseline" values. Differences in concentrations of blood parameters at baseline and at follow-up were assessed with a 2-factor repeated measures ANOVA (3 measurements x 3 treatment groups) that included the time x treatment interaction. Tukey's post hoc tests were used to assess differences between intervention groups.

Data on cognitive function were presented as the neuropsychological domains of attention, construction, sensomotor speed, memory, and executive function. The domains of attention and construction were assessed with the use of a single cognitive test, while the other domains were assessed with the use of multiple tests. All crude test scores were transformed to z-scores by: z-score = (individual result – mean result at baseline)/SD at baseline). For most of the individual neuropsychological tests, higher scores indicate a better cognitive performance, except for all tests of sensomotor speed, motor planning task 3, the Stroop test (part C/partA), and the trail making tests (part 3/part2). To achieve consistency in interpretation of results, we multiplied the crude test scores from these tests with -1 before transforming them into a z-score. The multiple tests for the domains of sensomotor speed, memory, and executive function were clustered to provide compound z-scores to reduce the effects of chance findings and to simplify interpretation of the cognitive data:

Attention = $Z_{\text{Digit Span Forward}}$; Construction = $Z_{\text{Rey, copy}}$; Sensomotor speed = $(-Z_{\text{Motor Planning(2)}} + -Z_{\text{finger tapping}} + -Z_{\text{trail making, part A}})/3$; Memory = $(Z_{\text{15WordLearning, immediate}} + Z_{\text{15WordLearning, delayed}} + Z_{\text{15WordLearning, recognition}} + Z_{\text{Rey, immediate}} + Z_{\text{Rey, delayed}} + Z_{\text{DigitSpan backward}})/6$; Executive function = $(-Z_{\text{Motor Planning(3)}} + -Z_{\text{Trail Making (partC/partA)}} + -Z_{\text{Stroop(part3/part2)}} + Z_{\text{Similarities (WAIS)}} + Z_{\text{Raven}} + Z_{\text{WordFluency(Animals)}} + Z_{\text{WordFluency(Letter)}})/7$.

Tests that were clustered for each cognitive domain correlated well with each other. Spearman rank correlation coefficients varied from 0.43 to 0.50 (all P values <0.0001) within the domain of sensomotor speed, from 0.37 to 0.82 (all P values <0.0001) within the domain of memory, and from 0.28 to 0.57

(all P values <0.0001) in the domain of executive function. Some participants were unable to complete all tests because of performance difficulties, e.g. tiredness. Compound z-scores were calculated when data for at least 2, 4, and 5 tests for the domains of sensomotor speed, memory and executive function respectively were available. The compound z-scores served as 'internal' z-scores from which z-scores at baseline and 24 weeks by study treatment were derived.

To determine potential treatment effects within and between intervention groups for each cognitive domain, we performed a 2-factor repeated measures analyses (2 measurements x 3 treatment groups) that included a time x treatment interaction. These analyses were performed with mixed models (SAS PROC MIXED procedure[41]), an extension from the linear regression model that includes random effects. Possible inter-investigator bias of the six neuropsychologists was entered as random effects. Tukey post hoc tests were used to compare mean changes in z-scores between treatment groups. All analyses were conducted using SAS statistical software (version 9.1; SAS Institute Inc., Cary, USA) and the graph was performed by GraphPad Prism (version 4; GraphPad Software Inc., San Diego, USA).

RESULTS

We found that 25% (232 out of 896) of the older people who were not supplemented had mild co-balamin deficiency. The recruitment, enrollment, and flow of participants during the trial are depicted in Figure 1. Two of the 195 participants who underwent random assignment dropped out during the run-in period, which left data for 193 participants who started supplementation. Thirty-one participants (16%) were unable to complete the trial, mostly due to illness, and the dropout rate was slightly higher in the cobalamin + folic acid group than the other groups. There were no significant differences between the participants who withdrew from the trial and the participants who completed the trial. However, the participants who withdrew from the trial were slightly more depressed than were those who completed the trial. On the basis of the number of unused capsules in returned dispensers, mean compliance was 99% and 4 participants had a compliance of between 80% and 90%. No adverse effects from study treatment were reported.

Characteristics of participants

Table 2 shows a summary of the demographic, lifestyle, co-morbidity, and hematological character-istics of the participants. These characteristics were not different across the treatment groups. The randomization procedure was successful since age, male/female ratio, scores on MMSE (Table 2), and concentrations of plasma MMA (Table 3) did not differ between the treatment groups. Anemia, defined as hemoglobin (Hb) concentrations ≤ 8.1 mmol/L in men and ≤ 7.4 mmol/L in women was present in 7% of the participants. Macrocytosis, defined as mean cell volume (MCV) ≥ 100 fl, was present in 5% of the participants. Neutrophil hypersegmentation, defined as > 5% of the neutrophils with five or more lobes, or the presence of at least one neutrophil with six or more lobes[42], was present in 54 % of the participants. There were no differences in prevalence of anaemia, macrocytosis and hypersegmentation across the treatment groups.

Table 2: Characteristics of older participants with mild cobalamin deficiency by treatment group[1]

		Cobalamin	Cobalamin + folic acid	Placebo
Demography	Age, mean (SD) years	82 ± 5[2]	83 ± 6	82 ± 5
	Sex, Male, n (%)	15 (23)	17 (26)	14 (22)
	Living, institutionalized, n (%)	37 (58)	35 (53)	39 (60)
	Education, n (%)			
	Low	24(38)	28 (47)	23 (35)
	Intermediate	31 (48)	26 (29)	35 (44)
	High	9 (14)	12 (18)	7 (11)
Lifestyle	Ex-smokers/ Smokers, n (%)			
	Ex-smoker	22 (34)	18 (27)	20 (31)
	Smoker	2 (3)	8 (12)	6 (9)
	Social drinking, n (%)	19 (30)	30 (46)	33 (51)
	Vegetarian, n (%)	4 (6)	2 (3)	3 (5)
	Multivitamin use, n (%)	14 (22)	11 (17)	14 (22)
Medical history	Myocardial infarction, n (%)	9 (14)	7 (11)	12 (18)
	Coronary bypass, n (%)	1 (2)	5 (8)	5 (8)
	Stroke, n (%)	3 (5)	6 (9)	1 (2)
	Transient ischemic attack, n (%)	9 (14)	16 (25)	14 (22)
	Angina Pectoris, n (%)	10 (16)	12 (18)	13 (20)
	Diabetes Mellitus, n (%)	5 (8)	4 (6)	9 (14)
	Hypertension, n (%)	15 (23)	22 (33)	16 (25)
	H_2 antagonist/proton pump inhibitors, n (%)	20 (31)	11 (17)	17 (26)
Neurological symptoms	MMSE	26.7 ± 3.1	26.7 ± 3.0	26.8 ± 2.9
	19 - 24 points (cognitive impairment),n (%)	9 (14)	10 (15)	8 (12)
	GDS	2.8 ± 2.6	3.2 ± 2.5	2.7 ± 2.7
	> 5 points (depression), n (%)	14 (22)	16 (25)	11 (17)
	CDR = 0, no cognitive impairment, n (%)	38 (59)	38 (59)	34 (66)
	CDR = 0.5, mild cognitive impairment, n (%)	19 (30)	16 (25)	16 (25)
	CDR = 1, moderate cognitive impairment, n (%)	7 (11)	8 (13)	6 (9)
	CDR = 2, severe cognitive impairment, n (%)	0 (0)	2 (3)	0 (0)
	Self perceived memory impairment, n (%)	35 (55)	40 (61)	38 (58)
	Self perceived depression, n (%)	22 (34)	20 (30)	16 (25)
Hematological symptoms	Hemoglobin, mmol/L	8.5 ± 0.7	8.5 ± 0.8	8.5 ± 0.7
	Mean Cell Volume, fl	91 ± 5	91 ± 6	92 ± 6

[1]MMSE, Mini-Mental State Examination; GDS, Geriatric Depression Scale; CDR, Clinical Dementia Rating Scale. No significant differences between the 3 treatment groups were observed, P>0.05 (one-way ANOVA for continuous variables and chi-square analysis for categorical variables)

[2]mean ± SD (all such values)

Blood biochemistry

Concentrations of cobalamin, MMA, and holoTC were not different, while tHcy concentrations increased slightly and RBC folate concentrations decreased slightly between the screening and randomization visit. Table 3 presents the concentrations of cobalamin, MMA, holoTC, tHcy, and RBC folate at baseline and at 12 and 24 weeks of supplementation. In the placebo group, no significant changes of blood parameters were observed during the study period. There was a significant time x treatment interaction for cobalamin, MMA, holoTC, tHcy, and RBC folate ($P < 0.0002$ for all biochemical markers). Mean MMA concentrations were reduced to normal levels, being < 0.26 μmol/L, after 12 weeks of supplementation with cobalamin. Cobalamin and cobalamin plus folic acid supplementation lowered mean tHcy concentrations by 16% and 37%, respectively. Concentrations of MMA and tHcy remained stable between 12 and 24 weeks of supplementation with cobalamin and folic acid, whilst concentrations of cobalamin, holoTC (borderline significant) and RBC folate further increased during the last 12 weeks.

Folate deficiency, defined as RBC folate < 305 nmol/L, was present in 2 (cobalamin-group), 6 (cobalamin plus folic acid-group), and 3 (placebo-group) participants at baseline, and in 2 (cobalamin-group), 0 (cobalamin plus folic acid-group), and 4 (placebo-group) participants after 24 weeks of supplementation.

Table 3: Mean (± SD) cobalamin, MMA, holoTC, Hcy, and RBC folate concentrations in participants with mild cobalamin definciency by treatment group at baseline, 12 and 24 weeks of supplementation[1]

Marker	Cobalamin	Cobalamin + folic acid	Placebo
Cobalamin (pmol/L)			
Baseline	186 ± 56 (52)	199 ± 50 (49)	188 ± 56 (55)
12 weeks	477 ± 194[2] (52)	538 ± 167[2] (49)	200 ± 76[3] (54)
24 weeks[4]	530 ± 210[2] (52)	627 ± 209[2,3,5] (51)	185 ± 62 (54)
MMA (µ mol/L)			
Baseline	0.47 ± 0.41 (52)	0.43 ± 0.22 (50)	0.46 ± 0.28 (55)
12 weeks	0.23 ± 0.06[2] (52)	0.25 ± 0.09[2] (50)	0.46 ± 0.30[3] (54)
24 weeks	0.22 ± 0.06[2] (52)	0.25 ± 0.10[2] (50)	0.48 ± 0.33[3] (53)
HoloTC (pmol/L)			
Baseline	58 ± 21 (52)	68 ± 33 (50)	70 ± 39 (54)
12 weeks	183 ± 124[2] (52)	222 ± 133[2] (49)	65 ± 43[3] (54)
24 weeks[4]	212 ± 118[2] (52)	282 ± 183[2] (51)	64 ± 42 (54)
Homocysteine (µ mol/L)			
Baseline	15.6 ± 6.6 (52)	14.5 ± 4.4 (50)	15.8 ± 5.6 (55)
12 weeks[5]	13.4 ± 5.7[2] (52)	9.7 ± 2.5[2,6] (49)	15.5 ± 5.6 (54)
24 weeks[5]	12.8 ± 4.9[2] (52)	8.9 ± 2.4[2,6] (51)	16.1 ± 6.8 (54)
RBC Folate (nmol/L)			
Baseline	578 ± 172 (52)	591 ± 203 (48)	680 ± 280 (55)
12 weeks[5]	694 ± 250[2] (52)	1179 ± 333[2,6] (49)	745 ± 353 (54)
24 weeks[5]	696 ± 271[2] (52)	1433 ± 418[2,5,6] (51)	670 ± 276 (54)

[1]All values are mean ± SD; n=parentheses. A significant time x treatment interaction was observed for all biochemical markers, P< 0.0002 (ANOVA). No significant differences between the 3 treatment groups were observed at baseline for all biochemical markers, P> 0.05 (ANOVA with Tukey's post-hoc tests)
[2]Significantly different from baseline, P> 0.05 (repeated-measures ANOVA with least squares means)
[3]Significantly different from the cobalamin and cobalamin + folic acid group, P> 0.05 (ANOVA with Tuckey's post hoc test)
[4]Significantly different between the 3 treatment groups were observed, P<0.05 (ANOVA with Tuckey's post hoc test)
[5]Significantly different from 12 weeks, P> 0.05 (repeated-measures ANOVA with least squares means)
[6]Significantly different from the placebo group and the cobalamin group, P> 0.05 (ANOVA with Tuckey's post hoc tests)

Cognitive function after B-vitamin supplementation

Table 4 describes the mean crude scores of the individual tests at baseline and 24 weeks of supplementation for the 3 intervention groups. Cognitive function improved slightly in all 3 groups, but most changes were not statistically significant. Significant improvement occurred mainly in the domain of memory for all three treatment groups.

The mean baseline scores for all the compound cognitive domains did not differ between allocated treatments. There were no significant changes in cognitive function for the domains of attention and construction between the intervention groups after 24 weeks of supplementation. Of the 162 participants who completed the trial, data for 141, 158, and 151 participants were included for analysis on the domains of sensomotor speed, memory, and executive function, respectively, because some participants were unable to complete all tests. Figure 2 shows changes in these domains after 24 weeks supplementation. There was a significant time x treatment interaction for the domain of memory (P = 0.0142). The figure confirms the improvement in memory function in all three treatment groups. The improvement in the placebo group was significantly better than the improvement in the cobalamin group (P=0.0036). However, separate analysis on the 6 memory tests indicated that only function on the Digit Span Backward (P=0.0014) and the 15 Word Learning (recognition) test (P=0.0376) showed this effect. Cobalamin with or without folic acid supplementation did not result in improved function on the domains of sensomotor speed and executive function. With respect to emotional status, there were no significant changes in GDS scores between the intervention groups after 24 weeks of supplementation (P=0.316).

Table 4: Crude scores from neuropsychological tests of cognitive function at baseline and after 24 weeks of B–vitamin supplementation in older people with mild cobalamin deficiency, by treatment group[1]

Test, maximum score		Cobalamin	B12 + folic acid	Placebo
Construction				
Complex Fig. of Rey - copy, 36 points	Baseline	28.0 ± 8.7 (54)	27.5 ± 9.5 (50)	27.7 ± 8.7 (56)
	24 weeks	30.0 ± 7.5 (47)	28.5 ± 9.0 (48)	29.2 ± 7.0 (49)
Attention				
Digit Span Forward, 16 points	Baseline	7.4 ± 1.5 (54)	7.5 ± 1.7 (51)	7.6 ± 1.7 (57)
	24 weeks	7.5 ± 1.7 (53)	7.4 ± 1.5 (51)	7.8 ± 1.6 (56)
Sensomotor Speed				
Motor Planning 2, milliseconds to press a button[2]	Baseline	627 ± 330 (49)	606 ± 233 (46)	673 ± 318 (53)
	24 weeks	647 ± 265 (48)	635 ± 295 (44)	618 ± 300 (50)
Finger Tapping, milliseconds to press a button[2]	Baseline	453 ± 270 (49)	442 ± 232 (46)	409 ± 230 (53)
	24 weeks	412 ± 175 (48)	425 ± 217 (44)	389 ± 168 (50)
Trail Making, part A, seconds to complete the task[2]	Baseline	75.4 ± 37.3 (54)	76.9 ± 51.9 (49)	72.0 ± 39.3 (56)
	24 weeks	77.5 ± 52.3 (53)	69.8 ± 49.0 (48)	73.9 ± 43.9 (56)
Memory				
15 Word Learning – Immediate recall, 75 points	Baseline	30.9 ± 11.7 (54)	30.1 ± 10.1 (51)	30.0 ± 10.3 (57)
	24 weeks	35.2 ± 12.1 (53)	36.3 ± 11.1 (50)	35.7 ± 11.1 (56)
15 Word Learning – Delayed Recall, 15 points	Baseline	4.8 ± 3.6 (54)	4.6 ± 3.7 (51)	5.1 ± 3.0 (57)
	24 weeks	5.5 ± 3.9 (53)	6.1 ± 4.2 (50)	6.1 ± 3.9 (55)
15 Word Learning – Recognition, 30 points[3]	Baseline	25.9 ± 3.6 (54)	26.6 ± 3.3 (51)	25.3 ± 4.4 (57)
	24 weeks	26.6 ± 3.7 (53)	27.4 ± 3.2 (50)	27.0 ± 3.6 (55)
Complex Fig. of Rey – Immediate recall, 36 points	Baseline	10.2 ± 7.1 (54)	10.8 ± 7.1 (50)	9.7 ± 6.8 (55)
	24 weeks	12.2 ± 7.7 (44)	14.0 ± 7.3 (43)	12.7 ± 7.4 (52)
Complex Fig. of Rey – Delayed recall, 36 points	Baseline	10.0 ± 6.6 (54)	10.3 ± 7.0 (50)	9.5 ± 6.3 (55)
	24 weeks	11.4 ± 7.0 (49)	12.2 ± 7.6 (43)	11.9 ± 7.3 (49)
Digit Span Backward, 14 points[3]	Baseline	4.9 ± 1.9 (54)	5.1 ± 1.3 (51)	4.7 ± 1.8 (57)
	24 weeks	4.6 ± 1.6 (53)	4.9 ± 1.3 (51)	5.3 ± 1.7 (56)

Table continued

Executive Function				
Motor Planning 3, milliseconds to press a button[2]	Baseline	898 ± 449 (49)	1053 ± 514 (44)	1012 ± 481 (50)
	24 weeks	863 ± 376 (45)	1066 ± 637 (43)	990 ± 696 (49)
Trail Making Test (PartC/PartA)[4]	Baseline	2.7 ± 1.2 (52)	2.7 ± 1.0 (47)	2.9 ± 1.0 (54)
	24 weeks	2.8 ± 1.2 (48)	3.1 ± 2.0 (46)	2.8 ± 1.0 (54)
Stroop (Part 3/Part2)[4]	Baseline	2.2 ± 0.6 (48)	2.2 ± 0.7 (42)	2.1 ± 0.6 (51)
	24 weeks	2.2 ± 0.9 (46)	2.2 ± 0.7 (43)	2.8 ± 1.0 (54)
Similarities (WAIS), 12 points	Baseline	5.3 ± 2.7 (54)	4.7 ± 2.9 (51)	4.8 ± 3.1 (57)
	24 weeks	6.1 ± 2.6 (52)	5.8 ± 2.5 (50)	5.4 ± 2.8 (56)
Raven, 24 points	Baseline	15.6 ± 3.6 (53)	15.2 ± 3.8 (49)	15.5 ± 4.1 (57)
	24 weeks	16.6 ± 3.5 (52)	16.7 ± 3.2 (47)	16.5 ± 3.9 (56)
Word Fluency – Animals, number of nouns	Baseline	17.6 ± 6.3 (54)	17.6 ± 5.4 (51)	17.4 ± 5.5 (57)
	24 weeks	17.6 ±5.5 (53)	17.3 ±5.3 (50)	16.5 ± 5.9 (56)
Word Fluency – Letter, number of nouns[3]	Baseline	16.2 ± 7.4 (54)	15.5 ± 6.7 (51)	15.2 ± 7.1 (57)
	24 weeks	15.5 ±7.9 (53)	16.0 ± 7.7 (50)	17.5 ± 8.8 (55)

[1]All values are mean ± SD; n=parentheses. The tests are described in Table 1. No significant differences between the 3 treatment groups were observed at baseline for any of the neuropsychological tests, P > 0.05 (mixed models with Tukey's post-hoc test)

[2]Higher scores indicate more time needed to complete a task, and thus poorer performance

[3]Treatment effects (changes from baseline within groups) are significantly different between the 3 treatment groups, as indicated by a significant time x treatment interaction, P < 0.05 (mixed models)

[4]Higher scores indicate poorer interference abilities

Figure 2: Mean changes (95% CI) in cognitive function after 24 weeks of B-vitamin supplementation in older people with mild cobalamin deficiency.
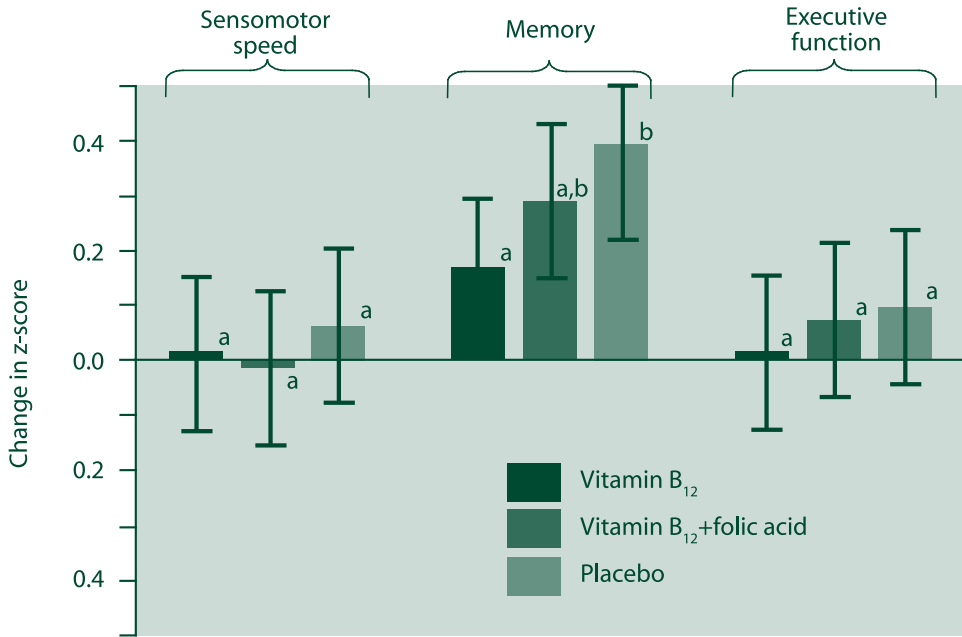
In the cobalamin group (n=54), data for 47 (sensomotor speed), 53 (memory) and 51 (executive function) participants were available for analysis. In the cobalamin + folic acid group (n=51), data for 44 (sensomotor speed), 50 (memory) and 46 (executive function) participants were available for analysis. In the placebo group (n=57), data for 50 (sensomotor speed), 55 (memory) and 54 (executive function) participants were available for analysis. Bars not sharing a common superscript letter are significantly different from each other. Mean difference in change of z-score between cobalamin and placebo group in the domain of memory was 0.22; 95% CI: 0.07 to 0.37 (MIXED MODELS with Tukey's post-hoc test)

DISCUSSION

The present randomized double blind controlled trial in older people with mild cobalamin deficiency did not show an improvement in cognitive function after 24 weeks of cobalamin supplementation when administered alone or in combination with folic acid compared with placebo.

Originally, a 2x2 factorial trial with placebo, cobalamin, folic acid, and cobalamin plus folic acid had been planned to assess the independent effects of cobalamin and folic acid and any interaction between them. However, it was not possible to conduct such a trial because of the theoretical risk of masking co-balamin deficiency and more rapid progression of neurological symptoms in individuals with cobalamin

deficiency who were treated with high-dose folic acid alone.[43] Therefore, a compromise was made by including an intervention arm with co-administration of cobalamin and folic acid. Since folate acts as a co-substrate and methyl group donor in the methionine synthase reaction, which is cobalamin dependent, additional folic acid supplementation would assure improved re-methylation of homocysteine to methionine.[7] Indeed, we observed an additional homocysteine lowering effect of combined treatment with both folic acid and cobalamin.

The present study was performed in order to confirm or refute the effects of cobalamin supplementation on cognitive function observed in previous smaller, non-randomized placebo controlled trials.[9, 19-22] We did this by using a larger sample size with a longer study duration and more rigorous methods to assess cognitive function. The results are consistent with previous randomized placebo controlled trials that reported null findings.[23-25] The latter trials included older people with mild to moderate cobalamin deficiency, and differed in criteria to identify cobalamin deficiency. The durations of treatment varied from 1 to 5 months, and treatment was administered either by injections[23, 25] or daily oral capsules with 10 to 50 µg cobalamin[24], which is considered to be an insufficient oral dose to normalize cobalamin deficiency.[28] One trial included older people with severe cognitive impairment.[23] All trials used some global neuropsychological tests to assess cognitive function such as the MMSE, the Alzheimer Disease Assessment Scale – Cognitive Subscale (ADAS-cog), or the Cambridge Cognitive (CAMCOG) Examination. The present study adopted an extensive battery of sensitive neuropsychological tests as the main outcome measure to assess the effects of supplementation. However, the comparison of study results was hampered by differences in the test batteries used in the different studies. The study by Hvas et al[25] showed that test scores on cognitive function improved slightly overall in both the treatment and the placebo group, and improvement was greater in the placebo group. This finding could be explained by a placebo effect and a learning effect of repeated cognitive testing. The learning effect is considered to be small when parallel versions of tests are used, which was the case in the present study. Interestingly, we observed a significantly lower improvement according to the compound z-score after cobalamin supplementation as compared to the placebo group in the domain of memory. However, the relevance of this finding is questionable because it was significant in only 2 of the 6 tests that measured memory function, which suggests that this is likely to be a chance finding. High-dose cobalamin supplements are considered as safe and no tolerable upper intake level has been set for cobalamin supplements in the United States[44] or Europe.[45] Moreover, no adverse effects from high-dose cyanocobalamin supplements have been reported in previous trials or in the present trial. We therefore assumed it to be unlikely that the supplements might have had short-term deleterious effects on memory.

The strengths of this trial are the double-blind randomized placebo-controlled design; its relatively large number of carefully selected participants; and its extensive assessment of cognitive function relative to most previous trials with similar objectives. To detect any potential beneficial effects of vitamin supplementation on cognitive function, the dose of vitamins, duration of treatment, characteristics of the study population, and methods of cognitive assessment are important. The oral doses given were effective in correcting mild cobalamin deficiency in peripheral blood, but it is still uncertain whether

normalization of impaired plasma cobalamin status reflects cobalamin status in the cerebrospinal fluid and cells in the central nervous system.

Magnetic resonance imaging (MRI) studies indicate that the repair of signs of demyelination may require treatment periods of greater than one year with-high dose cobalamin supplementation administered by injection, and resolution of clinical symptoms may require a longer period.[46] However, a pilot trial had shown beneficial effects of cobalamin injections on cognitive function after 5 months of cobalamin supplementation administered by injection in apparently healthy older people.[21] Hence, an intervention period of 24 weeks was chosen for the present trial, but these findings cannot exclude beneficial effects of cobalamin supplementation on cognitive function from longer term treatment.

The participants were selected on the basis of mild cobalamin deficiency, which may be associated with subtle cognitive impairment.[11, 12] The selection criteria were based on literature[28] and laboratory reference values, but there is no consensus on diagnostic criteria for mild cobalamin deficiency. Older people with severe cobalamin deficiency, as indicated by serum cobalamin < 100 pM, were excluded because of an assumed higher risk of progression to neurological damage.[43] When these individuals were identified during a screening visit (n=22), they were referred to their general practitioners for treatment and further follow up. Participants were not selected on the basis of mild cognitive or memory impairment. It is possible that subtle cognitive dysfunction may have been present for several years before the onset of clinically overt, severe cognitive impairment.[47] It has been hypothesized that individuals who have mild cognitive impairment for less than six months are more likely to respond to cobalamin therapy.[9] If the duration of cognitive problems is longer than 6 months, there may be a widespread neurological damage and loss of the ability to repair neurons.[27] A limitation of all studies so far is the lack of information on the duration of cognitive impairment.

Cobalamin deficiency is common in older people, mainly due to malabsorption.[3] The high prevalence of impaired cobalamin status is associated with cognitive impairment. However, such associations may not be causal. Furthermore, even though the expected proportion of true reversible dementia in patients with cobalamin deficiency is low[48], impaired cobalamin metabolism may contribute as one of many factors in the development of cognitive impairment and dementia and may modulate the course of the disease. The mechanisms by which this occurs are not completely understood and may include elevated concentrations of methylmalonic acid5 or homocysteine[49], or B vitamins in the maintenance of the integrity of the blood brain barrier (BBB)50, and reduced methylation capacity.[51]

Despite the strengths of the present study, 24 weeks of supplementation with cobalamin administered alone or in combination with folic acid did not show any improvement in cognitive function in the older persons with mild cobalamin deficiency.

ACKNOWLEDGEMENTS

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One carbon metabolites in relation to cognitive function in Dutch elderly people

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ABSTRACT

The role of compounds involved in the one-carbon metabolism other than homocysteine, such as methionine, choline, betaine and dimethylglycine (DMG), in cognition is unknown. We therefore explored the relation between plasma concentrations of these one-carbon metabolites and cognition. Elevated plasma homocysteine was associated with lower performance on the domains of attention, construction, sensomotor speed, and executive function. In addition, concentrations of betaine were positively associated with better performance on the domains of construction, sensomotor speed and executive function, whereas elevated concentrations of methionine were positively associated with sensomotor speed. Daily oral supplementation of 1,000 µg cobalamin with 400 µg folic acid for 6 months decreased homocysteine concentrations by 36%, and increased betaine concentrations by 38%. Participants with the largest increases in betaine concentrations showed a non significant (P = 0.07) higher increase in memory performance compared to others.

Key words: elderly, homocysteine, choline, betaine, DMG, cognition

INTRODUCTION

In order to prevent and possibly reverse age-related cognitive impairment in elderly people, a better understanding of the risk factors for cognitive decline is needed. In this respect, homocysteine is of interest because of its link with cognitive impairment and cardiovascular disorders because of the close association between cardiovascular health and function of the central nervous system (CNS)[,1,2] Moreover, homocysteine has been addressed in epidemiological studies as a potential modifiable risk factor for cognitive impairment.[3-13]

Homocysteine is located at a critical metabolic branch point with ramification to methyl- and sulfur group metabolism (Figure 1). Homocysteine is formed from the essential amino acid, methionine, via transmethylation reactions. Methionine is activated by its conversion to S-adenosylmethionine (SAM).[14] SAM is required for methylation of many acceptor substrates, such as DNA, RNA, lipids, proteins, phosphatidylethanolamine, creatine, myelin basic protein, and neurotransmitters.[15] Methylation of, for example, phosphatidylethanolamine, which results in phosphatidylcholine, is important to maintain myelin sheets of nerve tissue and thereby for CNS structure and function.

Homocysteine is remethylated to methionine via two remethylation pathways. In most tissues, the conversion of homocysteine to methionine is catalyzed by the cobalamin-dependent enzyme methionine synthase (MS). This enzyme uses 5-methyltetrahydrofolate as a methyl donor. In a few tissues, predominantly the liver and kidneys, homocysteine remethylation is also catalyzed by the enzyme betaine-homocysteine methyltransferase (BHMT). Methionine and dimethylglycine (DMG) are the products of this reaction. The methyl donor, betaine, is formed from choline, which also is a precursor for the neurotransmitter acetylcholine.[16]
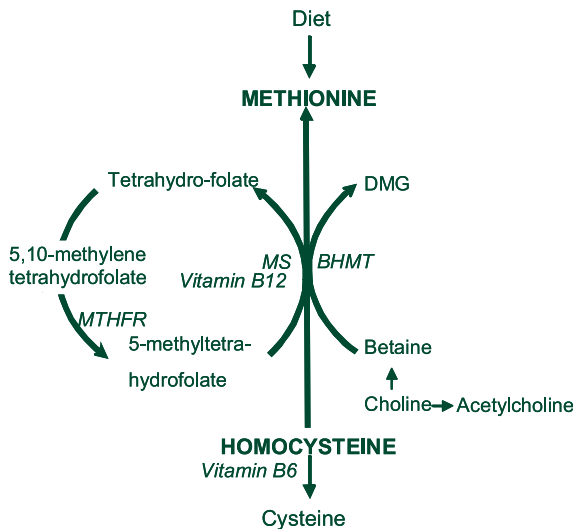
Figure 1: B-vitamins, homocysteine and the one carbon metabolism. The conversion form homocysteine to methionine can be catalysed via methionine synthase (MS) and betaine-homocysteine methyltransferase (BHMT). The conversion via MS requires vitamin B12 and 5-methyltetrahydrofolate (THF) as a methyl donor and couples folate metabolism to the choline-betaine pathway. Choline is a precursor for the neurotransmitter acetylcholine or it is oxidized to betaine. Betaine donates its methyl group directly to homocysteine for the conversion into methionine, and results in dimethylglycine (DMG) in the BHMT reaction.

Previous studies have focused on the associations of homocysteine, cobalamin and folate with cognitive performance[17-22], but the possible relation of other compounds of the one carbon metabolism with cognitive performance has not been explored yet. Considering the essential role of methylation in CNS structure and function[15], we explored cross-sectional associations between the metabolites choline, betaine and DMG with cognitive function. Secondly, we assessed whether supplementation with cobalamin and folic acid induced alterations in plasma concentrations of choline, betaine and DMG, and consequently, whether alterations in these metabolites were associated with improved cognitive function.

SUBJECTS AND METHODS

Participants

Elderly men and women aged 70 years or older were screened for participation in a randomized double-blind placebo-controlled trial that studied the efficacy of oral cobalamin supplementation on cognitive performance.[23] Individuals were included when they had mild cobalamin deficiency. Cobalamin deficiency was defined as either serum cobalamin concentrations between 100 and 200 pmol/L, or as serum cobalamin concentrations between 200 and 300 pmol/L in combination with plasma methylmalonic acid (MMA) concentrations ≥ 0.32 µmol/L. Participants had serum creatinine concentration ≤ 120 µmol/L to exclude severe impairment of renal function.[24] Other exclusion criteria were history of cobalamin deficiency, use of cobalamin (> 50 µg/day) or folic acid (> 200 µg/day) supplementation or injections, surgery or diseases of the stomach or small intestine, anemia, life-threatening diseases, severe hearing or visual problems, and severe cognitive impairment, which was defined by a score < 19 points on the Mini-Mental State Examination (MMSE). An additional sample of individuals with adequate cobalamin status and no severe cognitive impairment (n=40) was enrolled for cross sectional data analysis. Figure 2 presents the recruitment procedure, study design and flow of participants. The Medical Ethical Committee of Wageningen University approved the study protocol. Daily boards and client councils gave their consent for those individuals living in an institution, and written informed consent from all participants was obtained before the start of the study.

Study design of the intervention trial

Individuals who were included in the intervention trial, started with a 2-week placebo run-in period prior to randomization. Within this period, individuals were excluded from further participation if compliance (intake of capsules) was < 90%, or if they scored < 19 points on the MMSE. Eligible participants were randomized to receive 24 weeks of treatment in a parallel group design with daily oral doses of 1,000 µg cobalamin, a combination of 1,000 µg cobalamin and 400 µg folic acid, or a placebo capsule. Randomization was stratified according to MMA concentration at the screening visit (below and above 0.45 µmol/L), age (below and above 80 years), sex, and MMSE (below and above 24 points).

Figure 2: Recruitment procedure, study design and flow of dutch elderly participants

			Vitamin B12 group Start: n=64	Vitamin B12 group End: n=54

Fulfilled criteria for impaired vitamin B12 status: n=232 → Impaired vitamin B12 status group: **n=202**

Second thoughts (n=29)
Died (n=1)

Second thoughts during run-in period (n=7)

Vitamin B12 + folic acid group Start: n=66 → Vitamin B12 + folic acid group End: n=51

896 elderly people screened for impaired vitamin B12 status

Placebo group Start: n=65 → Placebo group End: n=57

Did not meet criteria for impaired vitamin B12 status: n=664 → Adequate vitamin B12 status group: **n=40**

Cross sectional associations

Effects of intervention on one-carbon metabolites, and effect of metabolites on cognitive performance

Data collection

Medical history, life style and anthropometry

A questionnaire was used to collect information on medical history and issues related to cobalamin status and cognitive function.[25] Individuals were asked to indicate "yes" or "no" to the following questions: history or presence of myocardial infarction, coronary bypass, stroke, transient ischemic attack, angina pectoris, diabetes mellitus, and hypertension, use of medication, subjective memory and depressive complaints. Also information on life style factors such as smoking habits, alcohol consumption, diet (vegetarian or vegan), and education were recorded. Education was classified as ''low'' (i.e. less than primary school or primary school), "intermediate" (i.e. less than low vocational training, low vocational training or mean vocational training) or "high" (i.e. high vocational training or university level). Body height and weight were measured in a standing position at the baseline visit.

Blood

A blood sample was collected at the screening and baseline visit and after 12 and 24 weeks of supplementation. A blood sample for measurement of methionine, total homocysteine (tHcy), choline, betaine, and dimethylglycine (DMG)[26] was collected into a 10 ml Vacutainer® tube containing EDTA. This blood sample was placed in ice water and centrifuged at 2600 rpm for 10 min at a temperature of 4 °C within 30 minutes of collection. All plasma samples were stored at –80 °C prior to laboratory analyses. Plasma concentrations were determined by a method based on methylchloroformate derivatization and gas chromatography-mass spectrometry (methionine and tHcy)[27] and a modification of a method based on liquid chromatography tandem mass spectrometry (choline, betaine and DMG).[28] Analytical coefficients of variation (CV) of the assays for methionine, tHcy, choline, betaine and DMG were <3.4%, <2.2%, < 10%, <10% and <10%, respectively.[27]

Neuropsychological test battery

Six trained and registered neuropsychologists performed cognitive testing of the participants during a 1.5 to 2 -hour session during the run-in period and at week 24 of intervention. The cognitive test battery consisted of tests that have been shown sensitive to the effects of B-vitamin treatment and aging in previous studies.[18, 29] The Finger Tapping Test[30] and Motor Planning Test[30] are computerised tasks in which measurements are obtained from a six-button panel, containing one red button and five white target buttons, laid out in a 180 ° arc, all at 6 cm distance from the red button. The participant is requested to press the red button as quickly and often as possible during 30 seconds (Finger Tapping), and afterwards to press the white button immediately adjacent (clockwise) to a white lit button, instead of the lid button itself (Motor Planning). Participants are asked to copy, draw immediate after copying (immediate recall, maximum score 36 points), and draw 30 minutes after copying (delayed recall, maximum score 36 points) the Complex Figure of Rey.[31] With the 15 Word Learning Task[32], a list of 15 words is read 5 times to the participant, and in between, the participant is asked to recall as many words as possible (immediate recall, maximum score 75 points). After 30 minutes, the participant is asked to recall as many words as possible (delayed recall, maximum score 15 points). This was followed by reading a list of 30 words to the participant whom has to indicate the 15 words (recognition, maximum score 30 points). In the Trailmaking Test[33], pseudo randomly placed circles with numbers (Trailmaking A), and with both letters and numbers (Trailmaking B) have to be connected with a line as fast as possible in a fixed order (score, seconds needed to complete the task). In the Digit Span, a subtest of the Wechsler Adult Intelligence Scale[34], participant are asked to repeat a string of digits in the original order (digit span forward, maximum score 8 points) and in reverse order (digit span backward, maximum score 7 points). Ravens' Coloured Progressive Matrices[35] consist of 24 figures. The principle on which a figurative matrix is constructed can be deduced from the design of the figure that is presented to the participant (maximum score 24 points). In the Stroop[36], participants are asked to read the name of colours (red, green, yellow, blue) (Stroop 1; score, seconds needed to complete the task), naming colour blocks (Stroop 2; score, seconds needed to complete the task), and naming the colour of the ink rather than the word (Stroop 3; score, seconds needed to complete the task). Within the WAIS[37], participants are asked to mention similarities between 5 pairs of nouns. Finally, the Verbal Fluency test[38] requests to list as many animals (Fluency Animal) and nouns (Fluency Letter) as possible within 2 minutes (score, number of items mentioned). Nouns beginning with letter P were asked at baseline whereas nouns beginning with letter G were asked after 6 months of supplementation. All these single tests were clustered into domains of attention, construction, sensomotor speed, memory and executive function, as indicated in the statistical methods. Since cognitive status can be influenced by depression[39], the presence of depression (defined as a ≥ 5 out of 15 points) was assessed by the Geriatric Depression Scale (GDS).

Statistical methods

One-carbon metabolites

The average concentrations of the biochemical parameters at the screening and randomization visits were calculated for each individual, and defined as "baseline" concentrations. Differences in concentrations between the three intervention groups were compared using a one-way analysis of variance (ANOVA). Tukey post hoc tests were used to assess differences between the intervention groups.

Cognitive function

Data on cognitive function were presented as the neuropsychological domains of attention, construction, sensomotor speed, memory, and executive function. The domains of attention and construction were assessed by a single cognitive test, while the other domains were assessed by multiple tests. All crude test scores were transformed to z-scores by: z-score = (individual result – mean result of study population at baseline)/SD of study population at baseline). The multiple tests for the domains of sensomotor speed, memory, and executive function were clustered to provide compound z-scores to reduce the effects of chance findings and to simplify interpretation of the cognitive data:

Attention = $Z_{\text{Digit Span Forward}}$; Construction = $Z_{\text{Rey, copy}}$; Sensomotor speed = $(-Z_{\text{Motor Planning(2)}} + -Z_{\text{finger tapping}} + -Z_{\text{trail making, part A)}}/3$; Memory = $(Z_{\text{15WordLearning, immediate}} + Z_{\text{15WordLearning, delayed}} + Z_{\text{15WordLearning, recognition}} + Z_{\text{Rey, immediate}} + Z_{\text{Rey, delayed}} + Z_{\text{DigitSpan backward})}/6$; Executive function = $(-Z_{\text{Motor Planning(3)}} + -Z_{\text{Trail Making (partC/partA)}} + -Z_{\text{Stroop(part3/part2)}} + Z_{\text{Similarities (WAIS)}} + Z_{\text{Raven}} + Z_{\text{WordFluency(Animals)}} + Z_{\text{WordFluency(Letter))}}/7$.

Tests that were clustered for each cognitive domain were highly correlated (P-values ranged from < 0.0001 to 0.04 for all tests). Some participants were unable to complete all tests because of performance difficulties, e.g. tiredness. Compound z-scores were calculated when data for at least 2, 4, and 5 tests for the domains of sensomotor speed, memory and executive function respectively were available. The compound z-scores served as 'internal' z-scores from which z-scores at baseline and 24 weeks by study treatment were derived.

Cross sectional analyses

Baseline information of our study population with mild cobalamin deficiency combined with an additional group of elderly with adequate cobalamin status enabled us to explore associations between compounds of the one-carbon metabolism and cognitive performance by means of partial correlation coefficients which were corrected for age and education.

Cognitive function and alterations in one-carbon metabolites due to intervention

Per protocol analyses were performed, including only the 162 participants (84%) who completed the trial. Changes in cognitive performance induced by alterations of the one-carbon metabolites were calculated by subtracting z-scores at the end of the intervention study by the z-scores at baseline. The potential effects of changes in one-carbon metabolites on cognitive function within and between tertile categories in biochemical changes were studied by a 2-factor repeated measures analyses (2 measure-

ments x 3 tertile categories) for each cognitive domain that included time x tertile category interaction. These analyses were performed with mixed models (SAS PROC MIXED procedure40), an extension from the linear regression model that includes random effects. Possible inter-investigator bias of the six neuropsychologists was entered as random effects. Statistical analyses were conducted using SAS statistical software (version 9.1; SAS Institute Inc., Cary, USA).

RESULTS

Characteristics of participants

The mean (SD) age of the participants was 82 (6) years, 74% of the participants were females, and 40% of the participants lived in a care facility home. Mean (SD) score on the MMSE was 27 (3) points which indicates no to mild cognitive impairment of the study population. A detailed description of the demographic, lifestyle, co-morbidity, and hematological characteristics of participants is presented elsewhere.[23]

Blood indices and cognitive function before supplementation

Mean concentrations for methionine, choline, betaine and DMG in the total screened population (n=896) were 25.1 µmol/L, 8.0 µmol/L, 32.2 µmol/L, and 3.7 µmol/L respectively. These concentrations did not differ from those concentrations observed in the segment of participants involved in the intervention trial (n=193; P values unpaired t-test > 0.05 for all indicators). In line with the inclusion criteria of mild cobalamin deficiency, participants of the intervention trial had lower cobalamin concentrations and higher tHcy concentrations compared to the total screened population (both P<0.0001).

Partial correlation coefficients, which were corrected for age and education revealed negative associations of tHcy concentrations with compound scores for the domains of attention, construction, sensomotor speed, and executive function. Methionine concentrations were positively associated with the domain of sensomotor speed, whereas betaine concentrations were positively associated with the domains of construction, sensomotor speed and executive function (Table 1).

Table 1: Partial spearman rank correlation coefficients between compound cognitive domains, vitamins and one-carbon metabolites in a Dutch elderly population. Analyses are adjusted for age and education.

		Vitamins			One carbon metabolites			
Cognitive domain	N	cobalamin	RBC folate	tHcy	Methionine	Choline	Betaine	DMG
Attention	238	-0.05	0.19**	-0.12*	0.01	-0.02	0.04	-0.07
Construction	236	0.09	0.08	-0.18**	0.01	0.06	0.19**	0.03
Sensomotor speed	222	0.15*	0.21**	-0.26**	0.16**	-0.07	0.14**	-0.03
Memory	237	0.08	0.15**	-0.07	0.02	0.00	0.01	-0.02
Executive function	233	0.16**	0.24**	-0.14**	0.10	0.00	0.13**	0.01

*P < 0.10, ** P < 0.05

Blood indices throughout 24 weeks of intervention

Table 2 presents the concentrations of vitamins and the one-carbon metabolites at baseline and at 24 weeks of supplementation. Concentrations of all metabolites in the placebo group remained stable throughout the study period. The time x treatment interaction was significant for cobalamin, tHcy, RBC folate (P < 0.0001 for these indices) and betaine (P = 0.0304), which indicates differences in effects between the intervention groups. The effects of treatment did not differ between the intervention groups (no significant interaction terms) for methionine, choline, and DMG. Nevertheless, concentrations of methionine and choline increased significantly by respectively 11% and 23% after combined supplementation.

Alterations in one carbon metabolites with alterations in cognitive performance

No differences in cognitive performance between intervention groups were observed at baseline. Since some participants were unable to complete all tests, data of 141, 158, and 151 participants were included for analysis on de domains of sensomotor speed, memory, and executive function respectively. The effects of treatment groups on cognitive performance have been described elsewhere.[23] Increases in both RBC folate and cobalamin concentrations were accompanied by significant improvements in the domain of memory. However, the differences in memory improvement with increasing cobalamin and RBC folate concentrations were not significant (P > 0.05 for interaction time x tertile category of change in B-vitamin concentration). No improvements in any of the other cognitive domains were observed after B-vitamin supplementation. Table 3 presents mean changes in cognitive scores according to changes in tHcy, methionine, choline, betaine and DMG, which are categorised into tertiles. Although mean changes in construction performance were not significant within changes in tertiles for betaine and DMG, we observed borderline significant tests for trend for these metabolites. Furthermore, the table shows significant improvements in the domain of memory of approximately the same magnitude within tertiles for all one-carbon metabolites. However, the time x tertile category interaction term was only significant for DMG (P = 0.04), and borderline significant for betaine (P = 0.07), which indicates differences in memory improvement between the tertiles. Participants with the largest increases in betaine concentrations (3rd tertile category; change in betaine concentrations > 6.88 µmol/L) showed the highest increase in memory performance compared to those in the 1st and 2nd tertile. For DMG, participants in the 2nd and 3rd tertile had a better memory performance compared to those in the 1st tertile. With respect to the other cognitive domains, no effects within and between tertiles were observed.

Table 2: Concentrations (Mean ± SD) of vitamins and one-carbon metabolites at baseline and 24 weeks after supplementation with cobalamin, cobalamin + folic acid or placebo in a Dutch elderly population.

		Cobalamin Mean ± sd	Cobalamin+folic acid Mean ± sd	Placebo Mean ± sd
Cobalamin (pmol/L)[1]	Baseline[3]	186 ± 56 (52)	199 ± 50 (49)	188 ± 56 (55)
	24 weeks[4]	530 ± 210[8] (52)	627 ± 209[8] (51)	185 ± 62 (54)
RBC Folate (nmol/L)[1]	Baseline[3]	578 ± 172 (52)	591 ± 203 (48)	680 ± 280 (55)
	24 weeks[4]	696 ± 271[8] (52)	1433 ± 418[8] (51)	670 ± 276 (54)
Homocysteine (µmol/L)[1]	Baseline[3]	15.6 ± 6.6 (52)	14.5 ± 4.4 (50)	15.8 ± 5.6 (55)
	24 weeks[6]	12.8 ± 4.9[8] (52)	8.9 ± 2.4[8] (51)	16.1 ± 6.8 (54)
Methionine (µmol/L)[2]	Baseline[7]	24.2 ± 4.0 (55)	24.5 ± 3.9 (50)	26.6 ± 5.1 (55)
	24 weeks[3]	25.5 ± 6.9 (54)	26.2 ± 5.5[8] (51)	26.2 ± 5.5 (54)
Choline (µmol/L)[2]	Baseline[5]	8.5 ± 1.9 (52)	7.8 ± 1.6 (50)	8.8 ± 1.9 (55)
	24 weeks[3]	9.0 ± 2.3 (52)	9.6 ± 2.4[8] (51)	9.0 ± 2.7 (54)
Betaine (µmol/L)[1]	Baseline[3]	32.9 ± 10.1 (52)	30.4 ± 6.4 (50)	33.4 ± 9.5 (55)
	24 weeks[4]	34.6 ± 12.0 (52)	40.9 ± 0.5[8] (51)	36.2 ± 12.8 (54)
DMG (µmol/L)[2]	Baseline[4]	4.2 ± 1.6 (52)	3.5 ± 0.7 (50)	3.8 ± 1.1 (55)
	24 weeks[4]	4.3 ± 2.2 (52)	3.5 ± 0.9 (51)	3.9 ± 1.1 (54

[1]Treatment effects (changes from baseline within groups) significantly different between the 3 treatment groups, as indicated by a significant time x treatment interaction, P < 0.05 (ANOVA)
[2]Treatment effects (changes from baseline within groups) not significantly different between the 3 treatment groups, as indicated by a non significant time x treatment interaction, P > 0.05 (ANOVA)
[3]No significant differences between the 3 treatment groups, P> 0.05 (ANOVA with Tukey post-hoc tests)
[4]Vitamin B12 + folic acid group differed significantly from the vitamin B12 group, P<0.05 (ANOVA with Tukey post-hoc tests)
[5]Significant differences between the vitamin B12 + folic acid group and the placebo group, P<0.05 (ANOVA with Tukey post-hoc tests)
[6]Significant differences between the 3 treatment groups, P<0.05 (ANOVA with Tukey post-hoc tests)
[7]Placebo group differs significantly from vitamin B12 and vitamin B12 + folic acid group, P<0.05 (ANOVA with Tukey post-hoc tests)
[8]Significantly different from baseline, P<0.05 (ANOVA repeated-measures analysis with LSMEANS)

Table 3: Mean (95% CI) cognitive changes in z-scores by tertiles in changes of one-carbon metabolites due to cobalamin with or withouth folic acid supplementation[1]

		Homocysteine	Methionine	Choline	Betaine	DMG
Attention	Tertile 1	0.03 (-0.18 to 0.25)	0.05 (-0.20 to 0.29)	0.01 (-0.19 to 0.21)	0.10 (-0.14 to 0.34)	-0.06 (-0.27 to 0.14)
	Tertile 2	0.02 (-0.26 to 0.31)	0.11 (-0.14 to 0.35)	0.24 (-0.03 to 0.50)	-0.04 (-0.27 to 0.20)	0.23 (-0.04 to 0.49)
	Tertile 3	0.10 (-0.13 to 0.34)	0.00 (-0.24 to 0.18)	-0.08 (-0.35 to 0.18)	0.08 (-0.17 to 0.33)	0.01 (-0.24 to 0.27)
	P trend	0.8991	0.7358	0.6289	0.7091	0.4811
Construction	Tertile 1	0.06 (-0.11 to 0.24)	0.13 (-0.07 to 0.32)	0.15 (-0.05 to 0.36)	0.21 (0.01 to 0.42)	0.03 (-0.16 to 0.22)
	Tertile 2	0.13 (-0.02 to 0.27)	0.12 (-0.06 to 0.30)	0.14 (-0.02 to 0.31)	0.12 (-0.07 to 0.31)	0.24 (0.05 to 0.42)
	Tertile 3	0.21 (-0.002 to 0.44)	0.16 (-0.03 to 0.35)	0.10 (-0.10 to 0.29)	0.05 (-0.11 to 0.21)	0.15 (-0.04 to 0.34)
	P trend	0.8494	0.1738	0.5745	0.0669	0.0602
Sensomotor speed	Tertile 1	0.07 (-0.08 to 0.22)	0.01 (-0.14 to 0.17)	0.03 (-0.11 to 0.16)	0.05 (-0.10 to 0.20)	0.05 (-0.10 to 0.20)
	Tertile 2	0.00 (-0.12 to 0.12)	0.02 (-0.11 to 0.15)	0.02 (-0.12 to 0.16)	0.01 (-0.12 to 0.14)	0.03 (-0.08 to 0.15)
	Tertile 3	-0.03 (-0.16 to 0.12)	0.01 (-0.12 to 0.15)	0.00 (-0.14 to 0.15)	-0.02 (0.15 to 0.12)	-0.03 (-0.18 to 0.11)
	P trend	0.9569	0.4994	0.7232	0.0857	0.4233
Memory	Tertile 1	0.25 (-0.02 to 0.52)	0.28 (0.20 to 0.40)	0.29 (0.17 to 0.40)	0.25 (0.13 to 0.38)[a,b]	0.17 (0.08 to 0.26)[a]
	Tertile 2	0.23 (0.00 to 0.45)	0.28 (0.16 to 0.40)	0.30 (0.21 to 0.39)	0.20 (0.09 to 0.31)[a]	0.35 (0.23 to 0.48)[b]
	Tertile 3	0.35 (0.07 to 0.63)	0.28 (0.14 to 0.42)	0.26 (0.13 to 0.39)	0.38 (0.29 to 0.50)[b]	0.33 (0.21 to 0.45)[b]
	P trend	0.1287	0.2767	0.3253	0.1000	0.4522
Executive function	Tertile 1	0.03 (-0.10 to 0.16)	0.04 (-0.08 to 0.15)	0.05 (-0.06 to 0.15)	0.08 (-0.02 to 0.19)	0.01 (-0.11 to 0.13)
	Tertile 2	0.03 (-0.06 to 0.12)	0.08 (0.00 to 0.17)	0.05 (-0.07 to 0.17)	-0.01 (-0.14 to 0.11)	0.14 (0.05 to 0.23)
	Tertile 3	0.12 (0.03 to 0.22)	0.06 (-0.06 to 0.18)	0.08 (-0.01 to 0.17)	0.10 (0.00 to 0.20)	0.03 (-0.08 to 0.15)
	P trend	0.2966	0.9950	0.2676	0.2518	0.9345

[1]Analyses are adjusted for age, education, and neuropsychologists. The cut-off points for tertile categories in changes of tHcy were -3.95 and -0.7 µmol/L; for methionine they were -1.32 and 2.88 µmol/L; for choline they were –0.17 and 1.48 µmol/L; for betaine they were -1.43 and 6.88 µmol/L; and for DMG they were –0.33 and 0.41 µmol/L. P values indicate tests for trend across median changes in concentrations for each tertile. Analyses are corrected for age, education and neuropsychologist. Mean (95% CI) changes in z-scores not sharing a common superscript letter are significantly different from each other.

DISCUSSION

This study showed that in addition to cobalamin, RBC folate and tHcy, also betaine concentrations are associated with various cognitive domains in cross sectional analyses. Daily oral supplementation of 1,000 µg cobalamin with 400 µg folic acid for a period of 24 weeks decreased tHcy concentrations and increased betaine concentrations. There was a tendency that participants with the largest increases in betaine concentrations showed the highest improvement in memory function.

Concentrations of the one carbon metabolites observed at baseline reflect long term biochemical status. Data on plasma concentrations of choline, betaine, and DMG are sparse and have only been reported in younger populations with[41] and without[28, 41, 42] B-vitamin deficiencies and renal diseases.[43] The reported concentrations of choline, betaine and DMG in these studies are similar to those observed in our older population. The present study observed comparable concentrations of choline, betaine and DMG in elderly with mild cobalamin deficiency and those with adequate cobalamin status. This result is in line with the finding that betaine concentrations were normal in most people with cobalamin and/or folate deficiency.[41] Moreover, spearman rank order correlations did not reveal significant associations between concentrations of cobalamin with any of the one-carbon metabolites, except with tHcy (data not shown). We therefore consider it unlikely that impaired cobalamin status modified the association of cognitive function with choline, betaine and DMG.

The two pathways which remethylate homocysteine are interrelated. This is reflected in our data showing that betaine concentration is inversely associated with tHcy, and is increased after combined supplementation of cobalamin with folic acid. In addition, both animal and human studies suggest a strong interrelationship between the BHMT and MS synthase pathways. Animals with a choline deficient diet had lower hepatic folate concentrations[44], and animals with folate deficiency had depletion of hepatic choline concentrations.[45] Human studies showed increased betaine concentrations after folic acid supplementation[46], depletion and subsequent repletion of folate intake affected plasma choline concentrations[47], and an inverse relation between betaine and tHcy concentrations was most pronounced at low serum folate concentrations.[48] Hence, diseases associated with high plasma tHcy concentrations may not only be linked to low concentrations of cobalamin and folate, but also with low concentrations of betaine and choline.

To our knowledge, the current study is the first one that explores associations of cognitive function with choline, betaine and DMG in an elderly population. Choline is an essential nutrient for humans. It is not only a source of methyl groups, but also a precursor for the neurotransmitter acetylcholine and several phospholipids, such as phosphatidylcholine and sphingomyelinin.[49] Thereby, choline may be related to neurodegenerative disorders through the one-carbon metabolism and other mechanisms. Thus, low concentrations of choline would affect cholinergic function in the central nervous system and thereby contributes to cognitive decline associated with ageing and dementia.[50] In accordance, degradation and dysfunction of neurons in the brain that contain acetylcholine are associated with Alzheimer's disease.[51] Furthermore, there is some evidence that supplementation with cytidinediphospho-choline (CDP-choline) could protect against, and prevent memory impairment in aging rats[52, 53], and has beneficial ef-

fects on memory and behavior in elderly people with cognitive problems.[54] However, a previous study observed that cholinergic precursors, such as choline, were not suitable for enhancing concentrations of acetylcholine in the brain.[55] This is in line with the absence of an association of choline with cognition in our study. Moreover, we did not observe a large variation in choline concentrations in the relatively small number of participants.

Betaine, a derivate of choline, is also important because it maintains water balance in kidneys[56] and provides methyl groups for homocysteine to form methionine in kidneys and the liver.[57] Despite the potential importance of the betaine pathway, not much is known about its role in various disease states. Although the enzyme BHMT is not present in the brain, the current study revealed positive associations of betaine concentrations with some of the cognitive domains at baseline, which could be explained by the interrelation of the two remethylation pathways. Participants with the largest increases in betaine concentrations (3rd tertile category; change in betaine concentrations > 6.88 µmol/L) showed the highest increase in memory performance compared to those in the 1st and 2nd tertile category, and not in any of the other cognitive domains. This is not in accordance with a non-placebo controlled pilot study.[58] In this trial, eight patients with Alzheimer's disease received 3 g oral betaine twice a day for 24 weeks. Seven participants completed the trial, from which four participants had worse scores on the Alzheimer Disease Assessment Scale-Cognitive Subscale test after 24 weeks. Possibly, cognitive impairment was irreversible in these elderly with Alzheimer's disease. Changes in concentrations of betaine and homocysteine during treatment were not reported, which does not enable us to compare changes in blood profile with results of the current study.

DMG is a product of the conversion of homocysteine into methionine via the BHMT dependent pathway. The present trial did not observe association of DMG with any of the cognitive domains at baseline. In addition, mean concentrations did not change after supplementation with folic acid and cobalamin. Although memory improvement of those participants with the highest increases in DMG concentrations was significantly higher than other participants, we consider this finding as a chance finding since mean DMG concentrations did not change after supplementation and the test for trend was not significant.

In summary, the present trial is the first to explore associations of cognitive function with choline, betaine and DMG, and revealed associations of homocysteine and betaine with cognitive domains. Furthermore, participants with the largest increases in betaine concentrations due to combined supplementation with cobalamin and folic acid showed a non significant higher increase in memory performance.

ACKNOWLEDGEMENTS

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General Discussion

Cognitive improvement after intramuscular cobalamin injections as shown by a non-placebo controlled pilot study[1] gave rise to the research described in this thesis. The primary aim of this thesis was to investigate the efficacy of oral cobalamin supplementation on cognitive performance in elderly people with mild cobalamin deficiency. In order to shed more light on the efficacy of oral cobalamin, we performed both a dose-finding study and an efficacy trial. These two trials enabled us to formulate additional research questions as addressed in the previous chapters. In this chapter, we summarize and further critically review the main findings.

MAIN FINDINGS

Figure 1 summarizes the main findings of the research questions addressed in the previous chapters. Our dose-finding study revealed that daily oral doses of 650 to 1000 µg normalize elevated MMA concentrations. Despite including a larger sample size, having a longer study duration, and using more rigorous methods to assess cognitive function compared to previous smaller, non-randomized placebo controlled trials[1-5], we could not confirm beneficial effects of cobalamin supplements on cognitive performance. The main conclusion of this thesis is that oral cobalamin with and without folic acid supplementation does not improve cognitive performance in elderly people. However, cross sectional analyses prior to the intervention showed associations between markers of cobalamin and folate status with cognitive function, which does suggest a role for these markers in cognitive function.

Figure 1: Summary of findings. The chapters within the frames indicate the main findings.
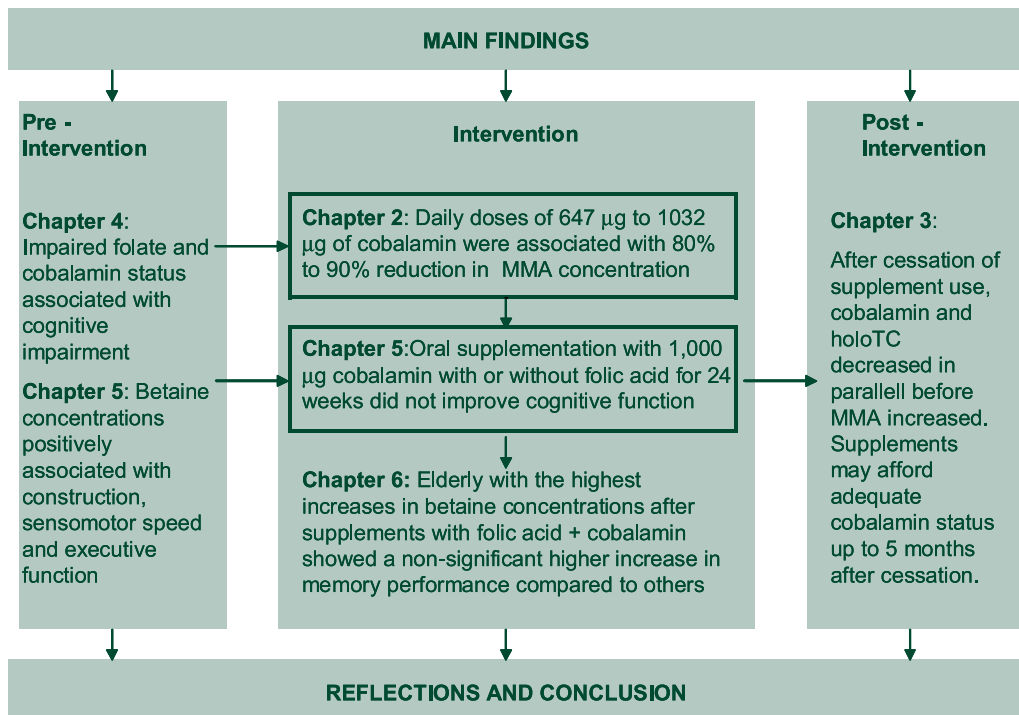
REFLECTIONS

Study design

Placebo group

We designed our study in order to confirm or refute beneficial effects of cobalamin supplementation on cognitive function as observed in previous smaller, non-randomized placebo controlled trials.[1-5] These previous trials did not include a placebo group in their design, probably because it was considered unethical to withhold cobalamin supplementation from individuals with impaired cobalamin status. Despite the lack of a placebo group, these trials concluded cognitive improvement after cobalamin supplementation. Although our trial revealed improved memory function in all three intervention groups, we could not conclude improved memory function after oral cobalamin supplementation relative to placebo capsules. This finding underpins the importance of a placebo group. It is not unlikely that we measured the so called 'placebo effect', which can be defined as any effect attributed to treatment, but not to its pharmacodynamic properties. It is a psychobiological phenomenon that can be attributable to different mechanisms, including expectation of clinical improvement.[6] During recruitment, the study protocol was explained as "investigating whether cobalamin supplementation would induce beneficial effects on memory performance". Although cognitive function comprises much more than memory performance alone, we did not use this term as elderly people would not understand the meaning. Therefore, participants might have expected improvement in the domain of memory due to our explanation, which is reflected in memory improvement in all three intervention groups.

Selection of intervention groups

Originally, a 2x2 factorial trial with placebo, cobalamin, folic acid, and cobalamin plus folic acid had been planned to assess the independent effects of cobalamin and folic acid and any interaction between them. However, it was not possible to conduct such a trial because of the theoretical risk of masking cobalamin deficiency and more rapid progression of neurological symptoms in individuals with cobalamin deficiency who were treated with high-dose folic acid alone.[7] Therefore, a compromise was made by including an intervention arm with co-administration of cobalamin and folic acid. Since folate acts as a co-substrate and methyl group donor in the cobalamin dependent methionine synthase reaction, additional folic acid supplementation would assure improved re-methylation of homocysteine to methionine.[8] Indeed, we observed an additional homocysteine lowering effect of combined treatment with both folic acid and cobalamin.

Study duration

We based the duration of our efficacy trial, which was 24 weeks, on the finding that daily oral supplementation normalizes mild cobalamin deficiency within 4 months[9] and cobalamin injections improved cognitive functions within 5 months.[1] On the one hand, some short-term studies suggest beneficial effects of cobalamin supplementation on neuropsychological cognitive tests.[1-3] However, on the other hand MRI-studies indicate that repair of signs of demyelination may require treatment periods longer

than one year with high dose cobalamin supplementation, and resolution of clinical symptoms may even require a longer period.[10] Thus, our findings cannot exclude effects of cobalamin supplementation on cognitive function or neuropathological processes from longer term treatment. Therefore, future studies might consider a longer study duration.

Selection of study population

Cobalamin status

In screening for mild cobalamin deficiency, we a priori defined criteria for the dose-finding study based on literature[11, 12] and laboratory reference values, as there is no consensus yet on which markers with corresponding cut-off values to apply.[13] For the efficacy study, we slightly narrowed the inclusion criteria for MMA, since subgroup analysis in the dose-finding study revealed more pronounced changes in cobalamin and markers of cobalamin status in those individuals with MMA concentrations ≥ 0.32 µmol/ L.[14] We considered this as a more valid cut-off value to identify cobalamin deficiency.

We aimed to include elderly people with mild cobalamin deficiency, since it has been previously suggested that this early stage of cobalamin deficiency is associated with subtle cognitive impairment.[15, 16] Reversibility of these early neuropsychological symptoms are believed to depend on early diagnosis of cobalamin deficiency and requires intensive cobalamin treatment.[3, 17] Indeed, our cross-sectional data analyses at baseline reveal lower cognitive performance in those elderly people with relatively unfavorable concentrations of the vitamins and its markers (chapter 4). The question arises from which turn-over point concentrations of cobalamin status are associated with cognitive impairment. Concentrations in the lowest tertile category (< 171 pmol/L) were associated with a cognitive performance below the average of the study population. In addition, the pilot study[1] showed beneficial effects of intramuscular cobalamin injections in elderly people with cobalamin concentrations < 150 pmol/L in combination with MMA concentrations > 0.32 µmol/L. Taken these results into account, it is conceivable that the null finding we found in our efficacy trial can partly be ascribed to too wide criteria for mild cobalamin deficiency. We therefore conducted secondary analysis in only those participants fulfilling the stricter criteria of the pilot study[1]. These analyses, however, did not reveal cognitive improvement after oral supplementation (n=64; data not shown).

Cognitive status

Cognitive status of individuals was not an inclusion criterion. However, we excluded those individuals with severe cognitive impairment (MMSE < 19 points)[18] in order to enroll elderly people with reversible symptoms of cognitive impairment. It has been proposed that reversibility depends on the duration of complications before interventions are initiated. Therefore, interventions should precede the stage of irreversible neuropsychological manifestations.[2, 3] Moreover, severely cognitively impaired elderly are not able to perform the used neuropsychological test battery, which would result in unreliable and missing data. Finally, conducting a trial with persons who do not understand the research aim and cannot clearly express one's free will would require a different approach in view of ethical considerations, logistics of

recruitment, and outcome measures for cognitive function for this specific group of people.

In order to combat severe cognitive problems, research in aging and dementia is focusing on the characterization of the earliest stages of cognitive impairment. The work of Eastley among geriatric elderly for example, revealed cognitive improvement after cobalamin therapy in individuals with cognitive impairment, but not in those with long-standing dementia.[2] Mild cognitive impairment (MCI) is considered as a transitional stage between normal aging and dementia. In this stage, persons experience memory loss to a greater extent than one would expect for age, but do not meet the criteria for clinical dementia.[19] Therefore, mild cognitive impairment is thought to be suitable for therapeutic interventions.

Originally, we aimed to include participants on the basis of both mild cobalamin deficiency and MCI. However, the tremendous logistics that would be involved in proper assessment of MCI as an additional screening tool were not feasible. Nevertheless, according to the mean MMSE score of 27 points it is likely that on average subtle or mild cognitive impairment was present in our study population.[18, 20] In addition, a prospective study showed that both low cobalamin and folate concentrations conferred a double risk of developing Alzheimer's disease, particularly in persons with baseline MMSE scores > 26 points.[21] Another aspect that has to be taken into account is the duration of cognitive problems. It has been hypothesized that individuals who have mild cognitive impairment for less than six months are more likely to respond to cobalamin therapy[3, 22], compared to those with a longer duration of symptoms.[22] If the duration of cognitive problems is longer than 6 months, there may be a widespread neurological damage and loss of ability to repair neurons.[23] We did not gain information on the duration of self perceived cognitive or memory problems, and can therefore not report secondary analysis on these two subgroups.

Recruitment procedure

Men and women aged 70 years or older living in service flats and in care facility homes were recruited from different parts from the Wageningen area in The Netherlands within a radius of approximately 100 km. Here we searched for villages and cities with at least 500 elderly people living in service flats and care facility homes to achieve feasible enrollment. Thus participants were not randomly selected. Furthermore, not much is known about why non-responders were not interested in participation, but it is likely that non-responders did experience more self perceived cognitive problems than responders did. The nature of the enrollment procedure and voluntarily participation could have introduced selection bias. Therefore, the results of cross sectional associations of markers for vitamin status (chapter 4) and one carbon metabolites (chapter 6) with cognitive function might not fully reflect associations that are present in the general elderly population. Nevertheless, we believe that a possible effect of selection bias did not affect the internal validity of the efficacy trial as treatment allocation occurred randomly and in a double blinded manner, and drop-out rates were comparable across treatment groups.

One of the inclusion criteria was a minimum age of 70 years, and in practice we enrolled a study population with a mean age of 82 years. The age of this population might partially explain our null finding since recent studies showed that predictive effects of homocysteine on cognitive decline have mainly been observed in the age category of 60 to 80 years.[24-26] In general, elderly people suffer from various chronic

diseases. Approximately 50% of participants in the efficacy trial lived in a care facility home compared to 10% in the general Dutch population. The elderly living in care facility homes suffered more frequently from several chronic disorders than free living elderly people did. However, correcting our cross- sectional and mixed model analyses for these co-morbidity factors did not modify our results.

Cobalamin supplementation

Application mode

We had to make a decision with respect to the form and route of cobalamin supplementation. Crystalline cyanocobalamin was selected as this synthetic form is used in the majority of oral supplements. Moreover, this form of cobalamin is widely available and more heat and light stable compared to hydroxocobalamin. However, in the human body, cyanocobalamin binds to serum proteins less well and is excreted more rapidly than hydroxocobalamin[27] in people with normal renal function. In a recent trial[28] the effects of hydroxo- and cyanocobalamin were compared, and results revealed a five times higher cobalamin concentration after intravenous injections with hydroxocobalamin compared to cyanocobalamin. Still, cyanocobalamin reached equal reductions in homocysteine concentrations as hydroxycobalamin did. We therefore assume that the use of cyanocobalamin resulted in optimal effects on the tissue markers homocysteine and methylmalonic acid in our trials.

With respect to the route of administration, we have chosen for oral supplementation as high oral doses have been shown to be as effective as injections. Moreover, the use of oral supplements was a more convenient strategy for both the participants and researchers. To our knowledge, the efficacy of injections versus oral supplements has not been studied before in the same study population. It would have been worthwhile to study the effects of a single 1 mg cobalamin injection at the end of the intervention period of the dose-finding study. This additional intervention could provide information on changes in concentrations of cobalamin markers 2-4 weeks after injection, and would enable us to compare and confirm the effects of oral supplements with intramuscular injections. When interpreting the results after injections as the gold standard, this information would have strengthened our findings with respect to the selection of the dose. Unfortunately, there were a number of barriers which could not be overcome to extend our dose-finding trial with a single injection and blood collection.

Not much is known about the kinetics within the first days after supplementation with injections and supplements. Consequently, the ratio of plasma cobalamin status after intramuscular injections versus oral supplementation is unclear. One study showed a 20 fold increase in serum cobalamin concentrations within the first day after intramuscular injections, and concentrations gradually flattened off.[29] Treatment with daily oral supplements results in a constant delivery in the blood stream and replete tissue stores. Although both routes of supplementation are effective in correcting cobalamin deficiency, it is possible that, unlike oral supplements, intramuscular injections results in a short high peak of cobalamin in serum. This situation may consequently overcome a certain threshold that may lead to a temporarily optimal cobalamin concentration in the cerebrospinal fluid and brain, where it is needed for brain-mediated functions such as cognitive functions. As indicated earlier, cobalamin injections did improve

cognitive performance in the pilot study[1], and it is possible that the discrepancy between results of this pilot study and the preset study may partly be explained by this reason.

Dosage of cobalamin supplements

The aim of supplementation was to optimize concentrations of cobalamin markers on the blood level and on the tissue level. Therefore, this paragraph addresses the selection of the dose, and changes in concentrations during and after supplementation.

The recommended daily intake (RDI) from food which was defined by the Institute of Medicine (United States 1998) is set to 2.5 micrograms of cobalamin per day.[30] This RDI accounts for adults, but does not meet requirements for elderly who have low cobalamin status. On scientific grounds there were no indications which dose would be optimal to normalize cobalamin deficiency prior to designing the dose-finding trial. The late Victor Herbert suggested that an oral dose of 100 µg would be sufficient to normalize cobalamin deficiency (personal communication, IUNS 2001). Furthermore, he proposed that all elderly would need a daily oral dose of 25 to 100 µg.[31] Within our dose-finding study, dose considerations were based on the recommended daily allowance (RDI: 2.5 µg per day) of The Netherlands for cobalamin intake, and on the amount of cobalamin in intramuscular injections (1,000 µg per injection on a monthly basis; which was considered to result in a maximum effect). To estimate a dose-response curve, the intermittent doses should, according to pharmacokinetics, approximately equally be distributed on a log scale. We therefore included 100, 250 and 500 µg per day as additional interventions for accurate curve-fitting. The point estimations of the effects of 2.5 µg and 1000 µg cobalamin are of major importance for optimal curve fitting, as are the amount of doses in between. Consequently, curve fitting, and thus estimation of the optimal dose, becomes more accurate with increasing amounts of doses included. Depending on how we assessed the dose that normalized cobalamin deficiency (closed test procedure or 80% vs. 90% of maximum estimated effect according to curve fitting), we found a dose of 500 to 1000 µg needed to correct elevated plasma MMA concentrations, which is much higher than the dose of 25 to 100 µg which was proposed by Victor Herbert. However, a recent study examined the relation between cobalamin intake and plasma markers for cobalamin status. This study showed that an intake of 6 µg cobalamin /day saturated markers of cobalamin status in postmenopausal women aged 41 to 75 years who did not have a cobalamin deficiency.[32]

This thesis presents changes in cobalamin markers during and after cessation of supplement use.

The dose-finding study (chapter 2) showed that the effects of treatment on different markers for cobalamin status depend on the dose and duration of supplementation. When comparing markers of cobalamin status of participants in the lowest dose group through the highest dose group, concentrations, concentrations of MMA and HoloTC demonstrate a plateau effect, whereas cobalamin and tHcy did not. It is likely that the fraction of cobalamin bound to transcobalamin II becomes saturated with increasing doses, which results in plateau effects for holoTC. Moreover, it might be hypothesized that the fraction of cobalamin entering the cell via holoTC is in favor for the methionine synthase reaction

rather than for the methylmalonyl-CoA mutase reaction. This could consequently explain the plateau effects for MMA and the linear effects for tHcy. Concentrations of MMA and tHcy did not change after two to three months of supplementation, whereas concentrations of cobalamin and holoTC increased in the latter part of the intervention studies after combined cobalamin supplementation with folic acid. Surprisingly, concentrations of cobalamin and holoTC increased more after combined supplementation than after cobalamin supplementation alone.

Once cobalamin enters the blood stream, cobalamin can cross the blood brain barrier, the blood cerebrospinal fluid barrier, and the cerebrospinal fluid brain barrier by active transport mechanisms.[33] This is reflected by the fact that the human brain is relatively rich in cobalamin.[34] A number of studies reported low cobalamin concentrations in both the serum and cerebrospinal fluid (CSF) in elderly with severe cognitive impairment.[35-42] Moreover, in cobalamin deficiency CSF cobalamin concentrations are low[35-42], and CSF concentrations of tHcy and MMA are elevated.[43, 44] These abnormalities could induce modifications in brain-mediated functions and processes. Our research showed that daily supplementation with 1,000 µg cobalamin corrected plasma markers of mild cobalamin deficiency. However, it is unclear if concentrations of these markers in CSF were corrected, and thereby optimised brain metabolism.

Our post supplementation study (chapter 3) revealed a parallel decrease of serum cobalamin and holoTC concentrations after cessation of supplement use. These decreases precede the attainment of tissue cobalamin depletion as indicated by increase in MMA concentrations. When monitoring changes in markers during supplement use and after cessation, plasma concentrations of the cobalamin and holoTC increase during supplementation and are the first to decline after cessation. In contrast, concentrations of MMA and tHcy remained stable during supplementation, indicating that tissue cobalamin status was replete, and that this status remained sufficient for a longer period than the plasma markers.

Compliance

Participants were advised to take their daily capsule with a meal, because some reports have suggested enhanced uptake with a meal due to simultaneously production of the intrinsic factor.[45] We believe that our efforts to keep participants motivated by regular telephone contact and newsletters resulted in high compliance of capsule intake and motivated participation in the cognitive performance sessions. Moreover, nurses monitored daily capsule intake by the participants who lived in a care facility home. Indeed, the high compliance of capsule intake according to returned capsules and diaries was reflected in changes in plasma concentrations. Furthermore, an evaluation after the trial indicated that elderly mainly participated in order to get more insight in their own health status, or to help future generations by the results of the trials.

Neuropsychological assessment

One of the strength of our trial lies in the composition of the cognitive test battery adopted in our research. This test battery covered a broad range of cognitive domains46 and included tasks that ranged from very easy to very difficult to perform.[47] Three out of the five domains were assessed by at least 2

tests that measured different aspects and/or degrees of complexities, which has been proposed as the preferred method of cognitive testing.48 In our trial, we calculated a compound cognitive score for each domain. The advantages of compound scores are the reduction of measurement errors by possible floor- and ceiling effects from difficult and easy tests respectively, to be able to account for missing data, data reduction and thereby reducing chance findings, and finally, a better sensitivity to measure cognitive changes.[48, 49]

Cognitive testing in a standardized way has several advantages. It is relatively easy and cheap to perform, it is generally well tolerated by participants, and it reflects functioning in general practice. For some individuals (n=7), cognitive testing was experienced as inconvenient and a reason to withdraw from the trial within the run-in period. Cognitive testing has also some limitations, including difficult comparison with norm populations, learning effects and interviewer effects. [49]

Within our intervention trial, we used parallel versions of tests whenever available. Although we cannot exclude improved cognitive performance as a result of repeated testing, we consider possible learning effects to be small. For example, we adopted a parallel version of the 15 Word Learning test, and assume it is unlikely that individuals remember the complex Figure of Rey after 6 months. If logistics would allow, future studies might consider a duplo measurement within a 2-4 weeks from the baseline measurement to get a better understanding of presence of learning effects and control for it in statistical analysis. The average of these two measurements can be used in analysis in order to minimize possible learning effects at the start of the intervention trial. In addition to learning effects, a duplo measurement could also reduce the effects of regression to the mean, but we consider these effects to be small since we used compound scores as an outcome for cognitive function. Results of cognitive testing may also partly depend on the interviewer and the way tests are administered. In our trial, assessment occurred in a standardized way, but due to the logistics we were not able to test each participant twice by the same neuropsychologist. Although we expected on beforehand the effects of the six different neuropsychologists to be negligible, cross sectional analysis revealed effects of the six neuropsychologists on test results. In order to analyze our data as properly as possible, the effects of cobalamin supplementation on cognition were analyzed by means of mixed models in which random effects of neuropsychologists were included. These random effects appeared to be negligible in studying the differences in effects from treatment groups.

Results from cognitive testing do not only depend on brain-mediated functions, but also on a number of factors other than cognitive functions. Age, sex, educational level, fatigue, use of medication, visual and hearing problems and co-morbidity factors also influence cognitive performance.[50] We therefore adjusted our cross sectional analysis for these factors and found that age and education were the main modifying factors. We did not correct our efficacy analysis for age, sex and education because age and sex were already taken into account in the randomization procedure, and education appeared to be comparable across intervention groups.

State of alertness, mood, physical well-being, and motivation also affect performance on cognitive tests.[46] We stressed the importance of motivation during the recruitment procedure. Unfortunately, modification by state of alertness, mood and physical well being could not be controlled for. This may have affected results from cross sectional data analysis, although we believe that these effects are negligible in the efficacy study, since the results in the intervention groups could be compared to those in the placebo group.

As outlined previously, cognitive functions are related to a variety of different brain-mediated functions and processes, and results of cognitive assessment depend on many other factors than cognition. Therefore it could be proposed to measure both neuropsychological and neuropathological signs. The neuropathological signs would be reflected in the effects of cobalamin supplementation on more 'hard' endpoints such as cerebral activity and changes. Previous studies indicated a lower cerebral activity in people with cobalamin deficiency compared to individuals with an adequate status.[36] Moreover, cobalamin supplementation improved cerebral activity.[1, 36, 51] We cannot exclude altered cerebral activity in our participants, in spite of the fact that we did not observe effects on cognitive performance.
Although results of previous studies on brain activity are promising for the effects of cobalamin treatment, no improvement for daily living is to be expected when cognitive improvement is not experienced or measured by a cognitive test battery. Nonetheless, future studies might take MRI scans and EEG's into account to actually measure changes in brain activity.

CONCLUSION

Our recruitment activities showed that 26.6% (367 out of 1382 screened) of the older people had mild cobalamin deficiency according to our criteria for mild cobalamin deficiency. Although cross sectional analysis revealed associations of impaired cobalamin status with poorer cognitive performance, correction of mild cobalamin deficiency was not accompanied by improved cognitive performance. The latter null finding may be explained by the high age of our study population and the relatively short study duration. Since our trial cannot exclude beneficial effects on disorders related to other functions of cobalamin, such as hematopoiesis and neuropathologic disorders, general practitioners should still monitor cobalamin status of elderly people and treat those with a cobalamin deficiency.
Taken all the pieces of evidence together from our research and recent other trials, the challenge for future studies which aim to unravel the clinical relevance of cobalamin deficiency is to pay due attention to the selection of the study population and study duration, application mode and dosage of supplementation, and outcome measures.

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Samenvatting

Mensen zijn voor hun vitamine B12 inname voornamelijk afhankelijk van dierlijke voedingsmiddelen zoals vlees, melk en eieren. Wie gezond eet, krijgt normaal gesproken voldoende vitamine B12 binnen. Desondanks kan de opname van vitamine B12 bij ouderen onvoldoende zijn. Zij hebben vaak problemen met het vrijmaken van vitamine B12 uit het voedsel, of met het opnemen van vitamine B12 in de darm. Dit probleem komt bij ongeveer 25% van de mensen ouder dan 65 jaar voor en ontstaat onder andere door veranderingen die optreden in het maagdarmkanaal tijdens het ouder worden. Als vitamine B12 niet meer in voldoende mate opgenomen kan worden in het lichaam ontstaat er op den duur een tekort aan vitamine B12. Dit tekort, ook wel vitamine B12 deficiëntie genoemd, zou kunnen leiden tot een verhoogde kans op bloedarmoede en cognitieve problemen (bijvoorbeeld geheugenklachten). Het onderzoek zoals beschreven in dit proefschrift had als doel de effectiviteit van oraal ingenomen vitamine B12supplementen op zowel het bloedbeeld als de cognitieve prestaties bij ouderen met een mild vitamine B12 deficiëntie te onderzoeken.

Vitamine B12 status voor, tijdens en na het gebruik van vitamine B12 supplementen

Volgens de meest recente inzichten zou het vaststellen van vitamine B12 status niet slechts gebaseerd moeten zijn op de serum vitamine B12 concentratie. Deze concentratie weerspiegelt namelijk zowel actieve als inactieve vormen van vitamine B12 in het bloed. Op basis van de gehanteerde norm voor een vitamine B12 deficiëntie (< 150 pmol/l) wordt een vitamine B12 deficiëntie niet voldoende herkend. Daarom is het van belang de concentraties van andere, nauw met vitamine B12 concentratie samenhangende indicatoren in het bloed te betrekken in de diagnose van een vitamine B12 deficiëntie. Deze indicatoren zijn methylmalonzuur, homocysteïne en holotranscobalamine. Zowel de methylmalonzuur als de homocysteïne concentratie zijn voor hun metabolisme afhankelijk van vitamine B12. Ze zijn daarom verhoogd als sprake is van een vitamine B12 deficiëntie. Homocysteïne-waarden zijn echter ook verhoogd als sprake is van een foliumzuurdeficiëntie. Vandaar dat van deze twee indicatoren methylmalonzuur momenteel wordt gezien als een meer betrouwbare marker voor vitamine B12 deficiëntie dan homocysteïne. Transcobalamine is het eiwit dat het actieve deel van vitamine B12 via de bloedbaan naar de weefsels transporteert, alwaar vitamine B12 nodig is voor biochemische reacties. Lagere concentraties van transcobalamine zouden eveneens kunnen duiden op vitamine B12 deficiëntie. Het onderzoek zoals beschreven in dit proefschrift heeft deelnemers geselecteerd op basis van vitamine B12 concentraties in combinatie met verhoogde concentraties methylmalonzuur. In totaal bleken 367 van de 1382 (26.6%) onderzochte mensen aan deze criteria te voldoen.

In hoofdstuk 2 staan de resultaten van een studie naar de minimaal effectieve dosering vitamine B12 die verlaagde concentraties van vitamine B12 en transcobalamine in het bloed enerzijds en verhoogde concentraties van methylmalonzuur en homocysteïne in het bloed anderzijds effectief 'normaliseert' bij mensen met een lage hoeveelheid vitamine B12 in het bloed. Alle van de in totaal 120 deelnemers ontvingen dagelijks een capsule met een bepaalde hoeveelheid vitamine B12 gedurende 4 maanden. De deelnemers werden willekeurig verdeeld over 5 groepen en iedere groep kreeg een andere dosering:

2.5 microgram, 100 microgram, 250 microgram, 500 microgram en 1000 microgram. Deze doseringen reduceerden de methylmalonzuur concentraties met respectievelijk 16%, 16%, 23%, 33% en 33%. Indien we onze conclusie zouden baseren op de toegediende doseringen, kan men concluderen dat 500 microgram de hoeveelheid methylmalonzuur effectief normaliseert. Echter, berekening van de geschatte maximum reductie in methylmalonzuur concentraties met 80% tot 90% wijst uit dat een dagelijkse hoeveelheid van respectievelijk 647 microgram tot 1032 microgram vitamine B12 nodig is voor een effectieve reductie in methylmalonzuur concentraties. Deze doseringen zijn meer dan 200 keer hoger dan de aanbevolen dagelijkse hoeveelheid van ongeveer 3 microgram. Dit grote verschil kan verklaard worden doordat dat ouderen met een vitamine B12 deficiëntie vaak het vitamine uit de voeding niet in voldoende mate kunnen opnemen en slechts 1% van de hoeveelheid vitamine B12 in de capsules opgenomen wordt. De conclusie van dit onderzoek heeft dan ook alleen betrekking op ouderen met een vitamine B12 deficiëntie en niet op de totale ouderenpopulatie.

Er is niet veel bekend over de duur van het effect van vitamine B12 suppletie. Daarom onderzochten we de veranderingen in vitamine B12, holoTC en methylmalonzuur concentraties in het bloed nadat deelnemers gestopt waren met het innemen van de capsules in hoofdstuk 3. Bloed werd afgenomen 3, 5, of 7 maanden nadat deelnemers gestopt waren met het innemen van de vitamine B12 capsules. De hoeveelheden vitamine B12 en holotransvitamine B12 daalden met respectievelijk 43% (P < 0.0001) en 55% (P < 0.0001) binnen de eerste 3 maanden. Na deze 3 maanden bleven de hoeveelheden vitamine B12 en transcobalamine stabiel. De hoeveelheid methylmalonzuur daarentegen nam met 15% (P = 0.01) toe binnen 5 maanden, en met 50% (P = 0.002) binnen 7 maanden. Daarom luidde de voornaamste conclusie van dit onderzoek dat gebruik van vitamine B12 supplementen een voldoende vitamine B12 status kunnen bieden tot een periode van 5 maanden na het stoppen van de supplementen.

De relatie tussen vitamine B12 status en cognitieve prestaties voor en tijdens het gebruik van vitamine B12 supplementen

Vitamine B12 deficiëntie kan zich op uiteenlopende wijzen manifesteren. Zo kan een tekort aan vitamine B12 zich niet alleen uiten in bloedarmoede, polyneuropatie of gecombineerde strengziekte, maar ook in neuropsychologische stoornissen, waaronder stoornissen in de cognitie. Het onderzoek beschreven in dit proefschrift richt zich op de relatie tussen vitamine B12 status en cognitie. Onder het begrip "cognitie" verstaan we alle processen die een belangrijke rol spelen bij de verwerking van informatie. Hierbij kan gedacht worden aan onder andere de cognitieve functies aandacht, snelheid, praxis, geheugen en executieve functies, zoals onderzocht in dit proefschrift.

Uit een toenemend aantal studies blijkt een relatie te bestaan tussen vitamine B12deficiëntie en cognitie. Echter, deze studies laten tegenstrijdige resultaten zien. Deze verschillen kunnen mogelijk verklaard worden door het feit dat deze studies verschillende indicatoren voor vitamine B12 status hebben onderzocht en tevens uiteenlopende cognitieve testbatterijen hebben gebruikt. Daarom onderzochten

we in hoofdstuk 4 of er een associatie gevonden kon worden tussen indicatoren voor vitamine B12 status en speficieke cognitieve functie domeinen. Om dit te onderzoeken hebben we een uitgebreide cognitieve testbatterij gebruikt. De resultaten van dit onderzoek wezen uit dat ongunstige hoeveelheden van indicatoren voor vitamine B12 status in het bloed samen hingen met verminderde cognitieve vaardigheden bij een aantal cognitieve functies.

De resultaten van voorgaande onderzoeken en de resultaten als beschreven in hoofdstuk 4 vestigen de aandacht op de mogelijke rol van vitamine B12 bij het ontstaan van cognitieve problemen. Een eerste pilotstudy van Dieneke van Asselt laat zien dat injecties met vitamine B12 bij ouderen zonder ernstige cognitieve problemen, maar wel met lage hoeveelheden vitamine B12 in het bloed, tot een verbetering in cognitieve functies kan leiden. Echter, deze studie was uitgevoerd bij 16 ouderen zonder dat er een controlegroep was opgenomen in het studiedesign. Daarom diende deze resultaten bevestigd te worden in een groter placebo gecontroleerd onderzoek zoals beschreven in hoofdstuk 5. De hypothese van dit onderzoek luidde dat orale vitamine B12 suppletie het cognitief functioneren kan verbeteren bij ouderen met een mild vitamine B12 deficiëntie. Aan dit onderzoek namen in totaal 195 ouderen met een vitamine B12 deficiëntie deel. De deelnemers werden willekeurig verdeeld over 3 groepen. Zij ontvingen gedurende 24 weken dagelijks een capsule met 1 milligram vitamine B12, of een combinatie van 1 milligram vitamine B12 met 400 microgram foliumzuur, of een placebo (=capsule zonder vitamine B12). Door middel van een uitgebreide testbatterij met gevoelige testen voor de cognitieve functies van aandacht, constructie, sensomotorische snelheid, geheugen en executieve functies onderzochten we het effect suppletie op deze cognitieve functie domeinen. De vitamine B12 bevattende capsules corrigeerden het vitamine B12 deficiëntie. Het geheugen verbeterde in alle drie de onderzoeksgroepen. Echter, deze verbetering bleek groter in de groep deelnemers die een placebocapsule ontvingen dan in de groep deelnemers die een vitamine B12 capsule ontvingen. Dit effect zou verklaard kunnen worden door het zogenaamde "placebo effect". Vitamine B12 had geen effect op de cognitieve functies van aandacht, snelheid, constructie en executieve functies. Daarom concluderen we dat het gebruik van vitamine B12 supplementen gedurende een periode van 24 weken de cognitieve functies van ouderen met een milde vitamine B12 deficientie niet verbetert.

Conclusie
De belangrijkste conclusie van dit proefschrift luidt dat vitamine B12 suppletie de cognitieve functies niet verbetert bij ouderen met een vitamine B12 deficiëntie. De meest voor de hand liggende verklaringen zijn de hoge leeftijd van de onderzochte groep deelnemers en de relatief korte studieduur. Mogelijk hebben "jongere ouderen" wel baat bij langdurigere vitamine B12 suppletie, maar dit dient toekomstig onderzoek uit te wijzen. Ondanks het feit dat ons onderzoek geen verbetering in cognitieve functies heeft aangetoond, is het toch belangrijk dat vitamine B12 status bij ouderen wordt gevolgd. Indien nodig, dient een vitamine B12 deficiëntie behandeld te worden gezien de andere belangrijke functies die vitamine B12 in het lichaam vervult.

Summary

With increasing life expectancy across the world, the number of elderly who suffer from cognitive impairment and dementia also increase. Knowledge of how to get older in good mental health may benefit quality of life of elderly people. The evidence to date suggests that, even in old age, improvements in nutritional status may improve cognitive functioning. Cobalamin deficiency is a particular problem in the aging population due to its high prevalence. It is associated with anemia, cerebrovascular diseases, and several neurological disorders, such as neuropathy, myelopathy, depression, and cognitive impairment.

Cobalamin status before, during, and after supplementation

We found that 26.6% (367 out of 1382) of the elderly people who were not supplemented had mild cobalamin deficiency. Reasons for the high prevalence of cobalamin deficiency are not fully understood, but include atrophic gastritis and bacterial overgrowth which affect the absorption of food-bound cobalamin. However, the ability to absorb crystalline cobalamin, e.g. the form found in fortified foods or supplements, remains intact in old age. Supplementation with high oral doses of crystalline cobalamin is as effective as cobalamin administered by intra-muscular injection to correct plasma markers of cobalamin deficiency. Increased concentrations of methylmalonic acid (MMA) and total homocysteine (tHcy), and reduced concentrations of holo-transcobalamin (holoTC) are established as useful diagnostic indicators of cobalamin deficiency. Although high doses of oral cobalamin are proven to be as effective as cobalamin injections, the efficacy of lower oral doses of cobalamin on these markers are uncertain. We therefore investigated the lowest oral dose of cobalamin required to normalise biochemical markers of cobalamin deficiency, with MMA was our main outcome measure. A randomized, parallel group, double-blind dose-finding trial assessed the effects of daily oral doses of 2.5, 100, 250, 500 and 1,000 µg of vitamin B12 on biochemical markers for vitamin B12 deficiency administered for 16 weeks in 120 people. Supplementation with vitamin B12 in daily oral doses of 2.5, 100, 250, 500 and 1,000 µg were associated with mean reductions in plasma methylmalonic acid (MMA) concentrations of 16%, 16%, 23%, 33% and 33%, respectively. Daily doses of 647 µg to 1032 µg of vitamin B12 were associated with a decrease of 80% to 90% of the estimated maximum reduction in plasma MMA concentration. These doses were over 200 hundred times greater than the recommended dietary allowance, which is about 3 µg daily (Chapter 2).

Little is known about the duration of the effects of oral treatment with cobalamin in elderly people with mild cobalamin deficiency. We therefore investigated the changes in markers of cobalamin status after cessation of oral cobalamin supplementation in participants treated with 1000 µg/day. In addition, this gave us a unique opportunity to compare the sensitivity of different markers of cobalamin status by monitoring early changes in markers after cessation of supplementation, during a period in which participants gradually attain a negative cobalamin balance. Participants provided one single blood sample at 3, 5 or 7 months after cessation of study supplements to determine concentrations of cobalamin, holoTC, and MMA. Cobalamin status was assumed to be replete at the end of the 6 month supplementation period. Plasma cobalamin declined by 43% (P <0.0001) and holoTC by 55% (P <0.0001) within

the first 3 months after cessation, with no significant further decline thereafter. Within the same period, mean MMA increased by 15% (P = 0.07) within the first 5 months, and by 50% (P = 0.002) after 7 months. There was a parallel decrease of serum cobalamin and holoTC concentrations, which preceded the attainment of tissue cobalamin depletion, as measured by increase in MMA concentrations. We concluded that oral supplementation may maintain adequate cobalamin status for a period of up to 5 months after cessation (Chapter 3).

Association of cobalamin status with cognitive function before and during supplementation

In both healthy and cognitively impaired elderly people associations between cobalamin status and cognitive performance have been observed. However, the published data on the associations between different markers for cobalamin and folate status and cognitive performance are inconsistent. These discrepancies could be explained by the fact that various markers of cobalamin status and various neuropsychological test batteries have been used in these studies. We therefore investigated whether there were any associations between cobalamin and folate status and specific cognitive domains by using sensitive markers for cobalamin and folate status and an extensive neuropsychological test battery. Our cross sectional analysis, adjusted for age, education and interviewers in 242 people aged > 70 years revealed significant associations (P for trend < 0.05) for cobalamin with sensomotor speed and executive function; holoTC with sensomotor speed; MMA with attention; tHcy with construction, sensomotor speed and executive function; and RBC folate with attention, sensomotor speed, memory and executive function. These results suggest that impaired folate and cobalamin status were associated with impairments of some cognitive domains (Chapter 4).

Although cobalamin deficiency is associated with cognitive impairment in older people, the evidence for the effects of cobalamin supplementation on cognitive function is limited and inconclusive. Therefore, the main purpose of our research was to investigate whether daily supplementation with high doses of oral cobalamin alone or in combination with folic acid has any beneficial effects on cognitive function in people aged 70 years or older with mild cobalamin deficiency. In a double-blind, placebo-controlled trial, 195 individuals were randomized to receive either 1,000 μg cobalamin, or 1,000 μg cobalamin plus 400 μg folic acid, or placebo for 24 weeks. Markers for cobalamin status were assessed before, and after 12 and 24 weeks of treatment. Cognitive function was assessed before and after 24 weeks of treatment using an extensive neuropsychological test battery that included the domains of attention, construction, sensomotor speed, memory and executive function. Cobalamin status was unchanged after treatment in the placebo group, and oral cobalamin supplementation corrected mild cobalamin deficiency. Improvement of memory function was observed in all treatment groups, and was greater in the placebo group than in the group who received cobalamin alone (P = 0.0036). Neither supplementation with cobalamin alone or in combination with folic acid was accompanied by any improvement in other cognitive domains (Chapter 5).

The role of compounds involved in the one-carbon metabolism other than cobalamin, folate and homocysteine in cognition function is unknown. We therefore also explored the relation between plasma concentrations of the one-carbon metabolites methionine, choline, betaine and dimethylglycine (DMG), and cognitive function. Cross sectional analysis revealed positive associations of betaine with the domains of construction, sensomotor speed and executive function, whereas elevated concentrations of methionine were positively associated with sensomotor speed. Daily oral supplementation of 1,000 µg cobalamin with 400 µg folic acid for 6 months increased betaine concentrations by 38%, which indicates that the two remethylation pathways to convert homocysteine into methionine are interrelated. Those participants with the largest increases in betaine concentrations showed a borderline significant higher increase in memory performance (P = 0.07) compared to others (Chapter 6).

Conclusion

The main conclusion of this thesis is that oral crystalline cobalamin supplementation with and without additional folic acid does not improve cognitive performance in elderly people. This finding was found, despite adopting a larger sample size with a longer study duration and more rigorous methods to assess cognitive function compared to previous smaller, non-randomized placebo controlled trials. Since our trial cannot exclude beneficial effects on disorders related to other functions of cobalamin, such as hematopoiesis and neuropathologic disorders, general practitioners should still monitor cobalamin status of elderly people and treat those with a cobalamin deficiency. Taken all pieces of evidence together from our research and recent other trials (chapter 7; general discussion), the challenge for future studies which aim to unravel the clinical relevance of cobalamin deficiency is to pay due attention to the selection of the study population and study duration, application mode and dosage of supplementation, and outcome measures.

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Simone

About the author

Curriculum vitae

Simone Eussen was born on February 13, 1975, in Sittard, the Netherlands. After completing secondary school in 1995 at the 'Open Leercentrum' in Sittard, she studied 'Human Nutrition' at Wageningen University. As part of that study, she carried out an internship at 'TNO Voeding' in Zeist where she carried out a literature study on functional markers of endothelial function (1998). Her first MSc thesis (1999) was carried out at the 'Department of Human Nutrition' in Wageningen in which she studied the relation between trans fatty acids on endothelial function. For her second MSc thesis (2000) she investigated the efficacy of different doses of iodised peanut oil on iodine status of lactating women and their infants in a rural area in Guatemala at the 'Centre for Studies of Sensory Impairment, Aging and Metabolism'. In November 2000, she received her MSc-degree. The Wageningen University appointed her in 2001 as a PhD-fellow to conduct research on mild cobalamin deficiency and cognitive function in elderly people, as described in this thesis. During this period, she attended several courses and conferences within the framework of the educational program of the graduate school VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences). Among a number of activities at the 'Department of Human Nutrition', she was also a member and secretary of the daily board of the committee of temporary scientific staff. In 2005, Simone was selected for the 11th European Nutritional Leadership Programme (ENLP). Currently she continues research on B-vitamins and homocysteine as a post-doc at the Section for Pharmacology, Institute of Medicine, University of Bergen, Norway.

Publications

Full papers

- Effect of oral vitamin B12 with or without folic acid on cognitive performance in elderly people with mild vitamin B12 deficiency: a randomized, placebo-controlled trial. Eussen SJPM, de Groot CPGM, Joosten L, Bloo R, Clarke R, Ueland PM, Schneede J, Blom HJM, Hoefnagels WHL, van Staveren WA. Am J Clin Nutr. 2006; 84(2):361-370
- Effect of supplementation with cobalamin carried either by a milk product or a capsule in mildly cobalamin deficient Dutch elderly people. Dhonukshe-Rutten RAM, van Zutphen M, de Groot LCPGM, Eussen SJPM, Blom HJ, van Staveren WA. Am J Clin Nutr. 2005;82:568-74
- Oral vitamin B12 supplementation in elderly people with mild vitamin B12 deficiency: a dose-finding trial. Eussen SJPM, de Groot CPGM, Clarke R, Schneede J, Ueland PM, Hoefnagels WHL, van Staveren WA. Arch Intern Med 2005; 165: 1167-71
- Vitamine B12 en cognitieve functies. Eussen SJPM, de Groot LCPGM, Hoefnagels WHL, van Staveren WA. Voeding Nu 2004;(4):29-31 (publication in Dutch)
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- One carbon metabolites in relation to cognitive performance in Dutch elderly people. Eussen SJPM, Ueland PM, Clarke R, Blom HJM, Hoefnagels WHL, van Staveren WA, de Groot CPGM. Submitted

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- Effect of daily oral vitamin B12 and vitamin B12/folate supplementation on cognitive performance in elderly people with vitamin B12 deficiency: a randomized placebo controlled trial. Eussen SJPM, de Groot CPGM, Joosten L, Bloo R, Clarke R, Ueland PM, Schneede J, Blom HJM, Hoefnagels WHL, van Staveren WA. Haematologica Reports 2005; 1(3):49 and J Nutr. Health and Aging 2005; 9(3):148
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- Efficacy of different doses of iodized peanut oil on iodine status of lactating women and their infants in a rural mountainous area in Guatemala. Eussen SJPM, Harbers MM, de Mutsert R, West CE, van der Heide D, Bulux J, Romero-Abal ME, Solomons NW. Ann Nutr Metab 2001;45(suppl 1):40 (Abstract)

Training and supervision plan
Discipline specific activities
- Meetings "International Academy Nutrition and Aging (IANA)"; Chicago (USA, 2006), Toulouse (France, 2004), and Albuquerque (USA, 2003)
- Meetings NWO Nutrition; Arnhem (NL), 2001, 2004 and 2005
- International Conferences "Homocysteine Metabolism"; Milan (Italy, 2005), and Saarbruecken (Germany, 2005)
- Annual Vitamin Meetings of the Homocysteine LOCUS Norway; Noordwijkerhout (NL, 2005) and Londonderry (Northern Ireland, 2004)
- Symposium "En ook nog dement"; Nijmegen (NL), 2004
- Masterclass Geriatric Nutrition: "Diet, functionality and disease"; Wageningen (NL), 2004

- Symposium "Add years to life: Nutrition Matters"; Wageningen (NL), 2004
- Conference "FASEB Folic acid, vitamin B12 & one carbon metabolism"; Snowmass (USA), 2002 and 2004
- ZON Implementatiedagen; The Hague (NL), 2003
- Twelfth Alzheimer Europe Conference; Maastricht (NL), 2002
- Symposium "Senioren en Voeding"; Wageningen (NL), 2002
- Symposium "Homocysteine, folate and vitamin B12 in cardiovascular and neurological diseases"; UMC St Radboud, Ravenstein (NL), 2001
- SENECA-HALE meeting; Wageningen (NL), 2001
- Seventeenth International Congress on Nutrition (IUNS); Vienna, 2001
- "Nutrition and Lifestyle Epidemiology"; VLAG advanced course, Wageningen (NL), 2001
- Symposium on Geriatric topics; Arnhem (NL), 2001

General courses
- Talent Classes "Media training" and "Subsidies"; NWO, The Hague (NL), 2005
- Course "Mixed Models and missing data"; Brussels (B), 2005
- Good Clinical Practise, NUTRIM, Maastricht (NL), 2005
- European Nutritional Leadership Programme (ENLP), Luxembourg (L), 2005
- Course "Cognitive Neuropsychology", Utrecht (NL), 2003
- Written English and Scientific Writing courses, Wageningen University, (NL), 2003
- Epidemiologic data analysis by K. Rothman; Bilthoven (NL), 2002
- Systematic literature research; NUTRIM, Maasticht (NL), 2001
- VLAG PhD week, Bilthoven (NL), 2001
- Organizing and supervising thesis work, Wageningen University (NL), 2001

Optional courses and activities
- Oldsmobiles, Wageningen University, 2001-2006
- Homocysteine Club, Wageningen University, 2001-2005
- Journal Club, Wageningen University, 2001-2004
- PhD study tour to Switzerland, Italy, Germany, and the United Kingdom, Wageningen University, 2001 and 2005

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