

# **Cognitive Performance in Relation to Folate and Cobalamin Status in Nondemented Patients at a Memory Clinic**

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## Abstract

One possible symptom of deficiency of folic acid (folate) or vitamin B<sub>12</sub> (cobalamin) is impaired cognition. Status of these two B-vitamins have been linked to cognitive performance also in various populations of subjects with no evident clinical deficiency (for example in “healthy elderly”). The present study investigated 60 nondemented patients (mean age 58 years) presented with mild cognitive impairment (MCI) or subjective memory complaints. Blood/serum folate, serum cobalamins, and total plasma homocysteine were determined and compared to age-normalized results on 14 cognitive tasks. 21 of the patients were re-examined one year later and follow-up data were compared to baseline data. Results for cobalamin and folate were inconclusive for baseline data, but for homocysteine significant associations were found for tasks of visual episodic memory and for perceptual speed and attention (higher concentration related to worse cognitive performance). In the longitudinal data cobalamin and folate were positively related to word fluency. The outcome is consistent with a negative long-term influence of homocysteine on cognition. However, from the present data it is not possible to conclude if the results are specific for the studied population.



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## Introduction

The importance of vitamins for the human body is well-known to most people. The name *vitamin*, proposed by the Polish chemist Casimir Funk in 1912 after he had identified a substance that interrupted the progress of the disease beriberi, comes from a combination of the Latin word *vita*, “life”, and *amine*, a member of the group of organic compounds (amines) that Funk’s substance belonged to. He had identified thiamine (vitamin B<sub>1</sub>), of which deficiency leads to the severe disease beriberi. Common to all vitamins is that they are essential for functions of the body, and that they must be supplied through dietary intake. Other well-known diseases due to vitamin deficiency is, for example, night blindness and scurvy, caused by shortage of vitamin A (retinol) and vitamin C (ascorbic acid), respectively. For general information about vitamins see Hambraeus (1996) or Machlin (1984) (for professionals).

### Vitamin deficiency and cognition

As already mentioned thiamine deficiency might result in beriberi, which symptom pattern includes neurological and psychiatric manifestations (for example psychosis) (VeriMed Healthcare Network (2002, August 2)). In people suffering from chronic alcoholism thiamine deficiency often results in Wernicke-Korsakoff’s syndrome, with amnesia as a prominent feature (Banich, 1997; VeriMed Healthcare Network (2002, July 28)). Dementia is a characteristic symptom of pellagra, a disease that might develop as a consequence of lack of the vitamin niacin (VeriMed Healthcare Network (2001, April 10)). It is also well known that a variety of mental symptoms, cognitive signs included, might develop in cases of deficiencies of the B-vitamins folate (folic acid) and vitamin B<sub>12</sub> (cobalamin). (For overviews see Hutto (1997); Rosenberg (2001); and Selhub, Bagley, Miller, and Rosenberg (2000).) This report is devoted to these two B-vitamins\*.

### Biochemistry of folate and cobalamin

#### Intake and uptake

Main food sources of folate (folic acid) are cereals and vegetables, but also meat products and fish contain considerable amounts. After intake the vitamin is absorbed in the small intestine and then transported in the blood plasma to the liver. In the liver it is converted to its bioactive forms and then released back to the bloodstream for transportation out to the cells around the body. (For more general information see for example Brody (1984).)

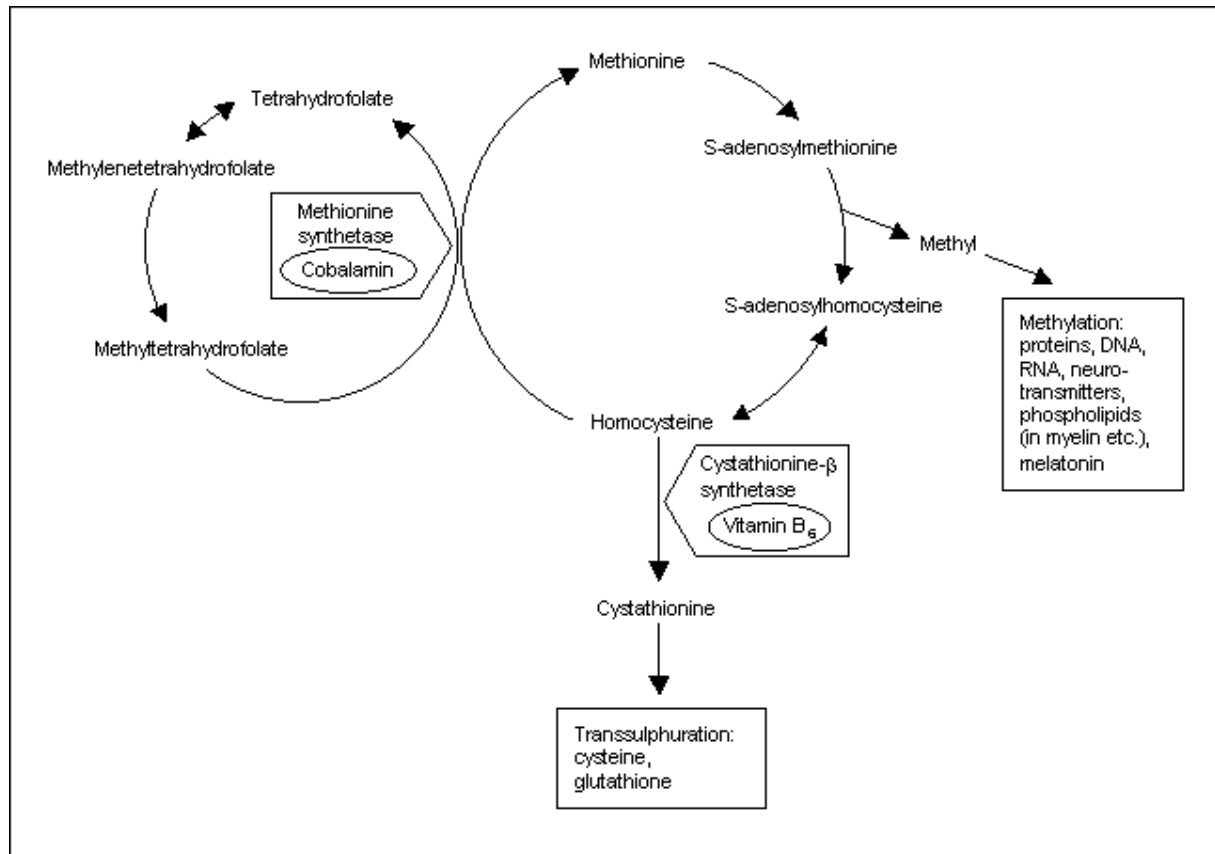
Cobalamin (vitamin B<sub>12</sub>), the last vitamin to be isolated (in 1948), is found in almost all animal (but not vegetarian) products. In difference to folate, the uptake of cobalamin depends on a complex mechanism. After ingestion cobalamin is split from dietary proteins by pancreatic enzymes. Next it binds to a molecule called intrinsic factor, which is produced in the parietal cells in the gastric mucosa. The cobalamin-intrinsic factor complex is then carried through the intestines to ileum, the distal section of the small intestine, and is there attached to special receptors in the intestinal wall. Finally, during passage through the ileal wall to the bloodstream, the complex is separated and the cobalamin binds to specific transport proteins (transcobalamin I, II, III, and haptocorrin), which transports the vitamin to places of utilization. (For more details see for example Ellenbogen (1984).)

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\* Throughout this report the terms *folate* and *cobalamin* will be used for *folic acid* and *vitamin B<sub>12</sub>*, respectively.

## One-carbon metabolism and homocysteine

In tissue both folate and cobalamin serves as coenzymes\* involved in the *one-carbon metabolism*. This metabolic cycle is schematically shown in figure 1. The name originates from that the role of these coenzymes is as acceptors and donors of one-carbon units.



**Figure 1.** Schematic figure of the one-carbon metabolism: Remethylation of homocysteine to methionine depends on the accessibility of both tetrahydrofolate, the bioactive form of folate, and cobalamin, as coenzyme in methionine synthetase. Also shown is the conversion of homocysteine to cystathionine, catalysed by the vitamin B<sub>6</sub> dependent enzyme cystathionine-β synthetase.

From figure 1 it can be understood that the one-carbon metabolism is very important in several ways. Methionine is converted to S-adenosylmethionine (SAM). Numerous transmethylation reactions depend upon SAM as a methyl donor. These reactions comprise production of proteins, DNA and RNA, neurotransmitters, phospholipids (in myelin et cetera), and melatonin. Therefore, S-adenosylmethionine is believed to be of crucial importance especially for brain tissue. Moreover, methylene-tetrahydrofolate is subsequently involved in the synthesis of thymidylate and purines (not shown in figure 1), which in turn are constituents in DNA and RNA synthesis. (For a review see Bottiglieri, Hyland, and Reynolds (1994).)

Tetrahydrofolate is the active form of folate, and insufficient delivery of folate implies that the left reaction-cycle in figure 1 cannot keep up with the right one, and therefore the remethylation of the amino acid homocysteine to methionine is impaired. When this happens a decrease in methionine, and consequently in S-adenosylmethionine, follows and homocysteine start to accumulate. Similar, a lack of cobalamin causes a disturbance of the remethylation of homocysteine to methionine, with corresponding result. Hence, homocysteine is a marker for intracellular deficiency of folate or

\* An active enzyme is a combination of a protein molecule, an *apoenzyme*, and an organic nonprotein molecule, a *coenzyme*.

cobalamin (confer for example Hultberg, Isaksson, Nilsson, & Gustafson, 2000; Lindenbaum, Healton, Savage, Brust, Garrett, et al., 1988; and Nilsson, Gustafson, & Hultberg, 1999.).

The one-carbon metabolism is discussed in Bottiglieri et al. (1994); Brody (1984); Calvaresi and Bryan (2001); Hultberg et al. (2000); Miller and Kelly (1996, November); Nilsson (2000); and Selhub et al. (2000).

Cobalamin also acts as a cofactor in another reaction, in which succinyl coenzyme A and methylmalonyl coenzyme A are converted into each other. Shortage of cobalamin leads to accumulation of methylmalonic acid, a successor of methylmalonyl coenzyme A. This reaction is described elsewhere (Ellenbogen, 1984; Hultberg et al., 2000; Nilsson, 2000).

## **Possible biological pathways from folate and cobalamin to cognition**

### **The hypomethylation hypothesis**

Figure 1 above shows that a disruption of the one-carbon cycle interfere with the synthesis of S-adenosylmethionine. As described S-adenosylmethionine serves as an essential methyl donor in a large number of reactions; in the central nervous system it is engaged in reactions involving neurotransmitters (catecholamines and indoleamines), especially monoamines such as serotonin, dopamine and noradrenaline (norepinephrine); phospholipids in myelin and on other neuronal membranes, which can alter membrane-bound receptors, second-messenger systems, and ion channels; and melatonin. Hence, it is obvious that a disturbance in availability of S-adenosylmethionine in the central nervous system potentially has a considerable impact on cognitive functioning, as well as on other psychological statuses. (The hypomethylation hypothesis is reviewed in Bottiglieri et al., 1994; Calvaresi and Bryan, 2001; Hutto, 1997; Miller and Kelly, 1996, November; Rosenberg, 2001; and Selhub et al., 2000.)

### **The homocysteine hypothesis**

The homocysteine hypothesis suggests that cognitive deterioration associated with low status of B-vitamins (in addition to folate and cobalamin also vitamin B<sub>6</sub>), is caused by the raised concentration of homocysteine *per se*. Homocysteine is a risk factor of vascular disease and even mild elevations of this amino acid in the plasma are associated with increased risk of occlusive vascular disease, stroke, and thrombosis. Impaired cognition related to high homocysteine might therefore be secondary to such kind of ailments. Further, increased homocysteine may lead to an excessive production of its successor homocysteic acid, which interferes with the function of the *N*-methyl-D-aspartate (NMDA) receptor. This receptor takes part in the function of the ion channels in the cell membranes, and in that way homocysteic acid is believed to have excitotoxic\* effects to neuronal cells. (The homocysteine hypothesis is reviewed in the reviews by Calvaresi and Bryan, 2001; Miller and Kelly, 1996, November; Rosenberg, 2001; and Selhub et al., 2000.)

There are experimental support for both the hypomethylation hypothesis and the homocysteine hypothesis (see the reviews by Bottiglieri et al., 1994; Calvaresi and Bryan, 2001; Hutto, 1997; Miller and Kelly, 1996, November; Rosenberg, 2001; Selhub et al. 2000; and also, for example, Bottiglieri, Parnetti, Arning, Ortiz, Amici, et al., 2001; Gottfries, Blennow, Lehmann, Regland, and Gottfries, 2001; Lehmann, Gottfries, and Regland, 1999; Nilsson, Gustafson, Fäldt, Andersson, Brattström, et al., 1996; Nilsson et al., 1999). Probably both hypotheses contribute to an explanation of cognitive decline linked to low status of cobalamin and folate. The main difference between their contribution to an explanation is concisely put by Calvaresi and Bryan (2001, p. P329): "It could be argued that studies examining direct or acute effects of B vitamins are consistent with the hypomethylation

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\* A substance is *excitotoxic* if it has the property of first exciting and then poisoning cells.

hypothesis and that those examining indirect or long term effects are consistent with the homocysteine hypothesis.”

## **Folate, cobalamin, homocysteine and cognition**

### **Folate and cobalamin deficiency**

#### **Causes**

The probably most common cause of folate deficiency is insufficient dietary intake, while this is only a rare cause of cobalamin deficiency. It is sometimes seen in undernourished elderly, in persons suffering from alcoholic abuse, and in long term use of strict vegetarian diet (cobalamin deficiency). Except for inadequate intake of folate, often deficiency is the result of dysfunctions in the uptake processes, or a consequence of increased utilization. The later is for example seen in the higher incidence of folate deficiency in pregnant women. Also, some drugs are known to interfere with the vitamins, and severe states of deficiency are seen in rare cases of congenital metabolic defects. (See Brody (1984) and Ellenbogen (1984) for a systematic going through of causes and factors related to deficiency of folate and cobalamin, respectively.)

Recently, researchers have put attention to age related alterations in the transport of cobalamins by the transcobalamins (Metz, Bell, Flicker, Bottiglieri, Ibrahim, et al., 1996; Sparring Björkstén, Dige, and Nexø, 2001). Such alterations in the delivery system might contribute to the high prevalence of tissue deficiency of cobalamin in the elderly population.

Other recent interest concerns the presence of genetic mutations related to the control of the one-carbon metabolism. Several genotypes predisposing development of impaired metabolism have been identified (see for example Bottiglieri et al., 2001; and Barbaux, Plomin, and Whitehead, 2000).

#### **Symptoms associated with deficiency**

Symptoms of either folate or cobalamin deficiency are very similar. This is not surprising while the two vitamins are metabolically intimately connected by the one-carbon metabolism. (For example, cobalamin deficiency leads to efficient folate deficiency by interrupting the folate cycle.) Symptoms of clinical deficiency can be divided into four categories: haematological, gastrointestinal, neurological, and mental.

This report focuses on cognition – reported examples of impaired cognitive functions involves memory, spatial abilities, problem solving, abstract thinking, attention, and word retrieval (Bernard, Nakonezny, & Kashner, 1998; Hart & McCurdy, 1971; Landis Evans, Edelson, & Golden, 1983; Larner, Janssen, Cipolotti, & Rossor, 1999; Lindenbaum et al., 1988; Louwman, van Dusseldorp, van de Vijver, Thomas, Schneede, et al., 2000; Martin, Francis, Protech, & Huff, 1992; Nilsson, Warkentin, Hultberg, Fäldt, & Gustafson, 2000; Nilsson, Gustafson, & Hultberg, 2001; Stojsavljević, Lević, Drulović, & Dragutinović, 1997). Other mental manifestations of deficiency are psychiatric as fatigue, depression, psychosis, delirium, and dementia (Botez, Botez, & Maag, 1984; Goggans, 1984; Hart & McCurdy, 1971; Landis Evans et al., 1983; Lassen & Ewald, 1985; Lindenbaum et al., 1988; Nilsson, Warkentin, et al., 2000; Stojsavljević et al., 1997; Zucker, Livingston, Nakra, & Clayton, 1981). The following articles review mental engagement in folate and cobalamin deficiency: Chiu, 1996; Hutto, 1997; Rosenberg, 2001; Selhub et al., 2000; and Zucker et al., 1981.

## **Cognitive relations to folate and cobalamin in "healthy elderly"**

In 1983 Goodwin, Goodwin and Garry published their study on *Associations Between Nutritional Status and Cognitive Functioning in a Healthy Elderly Population*, and by that a slight tradition of studies on relations between B-vitamins (and other nutrients) and cognition in "healthy elderly" subjects began. In these *healthy elderly studies* the age of the participants generally range from at least 60 years of age or older, and the meaning of the term "healthy" usually is that the physiological and psychological statuses of the participants are typical for individuals at their age, not suffering from severe diseases. In other words, inclusion criteria vary among studies, from "noninstitutionalised" to "physically healthy and cognitively intact". Typically individuals with cognitive decline severe enough to render them a diagnosis of dementia are excluded. However, subjects with mild cognitive impairment (both due to known and to unknown aetiologies), as well as subjects with subjective cognitive complaints, and subjects with perfect cognition most often fall within the realms of participating. Therefore the populations of these studies are quite disperse, as looked upon from a cognitive point of view. Nevertheless, the participants are at least to a major part free from evident deficiencies of folate and cobalamin, and generally with values of folate, cobalamin and homocysteine mainly within clinical reference limits. Hence, associations between the biochemical markers and tasks measuring various cognitive abilities would nevertheless be interesting, for the reason that it might implicate general concerns about the role of vitamins in the ageing population. However, incongruence is prevailing amongst the results of those researches, with most reporting some significant association – some not. Relations have been reported with verbal and nonverbal episodic memory, verbal semantic memory, short-term memory, verbal fluency, visuospatial construction, and tasks of perceptual speed and attention, as well as tasks on abstract thinking and problem solving. Probably there are some valid relations (though rather weak), and especially homocysteine seem to be of importance. (See Crystal, Ortof, Frishman, Gruber, Hershman, and Aronson (1994); Duthie, Whalley, Collins, Leaper, Berger, and Deary (2002); Goodwin et al. (1983); Hassing, Wahlin, Winblad, and Bäckman (1999); Hultdin, Wahlin, Bäckman, Edvardsson, Adolfsson, and Nilsson (2000); La Rue, Koehler, Wayne, Chiulli, Haaland, and Garry (1997); McCaddon, Hudson, Davies, Hughes, Williams, and Wilkinson (2001); Ravaglia, Forti, Maioli, Zanardi, Dalmonte, et al. (2000); Riggs, Spiro III, Tucker, and Rush (1996); Robins Wahlin, Wahlin, Winblad, and Bäckman (2001); Savaria Morris, Jacques, Rosenberg, and Selhub (2001); Tucker, Penland, Sandstead, Milne, Heck, and Klevay (1990); Wahlin, Bäckman, Hill, and Winblad (1997).)

## **Vitamin status and mild cognitive impairment and subjective memory complaints**

*Mild cognitive impairment* (MCI) is currently one of the most widely used concepts in clinical research in elderly individuals who run a high risk of developing dementia. However MCI is a heterogeneous clinical entity and no international consensus criteria have yet been established. In general the term mild cognitive impairment is used to describe the state of patients displaying objective impairment in cognitive functioning, as revealed by psychological testing, considered too severe to be part of normal ageing, but not severe enough to fulfil criteria for dementia. Several other names have been suggested for this dysfunction, such as "dysmentia" (see Lehmann et al. (1999)).

Another common terminology confronted with in clinical research on cognitive disturbances in elderly individuals is that of *subjective cognitive complaints*, especially *subjective memory complaints*. These expressions describe the state of patients with cognitive complaints, but without objective impairments seen in cognitive testing.

According to what is known from studies of vitamin deficient patients, and from research in cognitive associations with status of folate, cobalamin and homocysteine in "healthy elderly", one can hypothesize on a possible involvement of these substances in populations of patients with MCI and subjective cognitive complaints. However, very few articles have reported on cognitive relations to folate, cobalamin or homocysteine in comparable populations (without clinical evidence of deficiency of folate or cobalamin).

In a Canadian study by Ebly and colleagues (Ebly, Schaefer, Campbell, & Hogan, 1998) subjects older than 65 years were categorized into three groups: subjects without any cognitive loss, cognitively impaired but not demented, and demented subjects (those were further categorized according to type of dementia). The participants were assessed with the Modified Mini Mental State Examination, and their folate status was determined by a measure of serum folate. The Modified Mini Mental State Examination is similar to the Mini Mental State Examination (MMSE) and provides concise tasks of memory, abstract thinking, judgement, constructional abilities, language, and object recognition – a brief screening instrument for dementia. The population was then divided into the four quartiles of the folate distribution. Student's *t*-test and  $\chi^2$ -analyses were sequent used for comparisons among the quartile groups. It turned out that the frequency of demented subjects was highest in the quartile with lowest folate, followed by the second, and then third quartiles, and finally the quartile with highest folate status. Logistic regression showed that the differences were significant for every pair of folate quartiles. (Similar patterns were seen in subsequent analyses of the different categories of dementia.) Looked upon the quartiles from another point of view, being a member of the lowest quartile was more common in demented participants than in cognitively impaired but not demented subjects, than in cognitively intact (significant differences in all cases, according to logistic regression). Two more results relating to the group of cognitively impaired but not demented participants were stated. Within this group subjects with low folate values scored significantly lower on the Modified Mini Mental State Examination than did those with high folate status. For cognitively intact or demented subjects there were no corresponding effects. Further, also within the cognitively impaired but not demented group, short-term memory problems were more frequent ( $p = 0.004$ ) in the lowest folate quartile relative the highest quartile.

In a recent review by Calvaresi and Bryan (2001), a summary is given of a study carried out by Fioravanti and colleagues (published in 1997). The team around Fioravanti executed a placebo-controlled folate supplementing study of 30 elderly (70 – 90 years old) with subclinical levels of plasma folate (but without evident symptoms of deficiency) and "mild to moderate memory complaints". Before and after a period of two months, during which they received either folate or placebo, they were examined with tests measuring verbal and nonverbal episodic memory and attention. At baseline there were no significant relationships between folate status and cognition, as measured by the neuropsychological tests. Nevertheless, at retesting, the supplemented group performed better than the placebo group on measures of both memory and attention. Further, a difference in treatment sensitivity was noted. Participants with the lowest baseline folate status experienced the greatest cognitive improvement.

Levitt and Karlinsky (1992) examined the relationship between folate as well as cobalamin and cognitive impairment in patients with different dementias, and in patients with "mild cognitive deficits" (not demented). The mean age in the later group was  $71.0 \pm 9.9$  years (mean  $\pm$  standard deviation), and similar for the demented patient-groups. Biochemical values of serum cobalamin, serum folate and red blood cell folate fell essentially within normal ranges for reference values. MMSE scores of Alzheimer's dementia patients correlated (Pearson) with cobalamin status, but in the group of patients with mild cognitive deficits no significant correlation was seen. (In the group of Alzheimer demented patients, serum cobalamin levels explained as much as 17% of the variance in MMSE score.) Folate levels were slightly higher in the mild cognitive deficient group than in the groups of demented participants, but there were no significant group differences for either of the two measures of folate, or the measure of cobalamin.

Recently, Rebecca Eastley and co-workers (Eastley, Wilcock, & Bucks, 2000) performed a treatment study on elderly patients with low serum cobalamin, matched to controls with normal levels of the vitamin. All participants were classified either as demented or as cognitively impaired. The latter group included "patients who presented with memory impairment or cognitive impairment insufficient for a diagnosis of dementia". (Mean-ages were about 75/76 years in every subsample.) Serum values of the patients with low cobalamin fell below usual reference values. However, none of the subjects

presented with clinical symptoms of deficiency. Patients in the low vitamin status group were matched for age and diagnosis to the ones with normal cobalamin. Cognitive assessment with MMSE, a paragraph recall test (verbal episodic memory), the short-term memory test Digit Span from the Wechsler Adult Intelligence Scale – Revised (WAIS-R), word fluency (a measure of verbal fluency), and Kendrick’s Digit Copying Test (a test of perceptual speed) were performed; followed by a period of seven up to eleven months of cobalamin supplementation in the group with low initial status; and then cognitive reassessment (with the same tests) was accomplished. Group comparisons by *t*-testing revealed some interesting results. At baseline there were no significant differences between the treatment group and the control group within demented patients (in general slightly better performance by controls). In the cognitively impaired (not demented) group there was a significant difference for word fluency at baseline (means of the control group were higher for all five tests). At the follow-up evaluation, no significant improvement or deterioration was seen in the groups of dementia patients, neither in the treated nor in the controls. (Minor improvements was observed for paragraph recall and word fluency of the supplemented; for the remaining three tests of the treatment group, and in all tests of the matched control group, there were slight worsening effects.) In the group of cognitively impaired (not demented) patients similar results were noted, with two exceptions. In this case also an improvement, though not significant, was seen for the Digit Copying task in the treatment group; and, importantly, a substantial improvement took place for word fluency among the treated, a highly significant ( $p < 0.01$ ) change as compared to the matched controls. Noteworthy is also that among the 22 supplemented cognitively impaired (not demented) patients, two patients improved on all cognitive tasks.

In a study of homocysteine in relation to cognitive impairment among elderly, Lehmann et al. (1999) examined 336 consecutive patients (aged 50 years and above) at a memory clinic. Participants were classified according to the following diagnosis: Alzheimer’s disease with early onset (before the age of 65 years), senile dementia of Alzheimer type with late onset (at 65 years of age or older), vascular dementia, other dementias, minor cognitive dysfunction, and subjective memory impairment only. They reported a high prevalence of hyperhomocysteinaemia (as defined as a serum homocysteine value above the upper reference limit at their clinic, which was set to 15.0  $\mu\text{mol/L}$ ) in every one of the diagnosis categories. The distributions of high homocysteine values within respective groups were 11.1% in the early onset Alzheimer’s disease group (mean age  $\pm$  standard deviation: 63.7  $\pm$  6.9 years); 38.9% in the late onset senile dementia of Alzheimer type group (75.8  $\pm$  5.0 years); 57.6% in the vascular dementia group (76.7  $\pm$  6.3 years); 27.6% in the other dementias group (68.7  $\pm$  10.0 years); 38.9% in the minor cognitive dysfunction group (72.0  $\pm$  8.1 years); and 10.1% in the subjective memory impairment group (64.9  $\pm$  8.5 years). For patients with exaggerated homocysteine they also found a significant negative correlation between homocysteine values and MMSE scores, indicating an association between hyperhomocysteinaemia and cognitive performance. However, no subsequent analysis of such associations within every different diagnosis group was reported. In a later study (Gottfries et al., 2001), on subjects of the same age, carried out by the same team (now together with two other researches), the corresponding percentages of subjects with serum homocysteine above or equal to 15.0  $\mu\text{mol/L}$  were 45.2% in a dementia of Alzheimer type group (mean age  $\pm$  standard deviation: 72.6  $\pm$  9.3 years), no division due to early or late onset; 61.5% in a vascular dementia group (73.2  $\pm$  5.5 years); 31.3% in a mild cognitive impairment group (68.6  $\pm$  9.3 years); and 33.3% in a subjective memory complaints group (64.3  $\pm$  10.5 years). (Data for group of other dementia were not presented.) In the later study they also reported percentages of subjects with plasma cobalamin, plasma folate and blood folate below the lower reference values of their laboratory. However, these data were as expected in a healthy population, that is, a few percent.





## **Thesis aim**

Available for the present diploma work was prior collected data from patients having mild cognitive impairment (objectively stated by neuropsychological testing) or with subjective cognitive complaints, but without signs of clinical vitamin deficiency. These patients had been assessed with a large number of neuropsychological tests, measuring a variety of cognitive functions. Status of folate, cobalamin, and homocysteine had also been determined from blood samples. The aim of the study was to perform an exploratory search for statistical relations between the biochemical substances and different cognitive tasks within this population.



## Material and methods

### Subjects

The subjects included were patients referred to the Memory Clinic at the Department of Psychogeriatrics, Lund University Hospital. (A considerable portion of the patients was self-referred to the clinic.) All patients complained of memory dysfunction described as impaired day-to-day memory, word-finding difficulties, or a combination of impaired day-to-day memory and word finding difficulties. In addition to experiences of memory impairment, some of the patients also complained of impaired general cognitive functioning and impaired sense of spatial locality and attention. There were 110 consecutive cases of patients for which both cognitive assessment by neuropsychological testing, and measures of status of cobalamin (serum cobalamins, S-cobalamins), folate (blood folate, B-folate; or serum folate, S-folate) or homocysteine (total plasma homocysteine, total P-homocysteine) were available. Thirteen of these 110 individuals were removed because of absence of one or two of the measures of S-cobalamins, B/S-folate and total P-homocysteine. Further, twenty-seven of the patients were excluded due to ongoing vitamin supplementation or drugs on prescription or health food vitamins free from prescription (for example "multivitamins"). Among the remaining 70 individuals, seven more were excluded since they at a medical examination received a dementia diagnosis. Finally, three more patients, whose memory impairments were established to be associated with prior brain injury, were excluded from the sample. Thus sixty participants were left under investigation.

The age of the remaining 60 subjects (32 women and 28 men) was  $58.3 \pm 9.1$  years (mean  $\pm$  standard deviation), and ranged from 35 to 73 years. The mean age was approximately the same for women and men,  $58.0 \pm 10.2$  years (range 35 – 73) and  $58.6 \pm 7.8$  years (range 36 – 71), respectively. Number of years in formal education was  $13.3 \pm 2.9$  (range 7 – 20) for the whole sample,  $13.7 \pm 3.2$  (range 7 – 20) for women and  $12.8 \pm 2.4$  for men (range 9 – 17). (Note that in most cases the number of years of education was estimated from information about the form of education.)

Among the 60 participants analysed 21 (7 women and 14 men) had performed neuropsychological testing at a re-examination approximately one year after baseline testing. (There was no blood-sample analysis at follow-up.) It is unknown if any of these subjects had used vitamin supplementation during the year from the first examination to the follow-up, but the status on the residual exclusion criteria (as described above) were still the same.

### Neuropsychological testing

In the neuropsychological examination of the patients, the psychologists at the clinic use a considerable number of tests of different cognitive functions. Some of the tests are included in a "compulsory" standard battery of tests at the clinic, and some are optional. Only the compulsory tasks have been investigated in the present study. (All but three of the standard tasks are included; the three not included were rejected because of shortage in normalization data.) It was decided to use age-normalized values to avoid having age as a covariate in the analysis. The neuropsychological tests used are briefly described below.

#### Tests of verbal episodic memory

##### Free Recall of Sentences

These are two tests for episodic memory that are part of the Betula Battery (Nilsson, Bäckman, Erngrund, Nyberg, Adolfsson, et al., 1997). In *Free Recall of Enacted Sentences* the patient is told, in imperative form, to do something with an object. This is repeated in different enactments with different objects. Later on the examined is asked to recall the sentences. *Free Recall of Sentences*

(without enactment) is similar to Free Recall of Enacted Sentences. The difference is that in Free Recall of Sentences no objects and no acts are involved, instead each sentence is displayed printed on a card, and is simultaneously read aloud by the examiner. From a functional point of view, the dissimilarity between Free Recall of Enacted Sentences and Free Recall of Sentences, is that the first one involves a motoric component, while the second one only involves a verbal component. Hence, Free Recall of Enacted Sentences as well as Free Recall of Sentences measures verbal episodic memory, but the former task is supported partly by procedural memory.

### **Categorically Cued Recall of Sentences**

The objects (nouns) presented in the tests of free recall of sentences (described above) are selected from a number of semantic categories. In a task of categorically cued recall the subject is given the names of the semantic categories, as a cue for recalling the corresponding nouns presented before. *Categorically Cued Recall of Enacted Sentences* and *Categorically Cued Recall of Sentences* (also from the Betula Battery (Nilsson et al., 1997)) determines the number of correctly recalled nouns from the corresponding presentations of enacted and nonenacted sentences.

### **Verb-Cued Recall of Nouns**

In this task (Nilsson et al., 1997) the nouns from the previously enacted and nonenacted sentences should be recalled after a delay of approximately 45 minutes. Now the predicate part of the sentences is given as a cue, and the task is to recall the corresponding nouns. Two scores are estimated, one for cued nouns recalled originating from enacted sentences and one from nonenacted sentences. These tasks are supposed to measure delayed recall from episodic memory.

## **Tests of nonverbal episodic memory**

### **Face Recognition**

*Face Recognition* (Nilsson et al., 1997) is a test of visual episodic memory. Pictures of faces are presented to the respondent, who is instructed to try to remember the faces for a later recognition test. After a delay of approximately 25 minutes the examined is requested to identify faces presented before from a sequence of face pictures. This sequence consists both of faces presented before (“targets”), and faces not presented before (“distractors”). Raw-scores for target hits (faces correctly identified as re-presented) and distractor hits (faces incorrectly identified as re-presented) are then combined to a so-called  $d'$ -score.

### **Rey Complex Figure Test three-minutes recall**

The *Rey Complex Figure Test* (Meyers & Meyers, 1995) set is frequently employed as a neuropsychological test of visuospatial constructional ability and visuospatial memory. A free-hand copy drawing of the Rey Complex Figure (a figure constituted of a conglomerate of geometrical shapes) is carried out. Three minutes after the copy is finished, the figure should be redrawn from memory. This is the *3-minutes* or *immediate recall* task of the Rey Complex Figure Test, and provides a measure of visuospatial memory (incidental, because the subject is not told about later redrawing). Only the correct responses, not the time consumed, are used in the assessment at our clinic.

## **Tests of verbal functions**

### **Vocabulary**

The word comprehension test SRB:1 (Dureman, Kebbon, & Österberg, 1971). The test is in multiple-choice format constituted by a list of 30 words, each accompanied by five choices of words, whereof one is a synonym to the first word. The task is to select the synonyms. A test of vocabulary evaluates word comprehension as well as verbal semantic memory.

**Word fluency**

Two tests of *word fluency* (from the Betula Battery (Nilsson et al., 1997)): One for word fluency of words with the initial letter “a” (*letter fluency*); and one for word fluency of names of professions with the initial letter “b” (*category fluency*). In both cases fluency of speech is measured by the quantity of words, which fulfil the relevant criteria, produced within 60 seconds. Word fluency is a measure of verbal fluency and verbal semantic memory, and is part of verbal functioning.

**Test of visuospatial construction****Block Design**

This is the well-known *Block Design* subtest from the Wechsler Adult Intelligence Scale – Revised (Wechsler, 1999), used as a measure of visuospatial construction ability. Four or nine two-coloured cubic blocks are available. The task is to reproduce a number of 2-dimensional patterns (of increasing complexity) of arranged blocks, displayed as a picture on a card. Scoring is based on both accuracy and consumed time.

**Tests of perceptual speed and attention****Trail Making Tests**

The *Trail Making Tests* (TMT) are supposed to test visual conceptual and visuomotor tracking (Lezak, 1995). There are two tasks of “dot-to-dot-drawing”. In Trail Making Test A (TMT-A) the trail is drawn between dots marked with consecutive numbers; in Trail Making Test B (TMT-B) numbers and letters are altered. The task is to perform the drawings correct and as fast as possible. Hence the tests rely on perceptual speed and attention. (TMT-B is also supposed to include a component of working memory.)

**Normative data**

A majority of the tests used are taken from the Betula Test Battery (Nilsson et al., 1997), and are thoroughly described in the reference. Common for these tests are that the raw-scores are normalized to z-values using the age-scaled normative data from the Betula Prospective Cohort Study. The Betula Project, carried out at Umeå University in northern Sweden, has collected test results from cohorts of normal participants, divided into age groups for every five years span, from the age of 35 years to the age of 80.

The following tests were *not* normalized in accordance to data from the Betula Prospective Cohort Study (all the rest were).

Results from the three-minutes recall task of the Rey Complex Figure Test were scored in line with the procedure described in the test manual (Meyers & Meyers, 1995). In the development of the test-set, and in testing the reliability and validity of the same, 601 normal adult Americans at different ages (five-year-spans) were tested to obtain age-normative data (Meyers & Meyers). The neuropsychologists at our Memory Clinic apply these data on their patients too, for normalising raw-score to T-score\*.

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\* The T-score is a normalised score with mean 50 and standard deviation 10.

Age-differentiated data from Bergman, Bergman, Engelbrekton, Holm, Johannesson, and Lindberg (1988), in a study of healthy participants from Stockholm, has been applied in normalizing the raw-scores in the Trail Making tasks to T-scores.

The names of the tests, what abilities they are supposed to measure, and the normative data used, is summarized in table 1.

**Table 1.** Neuropsychological tests used in the study, what cognitive functions they are supposed to measure, and sources of age-normative data.

Test	Cognitive functions measured	Age-normative data from
Free Recall of Enacted Sentences <sup>a</sup>	Verbal episodic memory, supported by procedural memory	The Betula Prospective Cohort Study <sup>a</sup>
Free Recall of Sentences (without enactment) <sup>a</sup>	Verbal episodic memory	The Betula Prospective Cohort Study <sup>a</sup>
Categorically Cued Recall of Enacted Sentences <sup>a</sup>	Verbal episodic memory, supported by procedural memory	The Betula Prospective Cohort Study <sup>a</sup>
Categorically Cued Recall of Sentences (without enactment) <sup>a</sup>	Verbal episodic memory	The Betula Prospective Cohort Study <sup>a</sup>
Verb-Cued Recall of Enacted Nouns <sup>a</sup>	Delayed verbal episodic memory, supported by procedural memory	The Betula Prospective Cohort Study <sup>a</sup>
Verb-Cued Recall of Nouns (without enactment) <sup>a</sup>	Delayed verbal episodic memory	The Betula Prospective Cohort Study <sup>a</sup>
Face Recognition <sup>a</sup>	Delayed visual episodic recognition memory	The Betula Prospective Cohort Study <sup>a</sup>
Rey Complex Figure Test three-minutes recall <sup>b</sup>	Incidental visuospatial episodic memory	Rey Complex Figure Test and Recognition Trial <sup>b</sup>
SRB:1 <sup>c</sup>	Word comprehension and verbal semantic memory	The Betula Prospective Cohort Study <sup>a</sup>
Word fluency, letter fluency <sup>a</sup>	Verbal fluency and verbal semantic memory	The Betula Prospective Cohort Study <sup>a</sup>
Word fluency, category fluency <sup>a</sup>	Verbal fluency and verbal semantic memory	The Betula Prospective Cohort Study <sup>a</sup>
Block Design <sup>d</sup>	Visuospatial construction ability	The Betula Prospective Cohort Study <sup>a</sup>
Trail Making Test A <sup>e</sup>	Perceptual speed and attention	Magnus Huss Clinic, The Karolinska Hospital <sup>f</sup>
Trail Making Test B <sup>e</sup>	Perceptual speed and attention, working memory dependent	Magnus Huss Clinic, The Karolinska Hospital <sup>f</sup>

a) Nilsson et al., 1997

b) Meyers and Meyers, 1995

c) Dureman et al., 1971

d) Wechsler, 1999

e) Lezak, 1995

f) Bergman et al., 1988

## Laboratory assay

Blood samples from fasting subjects were collected in evacuated tubes containing EDTA for blood folate and total plasma homocysteine determination, and in empty tubes for serum cobalamin and serum folate determination. The laboratory assays were performed at the Department of Clinical Chemistry, Lund University Hospital.

### Total plasma homocysteine

The sample-tubes for homocysteine determination were, within 15 minutes after collection, centrifuged at  $3000 \times g^*$  for five minutes. The plasma was then stored at  $-20^\circ \text{C}$  until analysis. Homocysteine was separated with ion-pair chromatography on HPLC\*\* (after reduction of disulfide bond homocysteine) followed by further selective chemical reactions. Detection and quantification of total plasma homocysteine was then achieved photometrically. The procedure is described in Andersson, Silfverberg, Hultberg, and Lindqvist (2002). The reference interval for total P-Homocysteine used at Lund University Hospital at this time was 5.0 – 18.0  $\mu\text{mol/L}$ .

### Serum cobalamins

For serum cobalamin determination the AutoDELFLIA B<sub>12</sub> kit from Wallac Oy (Turku, Finland) was utilized. First, the cobalamins are set free from binding proteins. Then a competitive protein binding process follows. The sample cobalamins and a tracer of europium-marked cobalamin compete with each other to bind to a limited amount of intrinsic factor. Finally a solution is added, which dissociates the europium from tracer, and thereby a new chemical complex is created. The quantity of this complex is measured by fluorescence spectroscopy, and the proportional quantity of cobalamins in the original sample can be calculated. See Isaksson, Erreth, and Assarsson (1997) for a more detailed description. The reference interval at the time of the measurements was 110 – 650 pmol/L. (Note that these lower and upper reference limits are considerably low, as compared with most laboratories.)

### Blood and serum folate

For most of the participants blood folate were measured. However, during the time spanning the data collection, the standard measure for folate was changed to serum folate. Hence for part of the study-cohort S-folate were determined. Nonetheless, the process of the laboratory assays is the same. As for determination of cobalamin a competitive protein binding assay is used. Folate in the sample and folate marked by radioactive iodine-125 competes in binding to a folate binding protein. The binding protein is coupled to relatively heavy particles, and therefore the bound folate can be separated from the liquid by centrifugation. At last,  $\gamma$ -decay from the radioactive decay of the bound iodine-125 is detected by a gamma counter, and the concentration of folate in the original sample is then calculated. The process was carried out with the Dual Count Solid Phase No Boil Assay from Diagnostic Products Corporation, Los Angeles, California, USA. (See also Isaksson and Jörning (2001), and Lindström and Isaksson (1997).) Reference interval for B-folate and S-folate was 125 – 500 nmol/L and 7.0 – 40.0 nmol/L, respectively.

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\* This stands for a g-force of 3000 times  $g$  (the acceleration due to Earth's gravity) “experienced” by the sample in the centrifuged tubes.

\*\* HPLC, *high-performance liquid chromatography*, is a lab technique, which uses a combination of several separation techniques to separate substances at high resolution.

## Statistical analysis

Thorough statistical analysis on the data was applied: descriptive statistics, correlations and analyses of variance. Correlations were calculated with Spearman's nonparametric rank correlation. Univariate analysis of variance (univariate ANOVA), analysis of covariance (ANCOVA), and the nonparametric ANOVA correspondent Kruskal-Wallis  $\chi^2$ -test have been utilized. All statistical analysis was run with SPSS version 10.1 software from SPSS Incorporation, Chicago, Illinois, USA.

## Distribution assumptions

In the statistical analysis using parametric methods, all involved distributions of data have been checked according to requirements on normality and homogeneity of variances. In order to get objective comparisons of normality among the distributions *Shapiro-Wilk's Test of Normality* have been practised. In all instances of analysis of variance (and covariance) *Levene's statistics* were used as a mean of control for homogenous variance among the comparison distributions. (*Bartlett's Test of Sphericity* was applied in the repeated measures analysed with univariate ANOVA.)

## Correlations

Spearman's rank correlation's  $\rho$ -value (nonparametric test of significance) were calculated between every one pair-combination constituted of one of the blood components, that is S-cobalamins, B-folate/S-folate, and total P-homocysteine, and one of the cognitive test-variables as well as age and years of formal education.

## Group matching

To control for possible differences due to sex or education, group matching was applied for compared groups. In order to look for sex-differences, one-way ANOVAs and Kruskal-Wallis rank tests were evaluated for the vitamins, homocysteine, and the cognitive variables, with sex (women versus men) as dependent variable. Bivariate correlations were calculated between the test-variables and years of formal education. Furthermore, ANOVAs with sex as dependent variable and years of education as covariate (ANCOVA) were performed. In combining the results from these pre-analyses (see the sections *Results* and *Discussion*) it was considered as reasonable to perform group matching primarily for sex, in front of education.

In all statistical analysis based on group-comparisons (as described below), the groups were first of all divided according to levels of cobalamin, folate or homocysteine. If then there were differences in number of women and men within the groups, but moving subjects with successive levels of the biochemicals adjacent to border-values between the groups would give more equal distributions, those subjects were redirected (in order to obtain better sex-matching). Means and standard deviations for years of education were computed for the comparison-groups, in order to check that the groups were pretty good matched for education too. Finally one-way ANOVAs and Kruskal-Wallis tests were performed to see that there were at least no significant differences in sex and years of education among the groups, and that the groups differed significantly in the biochemical measure.



## Univariate analyses

For cobalamin and homocysteine, respectively, the participants were divided into two groups. Ideally, the laboratory values were about to be cut at the median, 30 values below and 30 above. If the values surrounding the median were equal, all the equal values were put in the same group (the one that implicated as equal sizes of the two groups as possible). In cases of unsatisfactory relative distributions of men and women within the two groups, subjects were further interchanged according to the sex-matching described above. With the division of cobalamin in “low”/“high” groups\* as dependent variable, univariate analysis of variance were applied for every cognitive test-variable. Without changing the dependent variable (the group-division) the corresponding Kruskal-Wallis evaluations were also done. The corresponding statistical calculations were then applied to homocysteine too.

In the case of folate the situation was more complicated, because B-folate was determined in 47 of the participants, and S-folat in the remaining 13. While the total number of subjects was quite small in relation to the statistical investigations, it was considered as a less good idea to exclude the 13 patients with measures of serum folate. Instead the laboratory values were transformed to sample z-values for B-folate and S-folate each individually, and then the two sets of z-values were put together. The z-values instead of the quantitative assay results were used to partition the subjects in a low and a high group, before statistical analyses similar to those for cobalamin and homocysteine were conducted.

## Repeated measurements

For the 21 subjects for whom cognitive follow-up data were available, repeated measurements analyses were performed. Partitioning in a low and a high group due to cobalamin, homocysteine and folate, respectively, followed the same procedure as described for univariate analysis above. For folate 20 participants had measures of blood folate and one for serum folate. The latter one was combined with the former by use of the z-values for B-folate and S-folate, calculated for the whole baseline-sample (47 subjects with B-folate and 13 with S-folate), as described for the combination of the two folate-subgroups of that whole sample. (In the case of cobalamin there was equal numbers of men and women, respectively, below and above the subject with the median value. Consequently, sex-differences between the low and the high group would be the same independently of what group the subject with the median value was assigned to. Hence, that subject was allocated to that group which thereby minimized the difference between the means of number of years of formal education.) Due to the small sample-size ( $n = 21$ ), and a considerable difference in number of men and women (fourteen and seven, respectively), group-match was worse here than in analyses of the whole baseline-sample. However, this is of less importance because here we are dealing with repeated measurements.

Repeated univariate ANOVAs were conducted. Though, it turned out that the sphericity requirements were in general not fulfilled. Therefore the alternative approach of ANCOVA was chosen. That is, instead of regarding the one-year follow-up as a repetition of the baseline examination, and performing a repeated univariate ANOVA on the data, the baseline data were held as a covariate when performing univariate analysis of variance on the follow-up data.

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\* In this report the names “low group” and “high group” represents the groups consisting of subjects with values mainly below and above the median value, respectively.



## Results

All statistical results with a  $p$ -value less than 0.10 are reported here. Note that most statistical outcomes do not fall within this range and are consequently not reported. The term *significant* always refer to a result with  $p < 0.05$ ; *near-significant*, or similar terms, refers to a result with  $0.05 \leq p < 0.10$ .

### Descriptive data

Descriptive data for the sample under investigation are displayed in table 2. Mean value, standard deviation and range is given for age, years of education, total plasma homocysteine, serum cobalamin, blood folate, and serum folate. Descriptives are specified for women and men respectively, and for the whole sample. The corresponding data, except for age and years of formal education, for the subsample of subjects provided with follow-up data is presented in table 3.

**Table 2.** Descriptive statistics for the studied sample. Descriptions are given for age, education and biochemical statuses, separated according to sex.

	Women ( $n = 32$ )			Men ( $n = 28$ )			Total ( $n = 60$ )		
	Mean	Standard deviation	Range	Mean	Standard deviation	Range	Mean	Standard deviation	Range
Age (years)	58.0	10.2	35 – 73	58.6	7.8	36 – 71	58.3	9.1	35 – 73
Formal education (years)	13.7	3.2	7 – 20	12.8	2.4	9 – 17	13.3	2.9	7 – 20
Total P-homocysteine <sup>a</sup> ( $\mu\text{mol/L}$ )	11.4	4.3	6.3 – 28.9	12.3	3.1	6.5 – 19.2	11.8	3.8	6.3 – 28.9
S-cobalamin <sup>b</sup> (pmol/L)	252	69	150 – 385	244	51	161 – 429	249	61	150 – 429
B-folate <sup>c</sup> (nmol/L)	320 ( $n = 25$ )	104	58 – 481	325 ( $n = 22$ )	103	101 – 551	322 ( $n = 47$ )	103	58 – 551
S-folate <sup>d</sup> (nmol/L)	14.3 ( $n = 7$ )	4.0	7.4 – 20.0	17.7 ( $n = 6$ )	7.5	9.0 – 30.0	15.9 ( $n = 13$ )	5.8	7.4 – 30.0

- Reference interval for total plasma homocysteine at Lund University Hospital at the time of measurements: 5.0 – 18.0  $\mu\text{mol/L}$ .
- Reference interval for serum cobalamin at Lund University Hospital at the time of measurements: 110 – 650 pmol/L.
- Reference interval for blood folate at Lund University Hospital at the time of measurements: 125 – 500 nmol/L.
- Reference interval for serum folate at Lund University Hospital at the time of measurements: 7.0 – 40.0  $\mu\text{mol/L}$ .

**Table 3.** Descriptive statistics for the subsample of participants provided with follow-up data. Descriptions are given for biochemical statuses, separated according to sex.

	Women ( $n = 7$ )			Men ( $n = 14$ )			Total ( $n = 21$ )		
	Mean	Standard deviation	Range	Mean	Standard deviation	Range	Mean	Standard deviation	Range
Total P-homocysteine <sup>a</sup> ( $\mu\text{mol/L}$ )	10.7	4.3	6.9 – 19.8	11.2	2.6	6.5 – 14.5	11.0	3.2	6.5 – 19.8
S-cobalamin <sup>b</sup> (pmol/L)	258	67	177 – 385	251	55	197 – 429	254	57	177 – 429
B-folate <sup>c</sup> (nmol/L)	384 ( $n = 6$ )	62	278 – 445	371 ( $n = 14$ )	85	259 – 551	375 ( $n = 20$ )	78	259 – 551
S-folate <sup>d</sup> (nmol/L)	17.0 ( $n = 1$ )	—	17.0	— ( $n = 0$ )	—	—	17.0 ( $n = 1$ )	—	17.0

- Reference interval for total plasma homocysteine at Lund University Hospital at the time of measurements: 5.0 – 18.0  $\mu\text{mol/L}$ .
- Reference interval for serum cobalamin at Lund University Hospital at the time of measurements: 110 – 650 pmol/L.
- Reference interval for blood folate at Lund University Hospital at the time of measurements: 125 – 500 nmol/L.
- Reference interval for serum folate at Lund University Hospital at the time of measurements: 7.0 – 40.0  $\mu\text{mol/L}$ .

## Age, sex and education

### Age and sex versus biochemical status

Neither S-cobalamins, B-folate, S-folate, nor total P-homocysteine correlated with age at the significant or near-significant level.

One-way ANOVAs and  $\chi^2$ -comparisons revealed no significant or near-significant sex-differences for homocysteine, cobalamin, blood folate or serum folate.

### Sex versus age and education

In the study-population there were no statistical sex-differences ( $p < 0.10$ ) for age or years of formal education, as evaluated by the methods of ANOVA and Kruskal-Wallis.

### Education and sex versus cognitive tests

#### Education

Years of education correlated significantly or near-significantly with Free Recall of Sentences (without enactment) (Spearman's  $\rho = 0.22$ ,  $p = 0.097$ ), word comprehension (SRB:1) ( $\rho = 0.25$ ,  $p = 0.055$ ), and word fluency of names of professions with the initial letter "b" ( $\rho = 0.25$ ,  $p = 0.054$ ), but with no other cognitive task.

#### Sex

One-way ANOVA and Kruskal-Wallis comparison of men and women were performed for every cognitive test. In three of the tests (Face Recognition, word comprehension (SRB:1), and categorical word fluency) women superseded men. This was seen both in the ANOVAs ( $7.42 \geq F \geq 3.33$ ,  $0.009 \leq p \leq 0.073$ ) and in the Kruskal-Wallis testing ( $5.80 \geq \chi^2 \geq 5.42$ ,  $0.016 \leq p \leq 0.020$ ). (Normality requirements on the ANOVA was violated for both word comprehension and categorical word fluency.) Details of these results are displayed in table 4.

**Table 4.** Comparison of women and men on cognitive tasks that showed significant or near-significant sex-differences.

Test	ANOVA <sup>a</sup>						Kruskal-Wallis <sup>b</sup>			
	$M_w$ (n)	$M_m$ (n)	$df_{win}$	$MS_{win}$	$F(1, df_{win})$	$p$	$M_w^{(rank)}$	$M_m^{(rank)}$	$\chi^2(1)$	$p$
FRec <sup>d</sup>	0.07 (32)	-0.46 (28)	58	1.12	3.77	0.057	35.6	24.7	5.80	0.016
SRB:1 <sup>e</sup>	0.74 (32)	0.39 (27)	57	0.53	3.33	0.073	34.8	24.3	5.42	0.020
WFb <sup>f</sup>	1.25 (31)	0.34 (28)	57	1.67	7.42	0.009	35.1	24.4	5.76	0.016

- $M_w$  = mean value for women;  $M_m$  = mean value for men;  $df_{win}$  = within-group (or "error") degrees of freedom;  $MS_{win}$  = within-group (or "error") mean squares
- $M_w^{(rank)}$  = mean rank for women;  $M_m^{(rank)}$  = mean rank for men
- FRec = Face Recognition (values in z-score)
- SRB:1 = word comprehension (values in z-score)
- WFb = word fluency of names of professions with the initial letter "b" (values in z-score)

## Cobalamin, folate and homocysteine versus tasks of cognition

Each of the 14 cognitive variables were analysed for the two vitamins and homocysteine. Spearman's correlation was calculated as well as one-way ANOVAs and the corresponding group comparisons by Kruskal-Wallis statistics (for every biochemical measure). Additionally, covariate analysis (ANCOVA) was executed on the cognitive follow-up data, with baseline data as covariate. Once again it should be noted that only results that turned out significant or near-significant are reported here, but all results for which that were true are reported. The parameter  $MS_{within}$  used below is the within groups mean square value.

### Tests of verbal episodic memory

#### Free Recall of Sentences

In Free Recall of Sentences, enacted as well as nonenacted, no relationship with  $p < 0.10$  was found in the correlation and group comparing analyses of the baseline data. However, one of the tests that showed significance in the longitudinal data analysis (the ANCOVA of the one-year-follow-up data with baseline investigation as covariate) was the folate-factoring of Free Recall of Enacted Sentences (ESFree). Higher folate status was related to higher result on the memory task ( $F(1, 18) = 5.01$ ,  $MS_{within} = 0.54$ ,  $p = 0.038$ ). The corresponding ANCOVAs for cobalamin and homocysteine, as well as all three ANCOVAs on Free Recall of Nonenacted Sentences were not significant or near-significant.

#### Categorically Cued Recall of Sentences

No results with  $p$  less than 0.10 were found for the two tasks of Categorically Cued Recall of Sentences (neither for enacted nor nonenacted).

#### Verb-Cued Recall of Nouns

The ANOVA of the task of delayed verb-cued recall originating from the enacted sentences (Verb-Cued Recall of Nouns from Enacted Sentences; for short ESVerb) turned out below the level of significance for cobalamin:  $F(1, 58) = 5.57$ ,  $MS_{within} = 1.12$ ,  $p = 0.022$ . This effect was also supported by the Kruskal-Wallis analysis:  $\chi^2(1) = 5.19$ ,  $p = 0.023$ . The effect was due to higher group-means on the cognition test for the low vitamin groups. These were the only outcomes with  $p$  less than 0.10 for Verb-Cued Recall of Nouns.

### Tests of nonverbal episodic memory

#### Face Recognition

Face Recognition (FRec) was related to homocysteine in several analyses. First there was a significant Spearman correlation ( $\rho = -0.28$ ,  $p = 0.029$ ). Secondly, in the univariate ANOVA low homocysteine was associated with higher result on the recognition trial ( $F(1, 58) = 6.20$ ,  $MS_{within} = 1.07$ ,  $p = 0.016$ ), and this result was repeated by Kruskal-Wallis examination ( $\chi^2(1) = 4.48$ ,  $p = 0.034$ ). No other statistical design reached below the level of near-significance.

#### Rey Complex Figure Test three-minutes recall

There was a significant relationship between the three-minutes recall task of the Rey Complex Figure Test (RCFT3min) and folate:  $F(1, 55) = 6.76$  ( $MS_{within} = 162.4$ ,  $p = 0.012$ ),  $\chi^2(1) = 6.25$  ( $p = 0.012$ ). The lower folate group scored higher, in mean, on the task, than did the higher group. No more statistics turned out with a p-value below 0.10.

## Tests of verbal functions

### Vocabulary

None of the twelve statistical runs on the data of the word comprehension task (SRB:1) reached the level of near-significance or better.

### Word fluency

The category fluency task (names of professions with the initial letter “b”; abbreviated WFb) correlated negatively to homocysteine at the near-significant level ( $\rho = -0.22$ ,  $p = 0.096$ ). In addition, the ANOVA, as well as the Kruskal-Wallis test, reported p-values below 0.10 in comparing two homocysteine groups (low homocysteine corresponded to better cognitive performance):  $F(1, 57) = 4.42$ ,  $MS_{within} = 1.75$ ,  $p = 0.040$ ;  $\chi^2(1) = 3.19$ ,  $p = 0.074$ . The corresponding situations for cobalamin and folate showed nothing of interest.

For the letter fluency task (words beginning with an “a” ); abbreviated WFa) no effects was observed when dealing with the baseline data. However, in the ANCOVA analysis of the follow-up data, both the low homocysteine group and the high cobalamin group outperformed their counterparts:  $F(1, 18) = 4.63$  ( $MS_{within} = 0.43$ ,  $p = 0.045$ ) and  $F(1, 18) = 4.49$  ( $MS_{within} = 0.43$ ,  $p = 0.048$ ), respectively. Further, the corresponding was true for folate on category fluency ( $F(1, 18) = 7.89$ ,  $MS_{within} = 0.66$ ,  $p = 0.012$ ).

## Test of visuospatial construction

### Block Design

In univariate analysis folate related to Block Design (BD). The low-group of folate had significantly higher scores on the Block Design test (as measured with ANOVA and Kruskal-Wallis  $\chi^2$ ). The statistical parameters follow:  $F(1, 58) = 5.95$  ( $MS_{within} = 0.96$ ,  $p = 0.018$ ),  $\chi^2(1) = 5.92$  ( $p = 0.015$ ). These were the only significant or nearly significant results for Block Design.

## Tests of perceptual speed and attention

### Trail Making Tests

The analysis of Trail Making Test A (TMT-A) brought about a number of significant or near-significant relationships, but for Trail Making Test B (TMT-B) not any one relation with  $p < 0.10$  was found. Relations to homocysteine were disclosed in the correlational analysis ( $\rho = -0.32$ ,  $p = 0.016$ ) as well as the group comparison ( $F(1, 53) = 6.09$ ,  $MS_{within} = 88.4$ ,  $p = 0.017$ ;  $\chi^2(1) = 5.62$ ,  $p = 0.018$ ). In both relationships there was an advantage in cognitive performance to have low levels of homocysteine. For folate and cobalamin the corresponding results all had p-values above 0.10.

Finally, with folate as independent variable the ANCOVA analysis on the follow-up data reached the near-significant range ( $F(1, 13) = 3.58$ ,  $MS_{within} = 24.42$ ,  $p = 0.081$ ). This difference appeared as a result of higher test-scores within the low folate subsample. The corresponding ANCOVAs for cobalamin and homocysteine did not reach below the near-significant level.

Every statistical analysis that reached a p-value below 0.10 is displayed in table 5. In that table mean value and size of every of the corresponding subsamples under investigation are shown too. A few of the analysed situations presented in table 5 involved distributions violating requirements on normality

or homogeneity of variances. That is, at least one of the subsamples differed significantly from normality as evaluated by Shapiro-Wilk's test, or there was significant in-homogeneity, as determined by Levene's statistics, among the groups. Because of the associated reliability problems for parametric statistics the corresponding designs are marked in the table.

**Table 5.** All statistical analyses of cognitive tasks versus biochemical measures with significant or near-significant outcome for at least one method. Cognitive task, statistical design, statistical method, effect, subsample mean-values, and statistical significance testing parameters are given.

Test <sup>a</sup>	Design <sup>b</sup>	Method <sup>c</sup>	Effect <sup>b,d</sup>	$M_{low}^e$ (n)	$M_{high}^e$ (n)	Ratio <sup>f</sup>	$\rho$
ESFree	2(fol: L, H)	ANCOVA <sup>g</sup>	+ fol	-0.09 (11)	0.73 (10)	$F(1, 18) = 5.01$	0.038
ESVerb	2(cob: L, H)	ANOVA	- cob	0.57 (29)	0.07 (31)	$F(1, 58) = 5.57$	0.022
		Kruskal-W	- cob	35.81 (29)	25.53 (31)	$\chi^2(1) = 5.19$	0.023
FRec	Corr. hcy	Spearman	- hcy	(60)		$\rho = -0.28$	0.029
	2(hcy: L, H)	ANOVA	- hcy	0.12 (33)	0.55 (27)	$F(1, 58) = 6.20$	0.016
		Kruskal-W	- hcy	34.8 (33)	25.2 (27)	$\chi^2(1) = 4.48$	0.034
WFa	2(cob: L, H)	ANCOVA	+ cob	0.39 (10)	0.95 (11)	$F(1, 18) = 4.49$	0.048
	2(hcy: L, H)	ANCOVA <sup>g</sup>	- hcy	1.01 (9)	0.44 (12)	$F(1, 18) = 4.63$	0.045
WFb	Corr. hcy	Spearman	- hcy	(59)		$\rho = -0.22$	0.096
	2(hcy: L, H)	ANOVA <sup>g</sup>	- hcy	1.14 (33)	0.41 (26)	$F(1, 57) = 4.42$	0.040
		Kruskal-W	- hcy	33.6 (33)	25.5 (26)	$\chi^2(1) = 3.19$	0.074
	2(fol: L, H)	ANCOVA	+ fol	1.21 (11)	1.68 (10)	$F(1, 18) = 7.89$	0.012
BD	2(fol: L, H)	ANOVA	- fol	0.56 (30)	0.05 (30)	$F(1, 58) = 5.95$	0.018
		Kruskal-W	- fol	36.0 (30)	25.0 (30)	$\chi^2(1) = 5.92$	0.015
RCFT3min	2(fol: L, H)	ANOVA	- fol	57.6 (28)	48.8 (29)	$F(1, 55) = 6.76$	0.012
		Kruskal-W	- fol	34.6 (28)	23.6 (29)	$\chi^2(1) = 6.25$	0.012
TMT-A	Corr. hcy	Spearman	- hcy	(55)		$\rho = -0.32$	0.016
	2(hcy: L, H)	ANOVA	- hcy	52.2 (29)	45.9 (26)	$F(1, 53) = 6.09$	0.017
		Kruskal-W	- hcy	32.8 (29)	22.6 (26)	$\chi^2(1) = 5.62$	0.018
	2(fol: L, H)	ANCOVA <sup>g</sup>	- fol	53.2 (8)	51.9 (8)	$F(1, 13) = 3.58$	0.081

- The test-abbreviations reads as follows: ESFree = Free Recall of Enacted Sentences; ESVerb = Verb-Cued Recall of Nouns from Enacted Sentences; FRec = Face Recognition; WFa = word fluency of words with the initial letter "a"; WFb = word fluency of names of professions with the initial letter "b"; BD = Block Design; RCFT3min = Rey Complex Figure Test 3-minutes recall; TMT-A = Trail Making Test A
- The used abbreviations reads as follows: hcy = homocysteine; cob = cobalamin; fol = folate; L = low; H = High; corr. = correlation. The design 2(X: L, H) symbolizes a comparison of two groups, one with low and one with high values on the variable X.
- ANCOVA always relates to analysis on follow-up data with baseline data as covariate; Kruskal-W = Kruskal-Wallis
- The sign (+ or -) describe the direction of the relationship, that is, if lower biochemical status is associated with lower cognitive scores, and higher status corresponds to higher scores, then there is a positive direction (correspondingly for a negative relation). The biochemical following the sign tells what blood measure the association holds for.
- Mean values of the low and the high subsamples, respectively, in comparison of groups. For Kruskal-Wallis analyses the rank means are given. For ANOVA and ANCOVA the means is for the normalised data.
- "Ratio" stands for the F-value in ANOVA and ANCOVA, the chi-square-value in Kruskal-Wallis, and the correlation coefficient  $\rho$  for Spearman correlations. The F-values and chi-square-values are given as functions of degrees of freedom.
- Analysis of variance/covariance involved distributions violating requirements on normality or homogeneity of variances.





## Discussion

An exploratory investigation for relationships between cognition, as assessed by neuropsychological testing, at one side, and blood status of cobalamin (vitamin B<sub>12</sub>), folate (folic acid) and homocysteine on the other side, was conducted on a sample constituted by patients with mild cognitive impairment and subjective memory complaints. Both group comparing and correlational statistical methods were applied. For several cognitive tasks there were significant associations with levels of the biochemical markers.

Before discussing the results of the present work two remarks are in place. First of all it must be pointed out that it is not advisably to draw distinct conclusions from an exploratory study like this one. Rather, it is recommendable to consider this work as a search for variables of special interest for further hypothesis testing research. For that reason all cognitive tasks in some way associated with any of the biochemical measurements with a p-value below 0.10 have been considered as interesting, in the sense of being first-choice candidates for further research.

Secondly, the 60 participants consist of two fractions, one with objectively stated (mild) cognitive impairment (based on neuropsychological testing), and one with subjective complaints only, that is, the neuropsychological testing was not able to conclude cognitive dysfunction.

### Considerations on the participant group and the statistical manipulation of data

#### Participants

It should be noted that among the sixty participants left in the cohort, some are certainly about to develop dementia, but diagnose criteria is not yet fulfilled. Unable to exclude (or identify) these probable subjects from the sample implies a possible interference with the statistical outcome if they not happened to be properly matched by luck. Anyway, this is not considered as a strong risk potential.

The evaluation of number of years of formal education might be connected to reliability problem. It is possible that these scores have been overestimated. However, the situation is approximately the same for all participants and therefore, at worst, a slight linear transformation from the actual values might have occurred – such does not affect the statistical outcomes.

#### Data screening and outliers

Due to the vast amount of data it was intractable to screen all involved distributions and to make special considerations of for example outliers. However, most distributions were quickly screened, just to realize the rather extensive variations in shapes, and, as recognized by ocular inspection, frequently occurring deviations from normality. That is the main reason why the strict mathematical test of normality by Shapiro-Wilk's was used in the check of normality requirements in the parametric statistics.

No outliers were treated individually; all data points were kept in all analyses. Notable is, however, that outliers were rare. For example, considering the distributions of cobalamin, folate and homocysteine in the whole sample, only one data point showed obvious deviation from the rest. That was the highest value of total plasma homocysteine (28.9  $\mu\text{mol/L}$  as compared to a rather homogenous distribution ranging from 6.3  $\mu\text{mol/L}$  to 19.8  $\mu\text{mol/L}$  for the remaining participants). (Noteworthy is that the corresponding subject in general performed better than most other participants on the cognitive tests.)

The main objective of this work was to identify cognitive abilities of special interest for further examination. Hence, potential statistical defects caused by "bad distributions" or outliers are not of greater importance, as far as it is reasonable to judge them as few.

### **About the group matching procedure**

The group matching of participants due to sex and education was conducted rather on group level than on individual level, which is more thorough. Anyway, all groups were checked by ANOVA and Kruskal-Wallis testing, to ensure that there were at least no significant differences in the group distributions of sex as well as years of education – that did not occur in any case. Furthermore, performing ANOVAs with sex as dependent variable and years of education as covariate (ANCOVA) did only alter the outcome slightly compared to the one-way ANOVAs without the educational covariate. Hence, this match on group level still kept the "core of the statistics".

It is here also appropriate to point out a caution on the method of combining blood folate and serum folate, based on the within-sample z-normalizations in the two subsamples – this is not an especially good approach! In fact, comparing respective within-sample means and standard deviations in relation to the corresponding clinical reference value ranges show some disagreement in the baseline data. Moreover, looking at correlation parameters ( $\rho$  and  $p$ ) for all calculated correlations (not only those with a p-value below 0.10) between cognition and B-folate and S-folate, respectively, there are considerable dissimilarities between the two measures of folate. Therefore the results of folate-factored ANOVAs and Kruskal-Wallis tests must be judged with special care. However, for the one-year-follow-up data the situation was much better. In this case there was only one subject with measure of serum folate and this value fell in the middle of the S-folate distribution of the whole sample as well as the clinical reference interval (that is, it was definitely not an outlier). Hence one can be confident that the reliability is maintained with regard to the combination of the two measures of folate in the follow-up data.

### **Validity and reliability problems associated with the biochemical measures**

There are a number of validity problems associated with status of serum cobalamin and blood/serum folate. These values does not always reflect the actual status of cobalamin and folate at intracellular level of utilization, they only gives a measure of the concentration of the vitamins in the bloodstream – this is especially true for cobalamin. However, homocysteine reflects the metabolic rate and is therefore considered as a better marker for tissue deficiency (Hultberg et al., 2000; Nilsson et al., 1999).

### **Saturation**

Cobalamin and folate are well represented in ordinary food sources, so with a balanced diet, and no pathological disturbances in the body's management of the vitamins, most normal people probably take up more of them than is necessary for maintaining normal body functions. Further, these vitamins are water-soluble and harmless when taken in also in amounts exceeding what is necessary\* – the excess just "travel around" in the bloodstream until it is utilized or excreted from the body. Hence, when studying blood levels of folate and cobalamin one must consider saturating effects.

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\* Remark: High dosages of folate are associated with some negative effects (see for example Brody (1984) or the discussion in Botez et al. (1984)).

## Age-variations

It is known that levels of cobalamin (but not folate) and homocysteine (independent of cobalamin and folate) vary with age, when measured in blood serum or blood plasma among healthy, normal subjects (Delport, 2000; Hultdin et al., 2000; Nexø, 1983; Wahlin, Bäckman, Hultdin, Adolfsson, & Nilsson, 2002). Hence, it would be appropriate to adjust clinical reference values for age, in the sense that reference ranges for cobalamin should descend with age and those for homocysteine ascend.

In the present study cognitive data were normalized according to age, while the values of cobalamin, folate and homocysteine were not. As a consequence one could fear an imbalance in age between the low and high groups of cobalamin and homocysteine, with older subjects in the low cobalamin group as well as in the high homocysteine group, at the same time as no such inequality is present in the folate division. This was not checked for! While the cognitive data is age-normalized an artefact of that kind would probably not implicate a severe drawback. However, the normalization data originates from healthy controls, and it is reasonable to expect the prevalence of pathological cognitive deterioration (independent of vitamin status) to increase with increasing age. Hence the possibility of an age in-equilibrium causing apparent associations where high homocysteine and low cobalamin relate to worse cognitive performance, at the same time as no relationships are present for folate, must be considered.

Fortunately, if that is the situation it can be argued that the effect at least is weak. For the first, a similar pattern of statistical outcomes from the group comparisons would be expected for homocysteine and cobalamin, but that is not the case. Secondly, in the investigated sample there were no correlations between age and the biochemicals.

Worth noting in connection to this is that both Trail Making Tests did in fact correlate negatively with age, despite the age-normative data: TMT-A  $\rho = -0.42$  ( $p = 0.002$ ) and TMT-B  $\rho = -0.27$  ( $p = 0.042$ ).

## Transport system

As reported by Metz et al. (1996), disturbances associated with transport proteins of cobalamin are common in the elderly. This is probably another reason that blood concentrations of cobalamin might not reflect tissue availability in a satisfactory manner.

## Genetic mutations

During the last years several mutations in genes controlling enzymes involved in the one-carbon metabolism have been determined. One of these mutations, named MTHFR gene C677T, affects the methylenetetrahydrofolate reductase enzyme that catalyses the reduction of methylenetetrahydrofolate to methyltetrahydrofolate, in such way that this reduction is impaired. As can be understood from figure 1 (in the introduction chapter) this might lead to increased homocysteine accumulation also if the availability of folate and cobalamin is good. Bottiglieri et al. (2001) reported a high prevalence (21.2%) of the mutation's genotype MTHFR TT in subjects with dementia (though not significantly higher than in nondemented controls). Among the patients with dementia this genotype was significantly associated with elevated total plasma homocysteine as well as low plasma folate (but no significant association with plasma cobalamin).

## Evaluation of the results

All the cognitive tasks displayed in table 5 in *Results* shall be considered as especially interesting candidates for further experimental research within the field of the present study. However, only the results left after exclusion of the results for analysis of variance and covariance involving distributions violating requirements on normality or homogeneity of variances, and exclusion of outcomes not reaching the level of significance (that is  $p < 0.05$ ), can be in the running for being established as significant relationships in the context of experimental psychological research. These results are discussed below.

### Associations to concurrent cobalamin status

#### Verb-Cued Recall of Nouns from Enacted Sentences

For the delayed verbal episodic memory task Verb-Cued Recall of Nouns from Enacted Sentences an unexpected *negative* relationship to cobalamin was seen. For a real effect it is reasonable to get similar outturns in more than one design, when analysing several. For example the same relation shows up as a correlation as well as group difference in a one-factor analysis. Because this is not the case for this task, it implicates us to put a question mark on the result.

The special task of Verb-Cued Recall of Nouns from Enacted Sentences has probably not been reported in association to cobalamin. Eastley and colleagues (2000) used another test of verbal episodic memory, a paragraph recall test, in their treatment study on “patients who presented with memory impairment or cognitive impairment insufficient for a diagnosis of dementia” and demented patients with levels of serum cobalamin below typical reference values (but without clinical symptoms of deficiency). She reported a slight but not significant improvement in the cobalamin-supplemented group, as compared to untreated matched controls with normal vitamin status.

Studies on “healthy elderly”, defined in the sense of the section *Cognitive Relations to Folate and Cobalamin in “Healthy Elderly”* in the introduction of this report, cannot be transferred to the population studied by us without further notice. Though, the characteristics of our population are more similar to those populations than to, for example, populations of patients with severe states of deficiency. Hence, it is of interest to compare our results with such results too. Recall of word lists or short sentences and paragraphs have been reported several times in that kind of studies. In general no relationships were observed (Crystal et al., 1994; Hassing et al., 1999; Savaria Morris et al., 2001), whilst Ravaglia et al. (2000) saw a significant positive correlation and Riggs et al. (1996) reported on a negative near-significant correlation. However, taking possible covariates into account in the latter two cases, took the results out of the regions of significance and near-significance, respectively.

Summing-up, no one else has reported a significant negative association between cobalamin and verbal episodic memory tasks, neither in populations similar to ours nor in populations of “normal” subject. Therefore the present result has to be strongly questioned.

### Associations to concurrent folate status

After excluding results as told above there were still two associations to folate – both in the opposite direction of what is anticipated. The tests are the Rey Complex Figure Test 3-minutes recall and Block Design.

#### Rey Complex Figure Test three-minutes recall

Both in the ANOVA and the Kruskal-Wallis statistics the three-minutes recall of the Rey Complex Figure Test related to folate. However, low folate was linked to better cognitive performance.

The folate treatment study on patients with "mild to moderate memory complaints" and subclinical values of folate (though not clinical symptoms of deficiency) by Fioravanti and colleagues (published in 1997, cited in Calvaresi and Bryan (2001)) reported significant improvements of memory. They used both verbal and nonverbal episodic memory tests, however, it is not known to the author of this report if there were improvements on the nonverbal tasks.

From studies of "healthy elderly" subjects, La Rue et al. (1997) reported that subjects taking folate supplements performed better on the three minutes delayed recall of the Rey Complex Figure Test than did those who did not supply (not significantly better). Spearman correlations to concurrent vitamin status showed nothing of value, but there was a near-significant correlation to intake of folate six years prior to the test.

### **Block Design**

The association to the Block Design test is very similar to that to the three minutes recall in the complex figure test. This might be suspected on theoretical basis, because both tasks strongly dependent on visuospatial abilities. From empirical data it is also known (see for example Meyers and Meyers (1995)) to be intercorrelations between these two tests; this was also the case in the present study ( $\rho = 0.27$ ,  $p = 0.046$ ).

In no one of the studies reviewed in the introduction, on patients with symptoms comparable to the ones in the present work, tests of visuospatial construction were applied. However, Block Design was included in the assessment batteries in the recent studies on "healthy elderly" by Duthie et al. (2002) and Robins Wahlin et al. (2001). In both investigations there were strong group-differences respective correlations due to serum/plasma folate ( $p < 0.0005$  and  $p < 0.01$ , respectively), with higher folate levels related to higher test results. (In the Duthie study this result fell out for a sample of 63 to 64 years old participants, but not for a sample of 78 to 79 years old subjects.) This is contradictory (but more expected) to the present data.

Our first choice of explanation to the negative relationships between folate and these two cognitive measures is that it is caused by artefacts contained in the data. It can be argued that the combination of values of blood folate and serum folate based on the two corresponding within-groups z-values may have a finger in the pie. This is not reasonable however, because if one look at the Spearman correlations between these two cognitive tasks and B-folate and S-folate, though not significant or near-significant ( $0.169 \leq p \leq 0.254$ ), each of the four separate correlation coefficients are negative.

### **Associations to concurrent homocysteine status**

Also for homocysteine there were significant relations to two cognitive tests left when the statistically uncertain results had been removed. This was the case for Face Recognition and for Trail Making Test A. In both these cases higher levels of homocysteine were related to worse cognitive performance. As far as the writer of the present report are aware, no study of homocysteine in relation to tests of specific cognitive abilities within a group of patients with a similar constitution as the one studied here (that is including individuals with mild cognitive impairment and subjective memory complaints), has been published before.

### **Face Recognition**

To the author's knowledge no prior investigation of possible associations between homocysteine and recognition of faces has been published – independent of study-population! (In a study on "healthy elderly" Hassing et al. (1999) compared cobalamin and folate to scores on the Face Recognition test, without finding any significant associations.) Tasks on immediate visual memory/recognition other

then faces have been applied in the works by Ravaglia et al. (2000) as well as Riggs et al. (1996) (on “healthy elderly”). None of them reported on significant or near-significant relationships.

### **Trail Making Test A**

No article reporting on search for links between trail making tasks and homocysteine is known to the author. (Neither in research on subjects with MCI or subjective memory complaints nor “normal” elderly subjects.) However, a few researchers have used other tasks involving similar cognitive functions in research on “normal” elderly. Riggs and partners (1996) used two such tasks, and Duthie et al. (2002) used the Digit Symbol test. While Riggs et al. did not achieve any significant or near-significant associations to homocysteine Duthie and co-workers reported a negative correlation in their sample of 63 and 64 years old.

### **Longitudinal data**

Important to remember is that the values for the biochemical variables in the follow-up analyses are from blood sample determinations at the baseline assessment one year earlier – measurements of cobalamin, folate and homocysteine were not repeated at the second visit.

Interestingly, in the ANCOVA of the one-year-follow-up data, word fluency of words on “a” were significantly associated with both cobalamin and homocysteine in the expected way. (High cobalamin and low homocysteine related to better performance.) But for homocysteine the subsamples violated distribution requirements, so that result lack in reliability. Further, folate was positively related, below the level of significance, to the task of fluency of professions beginning with a “b”. These outcomes might represent a valid influence of B-vitamin status on verbal fluency in the long run.

This is in agreement with Rebecca Eastley (Eastley et al., 2000) who reported on relations between cobalamin and word fluency in a placebo-controlled treatment study on a group of cognitively impaired (but not demented) patients with low cobalamin status. At baseline there was a significant difference between a group with low vitamin levels and one with normal. Further, during treatment with cobalamin a substantial improvement took place among the treated, but not among the untreated.

It is also in agreement with Robins Wahlin and colleagues’ (2001) study of “healthy elderly”, who also reported such relationships between folate and cobalamin and word fluency (not longitudinal data) among “healthy elderly”. Other researches (on “normal” subjects too) have not found similar connections (Ravaglia et al., 2000; Riggs et al., 1996; Tucker et al., 1990).

### **Survey of the results**

Exploring as many test variables that have been done in the present work implies the expectation of a few significant (and near-significant) associations by pure chance occurrence, also when no valid relationships are present. If that is the case the directions of these outturns is also expected to vary at random. Having a quick look at table 5 one realize that the number of presented results is not so few. However, looking at the distributions of the directions of the results, those give an inconclusive picture for both cobalamin and folate. For homocysteine the opposite is true – not any single outcome of homocysteine associations with  $p < 0.10$  is in the unexpected direction. This is an indicator for some rather overarching (though quite weak) valid relationship between status of homocysteine and scores on cognitive tests, in the studied sample!

The incongruence of the results for contemporary status of cobalamin is possibly due to a combination of unfortunate random distributions and the validity problem associated with measures of serum cobalamin (described above; consider also *A Remark on the Choice of Statistical Methods* below). This is most probably the cause of the revealed associations to folate too. It is possible to speculate

about the possibility that, especially, the negative relations between folate and both Block Design and Rey Complex Figure Test three-minutes recall originate from an prior unknown parameter present in the specific studied population. However, that is very unlikely, because no similar associations have been reported before in similar populations. Rather it is the result of hazard, and the reason why both tasks fell out significant is that they are strongly intercorrelated. One might argue that the interrelations between cobalamin, folate and homocysteine (see the result section) supported that low values of cobalamin was related to low values of folate, and the corresponding for the high values, while high homocysteine was associated to both low folate and low cobalamin, and vice versa for low homocysteine, and that this predicts that a valid relationship for one of the biochemical measures would implicate on such for the other two too. But those associations says nothing about individual variations between the vitamin values and homocysteine, and as might be supposed from the discussion on these measures validity problems such variations can be considerable. Contrary, the results for homocysteine are satisfactory and suggest a pathological, but weak, connection to cognition.

Also the significant outcomes of the ANCOVAs should be devoted extra interest. There were only two such reliable relations (one for cobalamin and one for folate), but in the expected directions and associated with two tasks closely related to each other (the word fluency tasks). In addition nearly significant relationships between concurrent homocysteine and category fluency are present (in the expected direction too). Further, with the support of Eastley's study over a treatment period (Eastley et al., 2000), it is tempting to propose that word fluency is sensitive to prior status of the vitamins and contemporary status of homocysteine. If so, the results give support to the homocysteine hypothesis (see *The homocysteine hypothesis* in the introduction) – a cognitive impairment with slow progress caused by long-term raised concentration of homocysteine.

### **A remark on the choice of statistical methods**

From clinical cases of deficiencies of cobalamin or folate it is well-known that when the deficiency start to develop, first not so much happens in the set of symptoms; next, not so much happens either; but then, when the deficiency reaches some level, a lots of symptoms occurs. On theoretical basis is it therefore reasonable to believe that comparisons of extreme groups are the best choice in studies on cobalamin and folate status as compared to cognitive scores. In the present study the compared groups was not especially extreme (approximately the lower and the upper half of the biochemical distributions), and this might be one additional reason to the lack of consensus between group comparisons and correlations in the analyses of the vitamin values. For homocysteine the situation is probably different. For example, because homocysteine is a risk factor for vascular disease, also lower concentrations might affect cognition, thought to a lesser extent.

### **Are the results specific for the studied population?**

On condition that the significant homocysteine results, and possibly the significant results of the longitudinal data are valid, representing a pathological pathway between status of the biochemical measures and cognition, it is now time to ask: Are the results specific for the studied group of patients with mild cognitive impairment and subjective memory complaints? Or are they just consequences of general pathologies associated with ageing – independent of the specific constitution of the studied population?

## The low intake hypothesis

Goodwin, Goodwin and Garry (1983) proposed a “low intake hypothesis”, suggesting that reduced capacity to attend to self-care and nutrition in impaired patients results in lower intake of vitamins. If so, a relationship between cognition and vitamin (and homocysteine) would follow. Several studies have also indicated that low levels of cobalamin or folate, and high values of homocysteine are frequent within populations of various types of dementia (Ebly et al., 1998; Gottfries et al., 2001; Lehmann et al., 1999; Nilsson et al., 1996; Nilsson et al., 1999; Nilsson, Gustafson, & Hultberg, 2000; also the review by Calvaresi and Bryan (2001)). However, experimental support for this hypothesis has not been established (confer Levitt and Karlinsky (1992)). Further, mild cognitive impaired patients are not that impaired that it does seem reasonable to believe such a hypothesis as relevant in this case.

## Prevalence of hyperhomocysteinaemia in patients with mild cognitive impairment and subjective cognitive complaints

Lehmann, Gottfries and colleagues (Lehmann et al., 1999; Gottfries et al., 2001) have reported on high prevalence of hyperhomocysteinaemia (defined as a serum homocysteine value above 15.0  $\mu\text{mol/L}$ ) in MCI patients as well as in patients with subjective memory complaints. In their article from 1999 38.9% of the MCI patients had high homocysteine values, in the study from 2001 the prevalence was 31.3%. Corresponding values for patients with subjective memory complaints were 10.1% and 33.3%, respectively. In samples of patients suffering from various dementia the highest prevalence is found in the group of vascular dementia (57.6% and 61.5% in their two studies), which is not surprising considering that homocysteine is a risk factor for cerebrovascular disease. Therefore one could expect a high prevalence of hyperhomocysteinaemia in MCI patients, and that there are some kind of association. However, in our sample only seven out of sixty participants, that is approximately 11.7%, had plasma total homocysteine above 15.0  $\mu\text{mol/L}$ , and only three (5%) had values above 18.0  $\mu\text{mol/L}$ , the upper reference value at our clinic. Hence, there appear to be no difference between spread of homocysteine status in the investigated sample and healthy controls. Note then that the prevalence of hyperhomocysteinaemia in our sample is considerably less than in their two studies. This might in part be explained by the fact that the subjects of the analogous subpopulations in Lehmann’s study and in Gottfries’ were approximately ten years older than our participants, and this difference possibly influence the homocysteine status.

## Explained variance in cognition

Considering the variance explained ( $R^2$ ) by homocysteine in the performed cognitive tasks, the values for the explained variance in the baseline data in the present study range up to approximately 0.10. This is analogical to typical results for homocysteine in studies on “normal” subjects (see Duthie et al., 2002; Hultdin et al., 2000; McCaddon et al., 2001; Ravaglia et al., 2000; Riggs et al., 1996; Savaria Morris et al., 2001). Hence, our data does not seem to deviate from data of “healthy elderly”. Nevertheless, the maximum  $R^2$  for the ANCOVAs on follow-up data reach approximately 30%. This is in fact noticeably high, but one has to remember that the explained variances for most of the cognitive tasks are much lower than that. Therefore it is impossible to draw any conclusions from this. (Corresponding data from the other reviewed studies on similar populations, see *Vitamin Status and Mild Cognitive Impairment and Subjective Memory Complaint* in the introduction, are hard to interpret with this regard.) Consequently, it is reasonable to assume that the homocysteine associations seen here is unspecific for the studied population, while no conclusions is appropriate for the longitudinal data.



## Speculations and further research

An important remark should be established: The participants in our study-sample are younger than the subjects in any of the studies on similar populations reviewed in the introduction. (That is also true with regard to the studies on “healthy elderly” referred to in the same part of this report.) Consequently, to the author’s knowledge, no one have conducted a prior study investigating status of cobalamin, folate and homocysteine and their relations to cognitive performance in a population consisting of patients with mild cognitive impairment and subjective memory complaints (or similar) that young (mean age less than 60 years)!

Though the results of this study are inconclusive in a couple of instances, there are nevertheless reasons to believe that valid associations were observed in other instances. The latter results give support for the involvement of a component of cognitive deterioration due to long-term raised homocysteine concentrations in the studied population. It is likely that the outcomes resembles those in similar studies on “normally ageing” (or “healthy elderly”) populations. However, there is an important difference – the age aspect.

One could speculate in the possibility that status of homocysteine is of different importance in this specific population, as compared to “normally” ageing individuals, and that this causes a negative homocysteine effect on cognition, present also in a normally ageing population, to become prominent at an earlier age. However, the total plasma homocysteine concentrations in the studied sample generally fell within the limits of clinical reference values, that is, status were mainly rather normal, also as compared to healthy individuals. Interestingly, the MTHFR C677T mutation mentioned above constitute a predisposition for developing hyperhomocysteinaemia (confer Bottiglieri et al. (2001)), but, as cited in the discussion of Barbaux et al. (2000), De Stefano and colleagues (published in 1999) reported that individuals bearing not only this mutation but also an allele called CBS 844ins68 did not have increased homocysteine concentrations. The 844ins68 mutation is related to the control of the vitamin B<sub>6</sub> dependent enzyme cystathionine-β synthetase. As shown in figure 1 (in the introduction) this enzyme catalyses the conversion of homocysteine to cystathionine via the so-called transsulphuration pathway in the one-carbon metabolism. Carriers of the CBS 844ins68 allele probably have a reinforced ability to clear out increased levels of homocysteine via the transsulphuration pathway, thereby maintaining quite normal homocysteine concentrations even so there are shortage of folate or cobalamin in the cells. What makes this interesting to the present study is that nonetheless this allele is associated to comparably lower concentrations of plasma homocysteine (as compared to what it else would be), at the same time it is linked to worse cognitive performance – at least in children. Barbaux and partners (2000) showed that the presence of this allele was significantly underrepresented in children with high IQ as compared to children with low IQ. One could therefore speculate in that the prevalence of this genetic mutation, or another genotype with similar effect, is increased in the specific population under investigation. Then a considerable fraction of the sample’s subjects might have only slightly increased homocysteine values but decreased cognitive functioning associated with the presence of the allele.

Indeed it would be interesting to type the studied sample for the CBS 844ins68 genotype. However, this would almost be vast of research foundation unless first establishing a deviation in status of the two investigated B-vitamins and homocysteine between the study-population and age-matched controls without subjective or objective cognitive impairment. (The presence of the allele might indirectly reduce a raised homocysteine concentration, but it would still be raised.) Prospective research on the same topic and in the same population should therefore be directed towards either such a study with normal controls, or, perhaps, a treatment study in which patients with mild cognitive impairment or subjective memory complaints are supplied with cobalamin and/or folate, to see if any improvement in cognitive performance would result. However, it must once be noted that the patients does in fact not show clinical symptoms of deficiency.

Anyway, it is not realistic to believe that the major cause of cognitive dysfunction (neither subjective nor objective) in the assessed population is due to B-vitamin functions. Nevertheless, in patients with

cognitive impairment all factors that might improve cognition, or at least slow down any progression, is of importance. Hence, if there is a homocysteine factor, though weak, contributing to the cognitive impairment in the studied sample, it is of significant interest to identify this factor and try to reduce it. Thus further research is motivated, and meanwhile the patients might be recommended to consider the use of low dosage supplement of folate and cobalamin.

## Conclusion

The first conclusion from the present material is that almost no conclusions can be made! Data for cobalamin and folate lack in reliability and are inconclusive. The outcome is satisfactory for homocysteine however, and for longitudinal one-year-follow-up too, and these results suggest a feature congruent with the homocysteine hypothesis. That is, a component in the cognitive deterioration might be due to a slowly progressing long-term negative influence of homocysteine.

From the available data it is not possible to draw conclusions about if the results are specific for the studied population of patients with mild cognitive impairment and subjective memory complaints, or if they are general within the normally ageing population. It is possible that the results are population-specific, but there are reasons to believe that this is not the case. However, in individuals suffering from cognitive dysfunction it is especially important to maintain, or, if possible, reinforce cognitive functioning. Hence further research is requested in order to conclude if associations between B-vitamin status and cognitive performance display anomalous characteristics in the study-population. In such a study those cognitive tests that showed significant or nearly significant relations to any of the measures of cobalamin, folate or homocysteine in the present study, would preferably be first-choice tasks of neuropsychological tests in the experimental set-up. Also if a study like that out-roles any population differences certain concern regarding status of cobalamin, folate and homocysteine is recommended for the investigated group of patients.



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