VITAMIN B$_{12}$ AND FOLATE DEPLETION IN THE ELDERLY DIAGNOSIS, CLINICAL CORRELATES AND CAUSES

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ABSTRACT

Subclinical vitamin B_{12} and folate deficiency is common in the elderly. The clinical significance remains unresolved. There is not a universally accepted set of laboratory criteria for diagnosis, however subclinical deficiency is important to diagnose since it is easy to treat. Currently available measures of vitamin concentrations are, except in pronounced deficiency, unreliable. Plasma tHcy and serum MMA are potentially more reliable markers of intracellular vitamin status.

The overall aim was to estimate the prevalence of B-vitamin deficiency and atrophic gastritis, to calculate health related reference intervals for plasma tHcy/serum MMA, to explore the dependence of glomerular filtration rate on these metabolites, and to study the effect of oral B-vitamin therapy both on biochemical and clinical outcome.

The thesis is based on a population-based study of 209 community-dwelling subjects, mean age 76 years. The study included a double-blind placebo controlled intervention with an oral daily combination of vitamin B_{12} (0.5mg), folic acid (0.8mg) and B_6 (3mg) during four months.

Elevated plasma tHcy and serum MMA was found in 53% and 11%. Vitamin B_{12} deficiency occurred in 7.2%, folate deficiency in 11%, atrophic gastritis in up to 14%. Health- related upper reference limits for the metabolites were higher than those commonly used. After adjustment for glomerular filtration rate also within it's normal range, the fraction of subjects with elevated plasma tHcy diminished significantly. Plasma tHcy and serum MMA correlated inversely with movement and cognitive performance. Vitamin therapy significantly decreased plasma tHcy (32%) and serum MMA (14%) but failed to improve movement or cognitive performance. Atrophic gastritis did not cause reduced vitamin absorption.

In conclusion, elevated levels of plasma tHcy and serum MMA were common and more frequent than actual B-vitamin deficiency. The prevalence of "elevated" plasma tHcy may be overestimated unless adjusted for glomerular filtration rate. Atrophic gastritis was not uncommon and correlated to inferior B-vitamin status. Short-term oral B vitamin treatment normalized plasma tHcy and serum MMA levels also in subjects with atrophic gastritis, but did not affect movement or cognitive performance.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I.   Catharina Lewerin, Herman Nilsson-Ehle, Michael Matousek, Göran Lindstedt and Bertil Steen. Reduction of plasma homocysteine and serum methylmalonate concentrations in apparently healthy elderly subjects after treatment with folic acid, vitamin B12 and vitamin B6: a randomised trial. Eur J Clin Nutr 2003; 57:1426-1436

II.   Catharina Lewerin, Susanne Ljungman, Herman Nilsson-Ehle. Glomerular filtration rate as measured by serum cystatin C is an important determinant of plasma homocysteine and serum methylmalonic acid in the elderly. Accepted J Int Med 060926

III.   Catharina Lewerin, Michael Matousek, Gunilla Steen, Boo Johansson, Bertil Steen, Herman Nilsson-Ehle. Significant correlations of plasma homocysteine and serum methylmalonic acid with movement and cognitive performance in elderly subjects but no improvement from short-term vitamin therapy: a placebo-controlled study. Am J Clin Nutr 2005;81:1155-62

IV.   Catharina Lewerin, Stefan Jacobsson, Göran Lindstedt and Herman Nilsson-Ehle. Atrophic gastritis and antibodies against Helicobacter pylori in the elderly. Implications for vitamin B12, folic acid and iron status, cognitive and movement performance, and response to oral vitamin therapy. Manuscript

TABLE OF CONTENTS

ABBREVIATIONS

AG	atrophic gastritis
BMI	body mass index
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
DNA	deoxyribonucleic acid
Fe	iron
GFR	glomerular filtration rate
Hb	blood haemoglobin
HC	haptocorrin
holo-TC	holotranscobalamin
H. pylori	Helicobacter pylori
HPAb	antibodies against Helicobacter pylori
IF	intrinsic factor
MCV	erythrocyte mean volume
n	number of participants
MEIA	micro particle enzyme immunoassay
MTHFR	methylentetrahydrofolate reductase
MMA	methylmalonic acid
tHcy	total homocysteine
PA	pernicious anemia
PLM test	postural-locomotor-manual test
	P phase postural phase
	L phase locomotor phase
	M phase manual phase
	SI simultaneity index
r	Pearson's correlation coefficient
rp	Pearson's partial correlation coefficient
RDI	recommended daily intake
RIA	radioimmunoassay
RNA	ribonucleic acid
RS	reference sample group
SD	standard deviation
SEM	standard error of the mean
TIBC	transferrin iron binding capacity
TS	transferrin saturation
TSG	total study group

INTRODUCTION

HISTORICAL REMARKS

The earliest recorded history of autoimmune gastritis was described in 1855 when Thomas Addison described mental and neurological symptoms of a pernicious (i.e. dangerous and life-threatening) anaemia (PA). Atrophy of the gastric mucosa in PA examined microscopically was reported 1870. Paul Ehrlich (1880) noticed megaloblasts in the blood in PA. Inspired by George Whipple, George Minot and William Murphy successfully treated a PA patient with raw liver. Liver concentrates were developed that could be given orally (Figure 1) and parenterally.
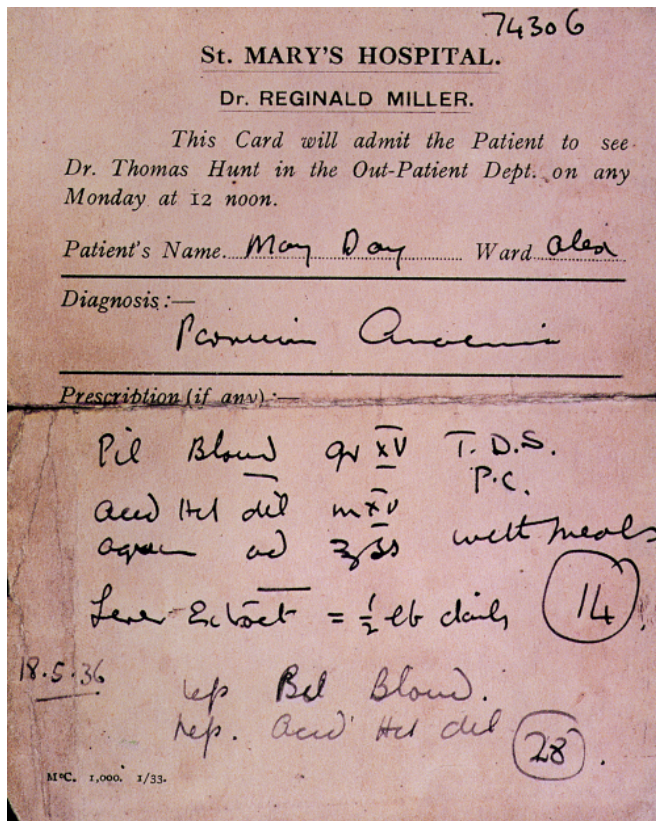
Figure 1:
Prescription of 230 mg of liver daily for PA in 1938
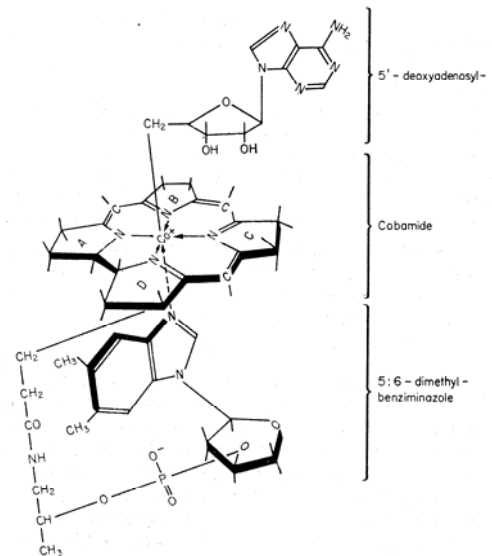
Figure 2:
The structure of deoxyadenosylcobalamin

It was noted that responding patients showed a rise in reticulocyte counts already after 4-5 days, before a clear rise in haemoglobin was seen. In 1934, Whipple, Minot and Murphy were awarded the Nobel Prize in medicine and physiology for this research. This was followed by the discovery, by William Castle, of intrinsic factor (IF), a cobalamin binding protein necessary for active intestinal absorption of the vitamin.

Vitamin B_{12} was first isolated in 1948, serum B_{12} assays based on microbiological methods (Euglena gracilis, Lactobacillus Leichmannii) were introduced in the early 1950´s. The Schilling test (indirect testing of a water-soluble trace dose of radiolabelled B_{12}) was first described 1953. The function of methylcobalamin as a co-factor for methionine synthetase, the enzyme catalysing the remethylation of homocysteine (tHcy) to methionine, was described in 1950. Deoxyadenosylcobalamin as a cofactor in the mitochondrial metabolism of methylmalonyl CoA was noted in 1957. Dorothy Hodgkin elucidated the structure of vitamin B_{12} (Figure 2) and was 1964 awarded the Nobel Prize. Parietal cell antibodies were described 1962. Transcobalamin (TC, formerly called TC II), the only carrier protein able to deliver vitamin B_{12} into the cells, was identified in 1965. The successful treatment with raw liver, followed by liver extracts and later vitamin B_{12} given parenterally or orally, of a previous fatal anaemia, represents one of the most significant achievements in medicine and haematology.

VITAMIN B_{12} AND FOLIC ACID - BACKGROUND

Severe clinical vitamin B_{12} and folate deficiency with haematological and/or mucosal damage is uncommon (prevalence 1-2%), the diagnosis is rarely problematic and the clinical consequences are obvious. Vitamin B_{12} deficiency, including the end stage of pernicious anaemia, occurs more commonly in the elderly due to an increased prevalence of atrophic gastritis (AG) and other factors that lead to a negative vitamin balance. The gold standard for the diagnosis of clinical deficiency of both vitamin B_{12} and folic acid is still an optimal clinical response to therapeutic doses of the vitamins. The megaloblastic anaemia seen in PA is usually associated with glossitis and neurological symptoms. The development of vitamin B_{12} deficiency may take several years from the onset of vitamin B_{12} malabsorption. Neuropathy due to vitamin B_{12} deficiency in the absence of megaloblastic anaemia (Lindenbaum *et al.* 1988) was noticed about two decades ago. This diagnosis depends on reliable laboratory tests for vitamin B_{12} deficiency. The sensitivity of total vitamin B_{12} in serum and blood folate concentrations merely below traditional reference intervals is limited. However, the specificity of undisputable low vitamin concentrations is high.
Serum/plasma concentrations of the vitamin B_{12} and folate dependent intermediate metabolites total homocysteine (tHcy) and methylmalonic acid (MMA) partly reflect the intracellular availability of these vitamins. They form the basis of the concept of functional or subclinical vitamin deficiency, i.e. the combination of decreased but not extremely low vitamin concentrations and elevated metabolites.

As regards any underlying gastrointestinal disorder, serum concentrations of the gastric derived functional markers gastrin and the pepsinogens are valuable complements to invasive diagnostic methods, and have in practice replaced the Schilling test.

Clinical sign and symptoms, i.e. incipient macrocytic anaemia and neurocognitive decline are often unspecific and might be caused by other reasons than vitamin deficiency. However, subclinical vitamin deficiency is important to diagnose since it is easily treatable.

Since there is no universally accepted set of laboratory criteria for upfront diagnosis of subclinical deficiency this might be a challenge. The inherent limitations of serum cobalamin assays are well known. First, total serum B_{12} reflects approximately 20-30% of biologically available serum B_{12}. Second, the distribution also in healthy subsets of the population is markedly skewed, making the calculation of reference intervals uncertain. Further, extremely low serum B_{12} has a high specificity for vitamin B_{12} deficiency, but concentrations well above the lower reference limits have also been proposed as compatible with deficiency. Elevated serum B_{12} levels are also seen in other diseases, e.g. myeloproliferative disorders and other malignancies, and the presence of intrinsic factor (IF) antibodies may cause false high values. Plasma folate levels reflect current and recent folate status and false elevated levels might be seen in vitamin B_{12} deficiency. Whole blood folate reflects folate status over the preceding last months, but the accuracy of whole blood folate assays has been questionable. Plasma tHcy is elevated in deficiency of vitamin B_{12}, folate and B_6, and is dependent on renal function. The health-related reference intervals are both age- and gender specific. Serum MMA is also affected by other factors than vitamin B_{12} deficiency, e.g. renal function, pregnancy and intestinal bacterial overgrowth.

The concept of health-related reference intervals for separating vitamin deficient from vitamin replete subjects thus faces serious challenges, and the laboratory criteria for the diagnosis of subclinical vitamin deficiency do in practice rely on measurements of vitamin and metabolite concentrations. Ideally, a cause for the vitamin depletion should be sought and found, be it malnutrition or malabsorption.

Most of the problems addressed above are present when diagnosing elderly patients, e.g. unspecific symptoms, borderline or grey zone laboratory results, influenced by factors not related to vitamin status. Further, there are often multiple factors rather than a single cause behind the vitamin depletion.

As a complement to vitamin B_{12} injections, oral vitamin treatment has a long tradition in Sweden, compared to other countries, based on the early vitamin B_{12} resorption data published by Berlin and co-workers (Berlin *et al.* 1968).

The treatment of this previously pernicious anaemia as well as other and earlier vitamin depletion states is nowadays simple and safe.

VITAMIN B$_{12}$ - FUNCTION, SOURCES AND RDI

Function

Vitamin B$_{12}$ is required to maintain normoblastic haematopoiesis, normal nerve tissue and normal levels of intracellular folate. Deficiency leads to defect DNA synthesis, probably due to the disturbed metabolism of folate. This results in disturbed division of cells with a rapid cell turnover i.e. bone marrow, mucosa and germinal epithelial cells.

Sources and RDI

Vitamin B$_{12}$ is supplied with food of animal origin. The main sources are liver, meat, fish, seafood, milk products and eggs. The daily recommended intake (RDI) of vitamin B$_{12}$ for adults is 2.0 µg per day (Sorenson 2004), corresponding to an intake of 3.0 µg. The body B$_{12}$ stores are 2.5-5 mg and last several years without vitamin B$_{12}$ supply.

VITAMIN B$_{12}$ DEPENDENT REACTIONS

In humans, there are two intracellular reactions that are dependent on vitamin B$_{12}$ as a co-enzyme. In the first reaction, adenosylcobalamin is a coenzyme in the conversion of methylmalonyl-CoA to succinyl-CoA, a metabolite in the citric acid cycle (Figure 3). Loss of methylmalonyl CoA mutase activity causes an accumulation of methylmalonic acid (MMA).

Metylmalonyl CoA (mitochondria)

L-metylmalonylCoA ←——— adenosyl-B$_{12}$ / *mutase* ———→ succinyl-CoA

↕

D-metylmalonylCoA

↓

Metylmalonic acid (MMA)
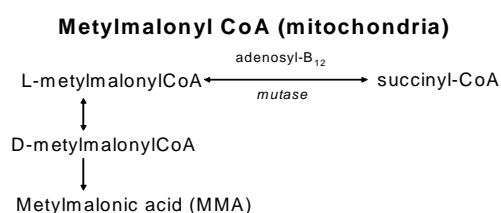
Fig 3. Vitamin B$_{12}$ dependent conversion of metylmalonyl CoA to succinyl CoA

The other vitamin B$_{12}$–dependent reaction is the remethylation of homocysteine to methionine (Figure 4). Methionine is an essential amino acid that is adenosylated to S-adenosylmethionine (SAM), an essential methyl donor for methylation of DNA, RNA, phospholipids and neurotransmittors. Methionine is metabolised to homocysteine.

Homocysteine is then either catabolised to cysteine, a reaction in which vitamin B_6 acts as a co-factor for the enzyme cystathione beta synthetase. More importantly, homocysteine is re-methylated to methionine in a reaction catalysed by methinone synthetase, which has methylcobalamin as cofactor. The substrate (i.e. methyl donor) in this reaction is 5-methyl-tetrahydrofolate, which in turn is derived from 5,10-methylene-tetrahydrofolate via a reaction dependent on a reductase. Thus, homocysteine levels increase when the supply of vitamins B_{12}, folate and B_6 is insufficient, or if any of the enzymes involved have reduced capacity.
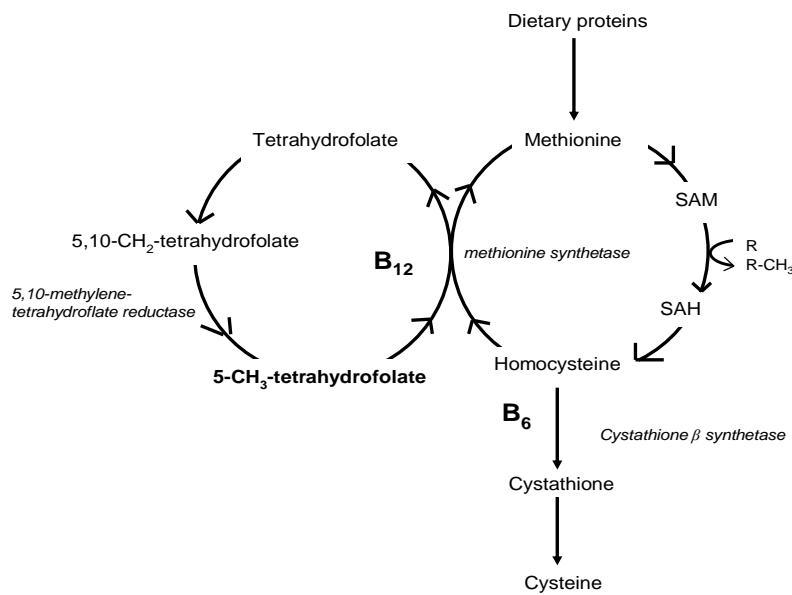
Fig 4. The vitamin dependent metabolism of methionine.

NORMAL VITAMIN B_{12} ABSORPTION

Vitamin B_{12} has first to be released from dietary proteins. In the stomach, this is accomplished by acid and pepsin, whereafter vitamin B_{12} is bound to haptocorrins. Vitamin B_{12} is thereafter liberated from haptocorrins by pancreatic enzymes and bound to IF. The B_{12} – IF complex is then absorbed in the terminal ileum. The IF-mediated absorption is an active transport of the small amounts of vitamin B_{12} from the food. Large oral doses, like sufficient amounts of raw liver, or in B_{12} tablets, are absorbed by passive diffusion in the intestine. Approximately 1.2% of orally administered crystalline B_{12} is absorbed by passive diffusion (Berlin *et al.* 1968).

VITAMIN B$_{12}$ DEFICIENCY

Prevalence of pernicious anaemia (PA) and atrophic gastritis

The prevalence of PA, the end-stage of autoimmune AG, is about 2% of individuals aged > 60 years and varies between populations (Nilsson-Ehle *et al.* 1989; Carmel 1996). Atrophic gastritis without obvious PA is more common and has been reported up to 30% in the elderly (Krasinski *et al.* 1986).

Prevalence of vitamin B$_{12}$ deficiency

The prevalence figures for vitamin B$_{12}$ deficiency varies between populations and depends further on the diagnostic criteria used. Prevalence figures from 3 to 41% have been reported (Baik *et al.* 1999). The prevalence in different elderly populations as defined by abnormal concentrations of vitamin B$_{12}$ and metabolites is approximately 5-20% (Lindenbaum *et al.* 1994; Nilsson-Ehle 1998; Clarke *et al.* 2004).

Symptoms of vitamin B$_{12}$ deficiency

Vitamin B$_{12}$ deficiency may cause neurological symptoms such as gait disturbances, impaired vibration sense, neuropsychiatric disturbances including depression, confusion and cognitive impairment, even in the absence of anaemia (Lindenbaum *et al.* 1988). Symptoms in the elderly are more often non-specific like tiredness or malaise (Lindenbaum *et al.* 1988). However, studies of older people indicate that only a small proportion of those found to have biochemical evidence of vitamin B$_{12}$ deficiency have anaemia, neuropathy or cognitive impairment (Hin *et al.* 2006). Vitamin B$_{12}$ deficiency is mostly caused by malabsorption, and is related to autoimmune disease, e.g. diabetes mellitus, thyroid disorders, vitiligo and Addison´s disease. Correlations between vitamin B$_{12}$, plasma tHcy and bone mineral density has been reported (Morris *et al.* 2005).

Subclinical cobalamin deficiency

Subclinical cobalamin deficiency in the elderly is, in addition to the approximately 2% with obvious clinical deficiency, found in approximately another 10-20% of the elderly. It is defined as an asymptomatic state in which metabolic insufficiency is demonstrable in patients and in seemingly healthy non-patients. There is no megaloblastic anaemia, no neurological signs or other clinical manifestations of vitamin B$_{12}$ deficiency (Carmel *et al.* 2003).

Serum vitamin B_{12} levels can be low normal (185-258 pmol/L) and at least one metabolite should be abnormal.

Causes of vitamin B_{12} deficiency

Malabsorptive disorders can be found in about half of the subjects with subclinical B_{12} deficiency. Food-cobalamin malabsorption syndrome (Döscherholmen *et al.* 1973) is due to hypochlorhydria and lack of pepsin leading to impaired release of vitamin B_{12} from food. It is caused by atrophy of the gastric mucosa, the atrophy may progress leading to low or absent IF secretion, which sets the stage for development of classical PA. This progress may be very slow and individuals could be asymptomatic for several years. Other causes for malabsorption of vitamin B_{12} are bacterial overgrowth in achlorhydric subjects (Suter *et al.* 1991), total or partial gastrectomy with inadequate secretion of IF and atrophy of the gastric remnant, celiac disease, ileocekal resection or Crohn´s disease in the distal ileum. Certain drugs interfere with vitamin B_{12} metabolism, such as gastric acid inhibitors (iatrogenic achlorhydria) (Laine *et al.* 2000), biguanides (intestinal malabsorption) (Adams *et al.* 1983) and slow release potassium chloride. Nitrous oxide (laughing gas) causes irreversible inactivation of methionine synthethase (Weimann 2003), which may be critical in vitamin depleted subjects or in repeated exposure/abuse. Insufficient dietary intake is less common except in vegetarians/vegans, but microwave heating of food destroys some of the B_{12} content (Watanabe *et al.* 1998). Deficiency or abnormality of TC leading to clinical deficiency is to date reported to be rare. However, genetic polymorphisms in the gene coding for the TC have recently been studied (Zetterberg *et al.* 2003)

FOLATE - FUNCTION, SOURCES AND RDI

Function

Folic acid and folate (the anion form) are a water soluble B-vitamins. The function of folate is to carry and transfer active carbon units for the novo synthesis of purines and pyrimidines required for DNA and RNA synthesis, e.g. to maintain normal haematopoiesis. Folate is required for the remethylation of Hcy to methionine (Figure 4). Deficiency of the cofactor vitamin B_{12} for methionin synthetase causes elevated levels of the substrate 5-methyltetrahydrofolate in serum and reduced amounts of intracellular tetrahydrofolate, i.e. increased plasma folate and reduced blood folate concentrations.

The fact that 5-methyltetrahydrofolate can not be reconverted to its precursor, methylentetrahydrofolate, is referred to as the methylfolate trap (Herbert *et al.* 1962; Smulders *et al.* 2006). Thus, elevated levels of plasma tHcy is a marker of both vitamin B_{12} and folate deficiency. Large doses of folate may normalise megaloblastic anemia but has no effect on neuropsychiatric symptoms caused by vitamin B_{12} deficiency (Neuhouser *et al.* 2001). In vitamin B_{12} deficient subjects, the safe upper dose of folic acid is less well defined, but appears to be in the range of 0.6-1.2 mg/day.

Sources and RDI

Folates are found in foods of both animal- and vegetable origin. The main sources are leafy green vegetables (like spinach and turnip greens), fruits (like citrus fruits and juices), and dried beans and peas. The daily-recommended intake of folic acid for adults is 300 µg per day and 500 µg for pregnant and lactating women (Sorenson 2004). Without supply, the body stores (approximately 5 mg) will last for 3-4 months.

NORMAL FOLATE ABSORPTION

Folic acid is absorbed in the upper part of jejunum and approximately 50% of the daily intake is absorbed. In subjects with intestinal malabsorption oral doses up to five mg folate is sufficient (Chanarin 1979d). The dose required to maintain normal homocysteine levels on a population basis is 0.5-5 mg folic acid daily (Clarke 1998).

FOLATE DEFICIENCY

Prevalence

Low blood and/or serum folate levels in the elderly have been reported in 5-19% (Joosten *et al.* 1993). The prevalence of folate deficiency increased with age to about 5% in people 65-74 years and 10% in people aged 75 years or greater (Clarke *et al.* 2004).

Clinical findings

Megaloblastic anemia and elevated MCV is a late phenomenon. Symptoms from the nervous system include dementia and depression but peripheral neuropathy like in vitamin B_{12} deficiency is usually not seen.

Causes

The most common cause is deficient intake e.g. some vegans, elderly, and chronic alcohol abusers. Excessive cooking of vegetables destroys significant amounts of folate in food (Chanarin 1979b). Malabsorption of folate may be seen following jejunal resection and Crohn´s disease affecting upper small intestine. Celiac disease may be diagnosed even in old patients. In a Swedish epidemiologic study, the highest prevalence, 178 per 100000 inhabitants, was found in patients aged 65-74 years (Midhagen *et al.* 1988). Deficiency of folate may occur when the need for folate is increased, e.g. in pregnancy, chronic hemolytic anemia, malignant disease and exfoliative skin disease. A number of drugs interfere with folate metabolism, i.e. phenytoin (James *et al.* 1997), sulfasalazine, trimethoprim and methotrexate (Refsum *et al.* 1989). The increased pH in the stomach and proximal small intestine seen in atrophic gastritis has been shown to lead to reduced folic acid absorption (Wolters *et al.* 2003).

MTHFR C677T POLYMORPHISM

Methylentetrahydrofolate reductase (MTHFR) catalyses the reduction of 5-10-methylenetetrahydrofolate, to 5-methyltetrahydrofolate, the methyl donor for the remethylation of Hcy to methionine (Figure 4). A polymorphism in the MTHFR gene, causing the enzyme to be thermo-labile and less functioning leads to elevated levels of plasma tHcy. Twelve % of the white population are homozygous (TT genotype) for this polymorphism (Brattström *et al.* 1998). It is associated with high concentrations of plasma tHcy, especially in subjects with low folate levels, and is associated with increased risk of neural tube defects. The T allele has been reported to protect against cancer in folate-replete subjects but increases the risk in subjects with impaired folate status. The TT genotype can predispose individuals to adverse effects of drugs with antifolate effects e.g. methotrexate (Ueland *et al.* 2001).

FOLIC ACID FORTIFICATION IN FOOD

In recent years, the role of folic acid for preventing neural tube defects has come into focus. This has led to folic acid fortification of food in some countries, e.g. the USA. This has lead to a decrease of approximately 20- 35% in neural tube defects (Williams *et al.* 2005). However, concerns relate to the potential risk of masking vitamin B_{12} deficiency by folate, especially in the elderly. There is as today no decision on folic acid fortification in Sweden.

VITAMIN B$_6$ (PYRIDOXIN)

Vitamin B$_6$ is a cofactor for cystathionine-β-synthetase in the catabolism of homocysteine to cysteine (Figure 4). A suboptimal vitamin B$_6$ status may thus lead to elevated levels of plasma tHcy, sometimes only revealed after oral methionine load (Ubbink *et al.* 1996).

The daily recommended allowance (RDI) in Sweden is 1.2 mg/day (women) and 1.6 mg/day (men) and the most common sources are meat products, egg and fruits (Sorenson 2004). Deficiency is not uncommon in the elderly (Haller *et al.* 1991) and mostly due to reduced intake (Bates *et al.* 1999). Low levels of vitamin B$_6$ are associated with depression (Hvas *et al.* 2004), neurological symptoms, Alzheimer dementia (Mulder *et al.* 2005), skin disorders, anemia and also seen in patients with asthma and rheumatoid arthritis (Sanderson *et al.* 1976). High doses (300-500 mg) of vitamin B$_6$ has been repereted to be toxic to the nervous system (Bässler 1989).

MATERIAL AND METHODS

STUDY POPULATION AND DESIGN

Study population

Participants in a previous study (Augustsson *et al.* 1994), the Johanneberg study (n=217), conducted to identify and evaluate social and medical risk indicators for mortality in an elderly urban population living in a local urban area in Göteborg, not reported to take B-vitamins, were invited by letter (n=120). Only 34 participants from this study were willing and/or qualified to participate. Further participants were recruited from the population registry. Every forth probands living in a defined area in the centre of the city of Göteborg (Johanneberg) were invited by letter. Letters were sent to about 1075 subjects aged 70-85 years. Subjects who accepted and were not primarily excluded due to vitamin medication were enrolled. Those who had taken any vitamin supplements during the last three months or pharmacological doses of vitamin B_{12}, folic acid and/or vitamin B_6 during the last three years were primarily excluded. No further exclusion criteria for entering the study were applied.

Study design

The number of participants needed to reach a p <0.05 significance level for the difference in serum MMA and plasma tHcy before and after the planned vitamin therapy in this placebo controlled intervention study was found to be 180 (vitamin group n=120, placebo group n=60), using previous pilot data. In total, 209 subjects entered the study and were assigned to vitamin or placebo therapy according to a double-blind randomised parallel group design. The mean age was 76,4 (SD $\pm$ 4.7) years, range 70-88 years (women) and 70-93 years (men). The proportion of females was 60%. In the total study group, 168/209 (80%) were treated with some kind of medication, 41/209 (20%) were on no medication. For the vitamin intervention study, 139 were randomised to active therapy (the vitamin group) and 70 to the placebo group.

Intervention

Treatment in the vitamin group consisted of a daily tablet containing 500µg cyanocobalamin, 800µg folic acid and 3mg pyridoxine hydrochloride, identical to the placebo tablets except for the vitamin content.

In both groups, one tablet was taken daily in the morning for four months. To ensure compliance, all subjects received a specified blinded number of tablets (n=130), and at the end of the study, the number of remaining tablets was compared with the initial number and planned intake during the study period. Height, weight and blood pressure were measured and all probands were interviewed regarding allergy, drug consumption and smoking habits. During follow-up, any new symptoms possibly related to the treatment given were recorded.

Reference sample groups (Paper I)

For the purpose of calculation of health-related reference intervals, three subgroups of the total study group (reference sample groups (RS) I-III) were defined. RS I (n=125), obtained by exclusion of subjects not meeting defined anamnestic and/or laboratory criteria indicating disease (table 2, paper I (Lewerin *et al.* 2003)) and was used for calculations of traditional baseline health-related reference intervals. RS II (66 for plasma tHcy and 115 for serum MMA) comprised subjects in the vitamin group *not* achieving a significant decline in plasma tHcys or serum MMA (arbitrarily defined as >3 SD of the change in the placebo group), thus presumably not vitamin deficient at baseline. RS III (n=115) comprised subjects in RS I who received active vitamin therapy. Data for these subjects were analysed at the end of study. Thus, RS III constituted a healthy *and* vitamin-replete subgroup.
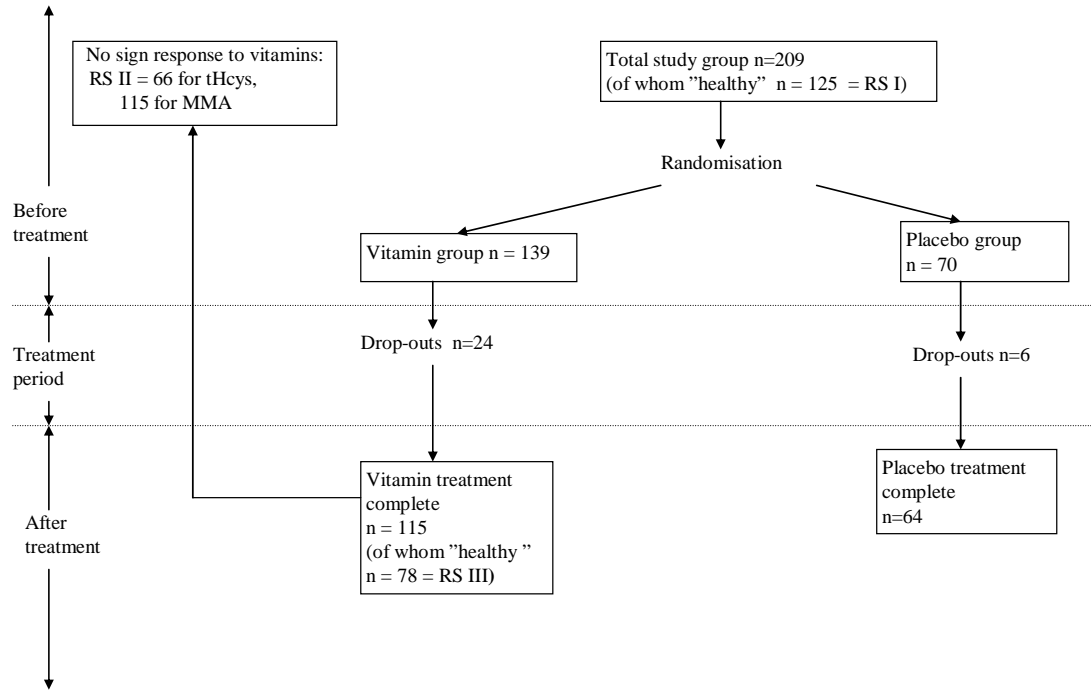
Fig 5. Study population and reference sample groups, paper I.

BLOOD SAMPLING AND LABORATORY METHODS

Blood sampling

Blood samples were collected at the start of the study and after one and four months. Samples were obtained with the subjects in a recumbent position, after an overnight fast. Vacuum tubes were used. Blood samples for determination of cell counts were collected in EDTA tubes, for plasma analyses in heparinised tubes and for serum analyses in tubes without anticoagulant. After venipuncture, serum and plasma samples were centrifuged and thereafter kept at room temperature for two hours before transport to the laboratory.

Blood hemoglobin and iron status

Blood haemoglobin and cell counts were analysed using a Technicon H2 flow cytometer, serum iron (Fe) and serum total iron-binding capacity (TIBC) using a Hitachi 917 analyser with ferrozine-ascorbic acid as chromogen.

Serum cobalamins

The concentrations of serum B_{12} were determined using an isotope dilution method with purified hog intrinsic factor as the binder and an alkaline pH, no-boil procedure (Solid Phase No Boil Dualcount, Diagnostic Products Corp., Los Angeles, CA, USA). Reference interval was 130-750 pmol/l. The detection limit (minimal detective dose) of the assay is approximately 25 pmol/L.

Whole blood and plasma folates

The concentrations of whole blood and plasma folates were determined by the same radio assays as for serum B_{12}, but using folate-binding protein as binders for folic acid (Solid Phase No Boil Dualcount, Diagnostic Products Corp., Los Angeles, CA, USA). Reference intervals for plasma folates were 6-35 nmol/l, for whole blood folates 100-450 nmol/l. The detection limit of the assay is approximately 0.7 nmol/L.

Serum methylmalonic acid (MMA) and plasma total homocysteine (tHcy)

Serum MMA was measured using capillary gas chromatography and mass spectrometry (Rasmussen 1989). Plasma tHcy was measured by high-performance liquid chromatography with fluorescence detection (Bald *et al.* 1994). The current health-related upper reference limits of the laboratory were for plasma tHcy 16µmol/l and for serum MMA 0.34µmol/l.

Serum cystatin C

Cystatin C was measured in serum using a particle-enhanced turbidometric assay method (Dako Cytomation AB art, number LX002), with Hitachi Modular P-module Roche Diagnostics (Kyhse-Andersen *et al.* 1994). The reference interval stated by the laboratory was 0.63-1.33 mg/L in individuals <50 years of age and 0.74-1.55 mg/L in individuals aged $\geq$50 years (Anonymous 1996). Serum cystatin C concentrations were determined four years after completion of the study using serum samples stored at - 70°C. Samples not clear by ocular inspection were centrifuged at 17 000 g for 15 min. Serum cystatin C was measured on one occasion during the study.

Serum creatinine

Serum creatinine was measured with a modification of Jaffe's method, a spectrophotometric kinetic method using Hitachi 917, reference intervals being 50-110 µmol/L for women and 60-120 µmol/L for men.

Holotranscobalamin

Plasma holo-TC concentrations were determined 9 years after completion of the study using serum samples stored at - 70°C. These samples were, for other purposes, thawed at two occasions before the present analysis. Holo-TC was only analysed on samples taken after one (n=195) and four months (n=162) due to limited supply of samples from study start. In 133 subjects of the vitamin group and 66 of the placebo group, holo-TC values were obtained. In the placebo group 56/66 and in the vitamin treated group 91 of 115 evaluable subjects had values both after one and four months. Holo-TC was determined by a new automatic method, AxSYM® HoloTC, a micro particle enzyme immunoassay (MEIA). There are as yet no validated clinical reference intervals.

Serum pepsinogen I and II

Serum pepsinogens I and II were determined by polyethylenglycol-assisted double-antibody radioimmunoassays (Sorin Biomedica 13040 Saluggia/Vercelli, Italy), the lower reference limit was 30 µg/L. As decision limits for AG, a pepsinogen I/II ratio below 2.9, and for antrum-sparing AG a serum pepsinogen I $\leq$30 µg/L in the presence of serum gastrin $\geq$75 µg/L, were chosen. Serum pepsinogen I, II, and gastrin were determined two years after completion of the study using serum samples stored at - 70°C.

Serum gastrin

Serum gastrin was determined by a gastrin double-antibody liquid-phase radioimmunoassay (Becton-Dickinson). The upper reference limits by laboratory standard was <50 pmol/L in individuals <40 years of age and <75 pmol/L in individuals aged >40 years and more.

Antibodies against H. pylori

Immunoglobulin G antibodies against H. pylori (HpAB) were determined by enzyme immunoassay (HM-CAP [TM] H. pylori immunoassay, Enteric Products Inc., Westbury, NY (Evans *et al.* 1989). A concentration $\geq$ 2.2 AU/L was defined as positive (HPAb+).

COGNITIVE TESTS (PAPER III)

Cognitive testing was conducted by the same psychologist at baseline and after four months. A comprehensive battery of cognitive tests was administered to characterize the overall level of cognitive abilities among the probands. Testing took about one hour. All tests except the memory test had time limits. Test scores were equal to the number of correct responses, except for Figure Grouping and Identical Forms, where corrections were made for guesses. In the analysis directed towards potential relationships across markers of vitamin status and movement performance we focused on tests measuring psychomotor ability and mental speed, e.g. the Digit Symbol Test and Block Design. The following tests were used on the two occasions.

Digit span forward/ backward, measuring short-term memory. The subject has to reproduce a series of digits, which increase gradually. In the backward version, the subject has to repeat the digits backward. The maximum (best) score is 9 in the forward and 8 in the backward subtests, respectively (Wechsler 1945, 1958).

Identical forms measures perceptual speed. This test contains 60 items of identification. For each item, a complex figure is compared with five other figures, and the one that is identical is marked. The maximum (best) score is 60 (Dureman _et al._ 1959).

Visual reproduction is a measure of visual memory using four drawings shown to the subject to be remembered and reproduced. The function is dependent on the memory for visuo-spatial relations but also to some extent to motor functions. The scoring followed the Wechsler Memory Scale (Wechsler 1945). The maximum (best) score is 14.

Synonyms, measuring verbal ability. The subject has to select, from among five words, a synonym for a given word. The maximum (best) score is 30 (Dureman _et al._ 1959).

Block design, measures spatial ability. This test consists of seven designs which have to be made out of red, white and red and white blocks. The maximum (best) score is 42. Bonuses are given for rapid performance (Wechsler 1958; Dureman _et al._ 1959).

 Digit symbol, a test of perceptual speed with a time limit of 90 seconds. The subject is asked to replace digits with symbols according to an existing code. This presumes concentration, sustained attention, learning, visual-motor coordination and cognitive flexibility. This test has also been used as a biomarker of aging in a number of studies. The maximum (best) score is 90 (Wechsler 1958).

Thurstone's Picture Memory Test measures long-term memory. The subject looks at 28 pictures consecutively, which are presented at a rate of one per five seconds, and is later asked to identify the picture among four similar pictures. The pictures were enlarged in order to minimize problems due to vision impairments in the subjects. The maximum (best) score is 28 (Thurstone 1938; Dureman _et al._ 1959).

Figure classification, measures inductive reasoning. In each item five figures are given. The figure that is different from the others is to be marked. The maximum (best) score is 30 (Thurstone 1938).

THE PLM TEST (PAPER III)

Movement performance (n =195) was measured by a Postural-Locomotor-Manual (PLM) test, a non- invasive optoelectronic technique using infrared light (Qualisys AB, Göteborg, Sweden). The PLM test (Matousek _et al._ 1994) consists of a complex motion during which the patient moves an object from the floor 1.5 meters forward and positions it upon a stand at the height of their chin (Figure 6). Six reflective markers are placed on the right side of the head, shoulder, elbow, hip, ankle and left foot of each subject. The seventh marker is placed on the test object, a metal handle fastened to a cylindrical horizontal plate weighing 550 grams. A camera system registers the infrared light pulses reflected from the markers. The position of the markers is calculated 50 times per second as two dimensional (x,y) Cartesian room co-ordinates and stored in a computer. The coordinate data are processed using commercially available software, the PLM program. Time spent for a) moving the object from floor to shelf (movement time, MT), b) raising the body after picking up the object (postural phase, P phase), c) moving the feet from start until stop in front of the stand (locomotor phase, L phase) and d) the goal directed active arm movement lifting up and placing the object on the stand (manual phase, M phase) were calculated. The overlap of the different phases is illustrated using the simultaneity index (SI) as calculated from the sum of the P, L and M phase durations divided by the movement time, SI=(P+L+M)/MT. A high value (near 2.0) of SI indicated good coordination of the P, L and M phases into a smooth and efficient body movement, whereas a value approaching 1.0 represented poor motor coordination with more sequential performance of the phases. Each subject performed five PLM trials, the last two were analysed, and the fastest was used for further analyses.

Data were selected from a representative file according to a previously described method (Matousek _et al._ 1994; Guo 2001; Guo _et al._ 2002). Movement time (MT) and simultaneity index (SI) were chosen as efficacy variables for statistical analyses.

Postural Phase   Locomotion Phase   Manual Phase

Postural Phase(P)

Locomotion Phase (L)

Manual Phase (M)

$$\text{Simultaneity Index} = \frac{P+L+M}{MT}$$

Movement Time (MT)
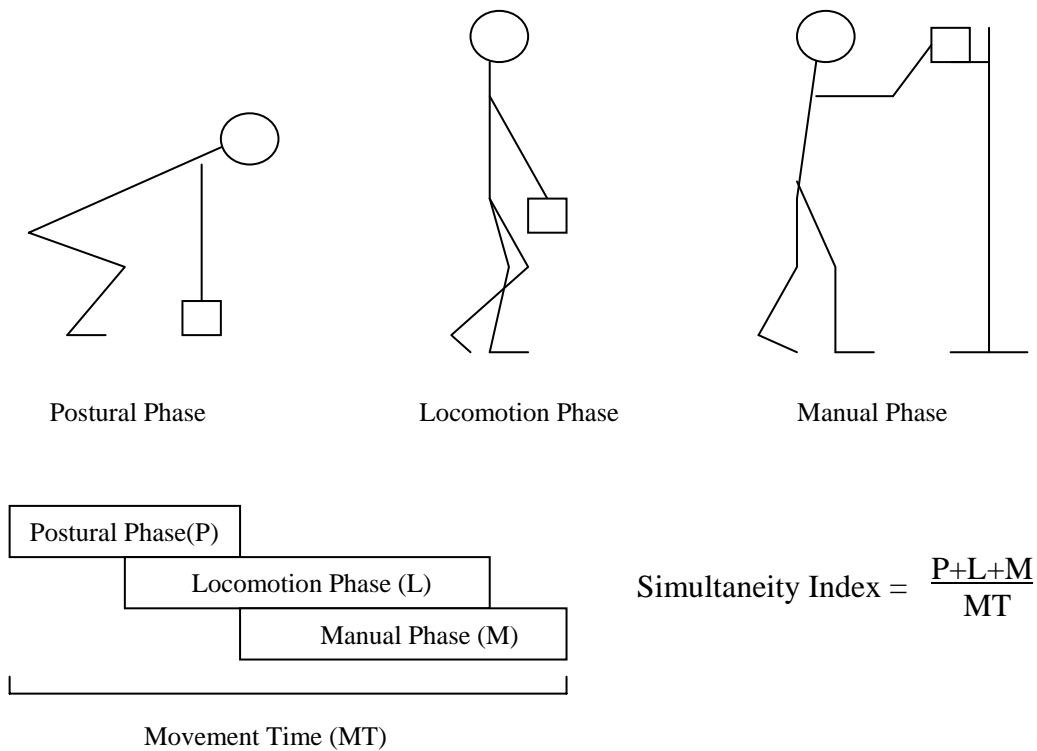
Fig 6. The Postural-Locomotor-Manual Test, paper III

STATISTICAL METHODS

Standard methods were used for calculation of mean, standard deviation (SD), Pearson's correlation coefficient (r), Pearson's partial correlation coefficient (rp) and multivariate linear least square regression coefficients and standard errors. T-tests were used for testing difference between two groups in linear scales, and Fisher's exact test for difference in proportions. Pair-wise t-tests were used for testing significance of change within single factors. Non-Gaussian distributions were log transformed in the regression models.
In testing for differences between two groups in more a general manner than achieved by the standard t-test, a method according to O´Brien was used (O´Brien 1988).
This test has good power to detect difference in both levels (or mean value) and shape (or standard deviation) between distributions. Stepwise linear regression models were used to find reduced sets containing the most importance predictors in the presence of correlation among the explanatory factors. Two-tailed tests were used throughout, and $p < 0.05$ was considered statistically significant.

Reference intervals, comprising the central 0.95 interfractile intervals, were calculated according to IFCC (International Federation for Clinical Chemistry) recommendations using a non-parametric method (Solberg 1983) after analysis and, if needed, transformation of the actual distributions. In subgroups containing < 100 subjects, the upper reference limits were calculated as mean + 1.97 S.D.

The software packages that have been used include SAS, SPSS, Statistica and, mainly, a statistical program system developed at the Department of Geriatric Medicine, Göteborg University, GIDSS.

COMMENTS ON STUDY POPULATION AND LABORATORY METHODS

Study population

The study population was not intended to be representative of the background population or any kind of potentially ill or vitamin deficient subset of the elderly, as evident from the selection process. Further, no formal analysis of the non-responders was conducted. The study subjects were all free-living individuals. The mean BMI in TSG at study start was 25.6, thus the studied population was not undernourished. We had the opportunity to compare some of the characteristics of the study group with contemporary study populations. Twenty % in the present study were without any kind of drug treatment compared to up to 7% in a representative cohort of 79-year-olds in 1995 (Lernfelt *et al.* 2003). There were no differences in mean blood hemoglobin, ESR or BMI as compared to a cohort of 75-year-old subjects (n=294) examined in 1990 in Göteborg (Kauppinen *et al.* 2002). Mean blood glucose were lower in the present study (4.8 vs. 5.5 $p<0.01$). In three out of four available cognitive variables, the present study population performed significantly better (digit span backward $p<0.05$, digit symbol $p<0.001$ and visual reproduction $p<0.05$).

Altogether, these data indicate that the present study population was healthier than representative population samples from the same geographical area.
We also compared the characteristics of our study population to that of the Skutskär study (Björkegren *et al.* 2001). In that study, the mean age was 77 years, 56% were women, mean

values for blood haemoglobin was 137 g/L, serum creatinine 90 µmol/L, serum MMA 0.21µmol/L, plasma tHcy 17.8 µmol/L, roughly comparable to our study.

Survival was not an endpoint, however we had access to survival data seven years after start of the study. Survival correlated with elevated levels of plasma tHcy (r=-0.19, p<0.01) and TIBC (r=-0.14, p<0.05) after adjustment for age and gender. No correlations between survival and blood haemoglobin, serum creatinine, serum MMA, serum vitamin B_{12} or blood folate were seen. There were no correlations between survival and movement/cognitive performance. Further analyses regarding the specific causes of death are planned.

Reference sample groups

The classification, in elderly populations, of healthy vs non-healthy individuals is difficult. Apart from health status, age and gender influence the distributions also in elderly populations. Ideally, traditional reference intervals should be validated prospectively, since they may or may not be equal to decision limits for investigation or treatment.
By applying different exclusion criteria to the TSG, a number of reference sample groups were established. RS I represents a traditional reference sample group. The exclusion criteria used to obtain this group were chosen both due to reference limits from the laboratory and on other significant circumstances, e.g. ongoing medication known to interfere with folate and vitamin B_{12} metabolism (Adams *et al.* 1983; Laine *et al.* 2000; Apeland *et al.* 2001). However, the upper limit of serum creatinine of 150 µmol/L chosen was probably too high in light of the later findings of correlations between plasma tHcy, serum MMA and renal function. Six subjects with previously recognised disease were still judged to be too ill and were excluded. A strength of the study was the thorough control of compliance during the intervention. Thus, we were able to utilise response data as criteria for "health" and vitamin status. RS II comprised subjects not showing a metabolite response to the vitamins and thus presumably not vitamin deficient at start, RS III comprised subjects healthy and vitamin replete after the end of the intervention. Altogether, the notion that the initial laboratory deviations found in this elderly, free-living population, obviously healthier than a representative sample or group of patients (paper I) represented an abnormal rather than an age-related healthy state was supported by these statistical calculations and to a further extent in the later analyses (papers II-IV).

Serum vitamin B12

Serum vitamin B_{12} was measured with the DPC Solid Phase No Boil kit with a specific binder (purified hog intrinsic factor), in order not to measure cobalamin analogues as well. The molecular weight of cyanocobalamin is 1355, thus a serum concentrations given in ng/L can approximately be converted to pmol/L according to the formula ng/L x 0.738 = pmol/L. However, serum B_{12} reflects the total amount of B_{12}, of which only 20-30% is bound to TC and thus available for transport into the cells. A markedly low value (<100 pmol/L) has a high specificity for the diagnosis of B_{12} deficiency, even though low levels of HC can give low serum B_{12} without actual deficiency (Carmel 2003). Serum B_{12} within the reference interval may still be compatible with deficiency, and values up to approximately the mean serum concentrations for the elderly have been proposed (Lindenbaum *et al.* 1994). Mean serum B_{12}, in a representative population of 81 years old men and women, was 248 pmol/L and 273 pmol/L, respectively (Nilsson-Ehle *et al.* 1991). Low levels without deficiency are also seen in multiple myeloma (Solomon 2006). High levels of serum B_{12} are seen in myeloproliferative disorders, due to elevated levels of haptocorrin, and in patients with liver disease (Ermens *et al.* 2003). Currently available cobalamin assay kits have some analytical errors. The no boil automated cobalamin immunoassay (Carmel *et al.* 2000) may show normal levels despite obvious B_{12} deficiency (Devalia 2006). This is due to high concentrations of intrinsic factor autoantibody levels (Hamilton *et al.* 2006) causing analytical interference leading to a false normal serum B_{12}. Such autoantibodies against intrinsic factor are seen in blood and gastric juice of patients with PA and autoimmune AG. In the current study serum vitamin B_{12} was measured with a method, DPC Solid Phase No Boil kit, were the alkaline denaturation technique is supposed to inactivate intrinsic factor antibodies. However, various methods might have various capacities performing this step (Hamilton *et al.* 2006).

Holo-transcobalamin

Serum B_{12} is bound to two carrier proteins, transcobalamin (TC) and haptocorrin (HC). Most of the cobalamin is bound to haptocorrin (80%), the exact function of which is unknown. Approximately 20-30% of total B_{12} is bound to transcobalamin and thus forms holotranscobalamin, holo-TC. Holo-TC is the only vitamin B_{12} compound that can be absorbed into cells from the circulation; this is mediated by a specific receptor with high affinity for holo-TC. The half-life of plasma holo-TC is only 1-2 h (Chanarin 1979a), but

holo-TC seems to reflect total vitamin B_{12} status rather than that newly absorbed (Hvas *et al.* 2005a).

Methods have been developed the last years (Nexo *et al.* 2002; Ulleland *et al.* 2002), competitive radio-binding assays and ELISA-assays, which are laborious and therefore less clinically useful. Another method, based on a mouse monoclonal antibody with high affinity and specificity for human holo TC has recently been developed (Orning *et al.* 2006). The holo-TC assay used in the present study is based on this antibody, and is an automated EIA for use on the Abbott AxSYM® analyzer. The method has an analythical range up to 240 pmol/L, and calculations of (health related) reference intervals for elderly populations is under way. If values were detected as >240 pmol/L, the value of 241 pmol/L was used for calculations.

Plasma and whole blood folate

The whole blood folate measures folate status over the preceding last months, the accuracy of whole blood folate assays has, however, been questioned. Plasma folate levels reflects current and recent folate intake and are considered more useful in detecting acute than long-term folate deficiency (Chanarin 1979c). The method for plasma folate has an analytical range up to 60 nmol/L. If values were detected as >60 nmol/L the value of 61 nmol/L was used for calculations. The corresponding upper limit for whole blood folate was 1200 nmol/L and in case of values >1200 nmol/L, the value of 1201 nmol/L was used.

Plasma tHcy

The HPLC method was used for measuring tHcy in this study. Elevated levels of plasma tHcy have a high sensitivity for vitamin B_{12} (Savage *et al.* 1994) and folate deficiency. However the specificity is less satisfactory. Elevated levels are also seen in vitamin B_6 deficiency (Ubbink *et al.* 1996), renal impairment (Arnadottir *et al.* 1996) but the inverse relationship between tHcy and GFR is seen throughout the whole range of renal function (Lewerin *et al.* 2006). Furthermore, plasma tHcy increases with age (Brattström *et al.* 1994) and there is a gender difference (Refsum *et al.* 2004). Lifestyle factors such as smoking habits, coffee consumption (Nygård *et al.* 1998; Christensen *et al.* 2001) and alcohol intake may also contribute to elevated levels.

Genetic factors such as a common mutation in the 5-MTHFR gene (EC 1.5.1.20, 677C→T) (Bailey *et al.* 1999; Strandhagen *et al.* 2004) and hypothyroidism (Nedrebø *et al.* 1998) may

result in hyperhomocysteinemia. In this study adjustment for age, gender and smoking habits was performed but MTHFR was not measured.

Sample collection and handling may influence. In the present study serum/plasma sample collection were standardized and samples immediately centrifuged. At room temperature plasma tHcy increases about 1 µmol/L per hour if not centrifuged (Fiskerstrand *et al.* 1993). The chromatographic method employed in this study is very robust.

Serum MMA

The method employed, capillary gas chromatography and mass spectrometry has a very good performance. Elevated levels of serum MMA have been proposed as a more sensitive and specific marker for B_{12} deficiency (Lindenbaum *et al.* 1990). However, the specificity of mildly elevated serum MMA for clinical symptoms of vitamin B_{12} deficiency has been questioned (Hvas *et al.* 2001). Serum MMA is associated with plasma creatinine even within the normal range of plasma creatinine (Hvas *et al.* 2000).

Serum cystatin C

Cystatin C, is a low molecular weight basic protein (13kDa) produced by all nuclear cells. Cystatin C is supposed to meet the criteria for an ideal filtration marker better than creatinine since it is produced at a constant rate, is freely filtered, not secreted, and metabolized after tubular reabsorption so that it does not return to the blood flow (Newman 2002). Serum cystatin C is extracted by the kidneys from the circulation at about the same rate as ^{51}Cr-EDTA (Tenstad *et al.* 1996). Cystatin C production is unrelated to gender, age, muscle mass, dietary factors, inflammation and creatinine formation (Kyhse-Andersen *et al.* 1994) but possibly related to thyroid function (Fricker *et al.* 2003; Jayagopal *et al.* 2003). Serum cystatin C has been shown to be a better marker of GFR than serum creatinine (Kyhse-Andersen *et al.* 1994), which is especially useful in elderly with mild GFR reduction (Coll *et al.* 2000; Fliser *et al.* 2001). A serum cystatin C value of 1.55 mg/L corresponds approximately to a GFR of 60-70 ml/min/1.73 m^2 (Randers *et al.* 1998; Rule *et al.* 2006). Serum cystatin C was measured on one occasion during the study. In 176 subjects (mean 1.35 mg/L) this was at the start of the study and in 30 subjects at the end (mean 1.16 mg/L) (p= 0.0067). However, there were no significant differences in serum creatinine before and after treatment either in the vitamin treated subjects or in the placebo group, indicating stable renal function throughout the study period.

Serum pepsinogens, gastrin and antibodies against H.pylori (HPAb)

The diagnosis of AG could either be done by endoscopy, including multiple biopsies from the duodenum and the gastric antrum and corpus or by serological markers. Pepsinogens are proteases, secreted into the gastric lumen and transformed into pepsin. Serum pepsinogen I and II are both synthesized in the chief cells and the mucosa neck cells of the gastric body mucosa, whereas pepsinogen II also is produced in the pyloric glands of the gastric antrum (Samloff 1971; Samloff *et al.* 1973). The serum levels of pepsinogen I and II increase with gastric mucosal inflammation and decreased levels of serum pepsinogen I and pepsinogen I/II ratio are seen as the atrophy progress (Kekki *et al.* 1991). The liberation of gastrin from the antral G-cells is controlled by the intragastric acidity. Hypochlorhydria caused by AG is associated with decreased g-cell inhibition, resulting in hypergastrinemia in individuals with preserved g-cell function as seen in subjects with autoimmune antrum-sparing AG. Pepsinogen I and II are filtered through renal glomeruli and metabolized in the kidney (ten Kate *et al.* 1989), but the pepsinogen I level is more prone to rise if renal function decreases. On the other hand, pepsinogen II is more readily increased by H. pylori infection due to inflammation and disturbance of the integrity of the gastric mucosa and circulation (Kuipers *et al.* 1996). Thus, when using pepsinogens for indirect assessment of gastric mucosal status, renal function must be taken into account (Tamura *et al.* 1999). Histology has been considered the gold standard of diagnosing AG, however influenced by mucosa sampling and intraobserver variability. Comparison between histology analysis, serological markers and morphometric image analysis of AG has been performed (Staibano *et al.* 2002; Nardone *et al.* 2005). The serological markers corresponded better with the morphometric diagnosis of AG than histology. However, a rather low sensitivity (23-44%) and high specificity 99-100% were seen for both pepsinogen I and pepsinogen I/II ratio when comparing both with histology and morphometric diagnosis (Nardone *et al.* 2005). In the present study only serological markers for AG were available, endoscopy with biopsies would have made the study too extensive and invasive. Thus, some cases of atrophic gastritis might have been missed, whereas the risk of over diagnosing atrophic gastritis with serological markers seems small.

In comparison with light-microscopic examination and the 13C-urea breath test, measurement of HPAb has superior diagnostic sensitivity in patients with AG. In AG, therefore, detection of HPAb is the method of choice for detecting H. pylori infection (Kokkola *et al.* 2000).

RESULTS AND COMMENTS

PAPER I

Results

The aims of the study were to investigate the effects of oral vitamin therapy on plasma tHcy, serum MMA, blood haemoglobin and MCV, to investigate appropriate decision limits for "high" metabolites, and to calculate the prevalence of vitamin B_{12} /folate deficiency by different laboratory criteria. For study population and design, see the Material and Methods section. The characteristics of the study population at start of the study is shown in Table 1, and the reference intervals for the sample groups are shown in Table 2.

Table 1.
Characteristics of the study population (n=209) at start of the study showing median values and means (SD)

	Median	Mean (SD)
Age, years	75	76 (4.8)
Women, %	-	59.3
Smokers, %	-	12.4
BMI, kg/m^2	25.1	25.6 (3.5)
Haemoglobin, g/L	137	138 (10.0)
MCV, fL	92.0	92.0 (4.2)
Transferrin saturation, %	28	29 (8.4)
Whole blood folate, nmol/L	327	351 (135)
Plasma folate, nmol/L	15.0	16.0 (6.6)
Serum vitamin $B_{12,}$ pmol/L	301	325 (159)
Plasma holo-TC, pmol/L[1]	71.8	79.2 (33.7)
Plasma tHcy (µmol/L)	16.3	17.2 (5.4)
Serum MMA (µmol/L)	0.19	0.22 (0.1)
Serum creatinine (µmol/L)	96.1	101 (20.6)

[1]n=66

Table 2.
Reference intervals at baseline for the total study group (TSG), for apparently healthy subjects (RS I) and for subjects *not* showing a significant decline in plasma tHcys or serum MMA during vitamin therapy (RS II). Values for apparently healthy subjects after completion of vitamin treatment (RS III).

		n	0.025	M	0.975	S.D.	Measurement
TSG	serum B_{12}	209	112	325	689	159	Baseline
	plasma folates	208	6.6	15.8	29.2	5.8	
	whole blood folates	207	171	343	598	110	
	plasma tHcys m	85	7.1	18.9	30.6	6.0	
	f	124	9.2	16.1	27.3	4.7	
	serum MMA	208	0.11	0.22	0.48	0.1	
RS I	serum B_{12}	125	148	333	691	154	Baseline
	plasma folates	125	8.6	16.6	28.8	5.4	
	whole blood folates	125	185	350	619	111	
	plasma tHcys m	46	8.2	17.8	27.4	4.9	
	f	79	7.9	15.6	23.2	3.9	
	serum MMA	123	0.12	0.20	0.38	0.07	
RS II	plasma tHcys m	21	9.3	16.5	23.6	3.6	Baseline
	f	45	8.1	13.8	19.4	2.9	
	serum MMA	115	0.12	0.22	0.55	0.09	
RS III	serum B_{12}	77	196	555	914	182	After vitamin
	plasma folates	78	41.9	56.5	71.1	7.4	therapy
	whole blood folates	77	537	844	1151	156	
	plasma tHcys m	24	7.6	13.1	18.6	2.8	
	f	54	6.7	11.2	15.7	2.3	
	serum MMA	78	0.02	0.18	0.34	0.08	

M = mean
In subgroups containing < 100 subjects, the central 0.95 fractile intervals calculated as mean $\pm$ 1.97 SD.
m = men
f = women

A considerable amount of the TSG showed elevated plasma tHcy ($\geq$16 µmol/L), 64% of the men and 45% of the women. 11% of the TSG showed elevated serum MMA ($\geq$0.34 µmol/L). Mean plasma tHcy was significantly higher in men (18.9 µmol/L) than in women (16.1 µmol/L), p<0.001. No gender difference was seen in serum MMA. Both plasma tHcy and serum MMA correlated with age, r=0.19, p<0.01 and r=0.27, p<0.001, respectively. Vitamin deficiency, as defined by serum B_{12} $\leq$258 pmol/L (Lindenbaum *et al.* 1994) and serum MMA $\geq$0.34µmol/L, was present in 7.2% of the TSG. Using plasma folate $\leq$ 10nmol/L (Brouwer *et al.* 1998) in combination with the upper reference limits for plasma tHcy in RS III, the number of folate deficient was 11%. 1.4% had both folate and vitamin B_{12} deficiency. After randomisation to four months of vitamin treatment with 500µg cyanocobalamin, 800µg folic acid and 3 mg pyridoxine hydrochloride, serum B_{12}, plasma and blood folate increased and serum MMA and plasma tHcy decreased significantly as compared to the placebo group.

Mean plasma tHcy decreased by 32% and mean serum MMA by 14% in vitamin treated subjects. There were no significant differences in blood haemoglobin or mean MCV either in the vitamin or in the placebo group during the study.

Independent variables for decrease in serum MMA and plasma tHcy were calculated in a multiple regression analyses. High baseline metabolite concentrations, low vitamin concentrations, "low" age (for serum MMA) and low transferrin saturation (for plasma tHcys) were independently correlated with metabolite decline.

Comments

Reference intervals are in general calculated as the 95% reference interval (2.5^{th}-97.5^{th} percentile interval) or the mean +/- 1.97 SD in presumable healthy individuals. The upper reference limits calculated in this manner (i.e. in RS I) were higher than those stated by the laboratory for both plasma tHcy and serum MMA. In healthy and vitamin replete subjects (RS III), the upper reference limits for plasma tHcy and serum MMA were considerably lower than for TSG and RS I and close to those stated by the laboratory, thus inadequate B-vitamin status is believed to be an important factor behind elevated metabolites in this study. This is consistent with recent findings in the Framingham study, in which low vitamin status or intake had a substantial impact on the prevalence of high homocysteine (Selhub 2006). Plasma tHcy and serum MMA concentrations above the reference limits were found to be very common. Adjustment for factors known to influence the metabolites was performed, such as age, sex and smoking habits. Certain drugs that might affect the B-vitamin metabolism were excluded by calculating health related reference intervals. A shortcoming of this study was the lack of information on the prevalence of polymorphism in the 5-MTHFR gene. The prevalence of vitamin B_{12} and folate deficiency was calculated to be 7.2% and 11%, respectively, which is noteworthy in a population at good health. The present study was not epidemiologically representative, on the other hand, probands were not patients selected by suspect laboratory deviations or clinical suspicion of deficiency. Thus, the bias for over- or under diagnosis of vitamin deficiency was judged to be small.

In spite of the randomisation procedure, the differences in serum B_{12} and plasma tHcy between the vitamin and placebo groups reached statistical significance, the placebo group showing higher serum B_{12} and lower plasma tHcy. However, this was probably not caused by a true significant difference in vitamin status, since neither folates nor serum MMA differed between the groups.

Based on early B_{12} absorption studies (Berlin *et al.* 1968), the vitamin doses were chosen to provide adequate vitamin treatment also in subjects with AG or other vitamin malabsorption. This was achieved in all cases, as judged from the post treatment vitamin concentrations. However, 11% had plasma tHcy $\geq$16 µmol/L and 3% had serum MMA $\geq$0.34 µmol/L post-treatment, probably due to decreased renal function. This points to the necessity of assessing renal function when using plasma tHcy and serum MMA as efficacy variables for vitamin treatment in the elderly.

Low transferrin saturation was correlated with a larger decline in plasma tHcy after vitamin treatment and could possible be associated with a poorer nutritional status. "Low" age was associated with a larger decline in serum MMA, and regarding clinical response; prevention of cognitive decline with B-vitamins has been proposed to be more successful in younger cohorts (Duthie *et al.* 2002).

In a study of 64 healthy, free-living elderly with a mean age of 76 years, the upper reference limit for plasma tHcy was 21µmol/L and for serum MMA 0.476 µmol/L, subjects with atherosclerotic disease, renal impairment and certain medicaments were excluded (Joosten *et al.* 1996). In a later publication the upper health related reference limits for plasma tHcy in elderly nonsupplemented subjects >65 years, is 20 nmol/L based on the literature (Refsum *et al.* 2004). Upper reference limits for healthy and vitamin replete elderly has been reported for plasma tHcy 12.7 µmol/L and for serum MMA 0.278 µmol/L (Joosten *et al.* 1996). The health related upper reference intervals as well as the reference limits for healthy and vitamin replete in the present study seemed to be higher than other published, maybe due to inferior renal function in the healthy sub sample.

Plasma tHcy and serum MMA levels above the reference limits have been reported to be common also in later studies. In a Swedish population study of 224 healthy subjects over 70 years of age, 58% of those not treated with B-vitamins had elevated plasma tHcy ($\geq$15 µmol/L) or serum MMA ($\geq$0.37 µmol/L) (Björkegren *et al.* 2001). In a recently published study of geriatric patients with a median age of 82 years and mean serum creatinine of 89 µmol/L, 40% had plasma tHcy concentrations above 20µmol/L (Raeder *et al.* 2006).

Decision limits for vitamin B_{12} and folate deficiency were chosen according to the literature (Lindenbaum *et al.* 1994; Brouwer *et al.* 1998) and the maximal prevalence was calculated in this population with no overt sign of vitamin deficiency. In the Framingham elderly cohort (n=458) 11.3% were judged to have cobalamin deficiency using the combined criteria serum B_{12} <258pmol/L and serum MMA >0.376µmol/L (Lindenbaum *et al.* 1994).

In a population-based study of >3500 people aged 65 years or older the prevalence of vitamin B_{12} deficiency, defined as serum B_{12} <200 pmol/L and plasma tHcy >20µmol/L, increased with age from about 5 percent at 65 years of age to 10-20% at the age of 80 years (Clarke *et al.* 2004). The prevalence of folate deficiency increased with age and was about 5% in people 65-74 years and about 10% in people aged 75 years or more. Decision limits were serum folate below 7 nmol/L in combination with a plasma tHcy above 20 µmol/L. Thus, metabolic evidence of vitamin deficiency was more frequent than low serum/plasma concentrations of the vitamins in the present as also in other studies (Joosten *et al.* 1993).

Age and gender differences were seen for plasma tHcy like in other studies (Refsum *et al.* 2004). In the present study there was an association between increasing serum MMA and age in agreement with several publications (Björkegren *et al.* 2001), (Lindenbaum *et al.* 1994; Joosten *et al.* 1996) but not with others (Nexø *et al.* 1994). No correlation between serum MMA and gender was seen, in agreement with others (Nexø *et al.* 1994).

The present study indicates, in agreement with many others, that inadequate B-vitamin status is an important factor for elevated plasma tHcy and serum MMA in the elderly. The reasons for vitamin depletion in the elderly are probably multifactorial and not restricted to malabsorption. Other factors, accounted for when calculating the reference intervals, was ongoing medication with drugs known to influence vitamin metabolism. However, the relative impact of the factors with a potential influence on vitamin status was not possible to measure. We did not obtain diet histories, but the rather high BMI in the population speaks against malnutrition. However, excessive cooking (Chanarin 1979d) and microwave heating (Watanabe *et al.* 1998) might still occur.

Vitamin treatment in the doses used produced significant biochemical responses, although they were lower than those traditionally used to treat overt deficiency. For oral vitamin treatment, dose finding studies are scarce, and tradition rests upon early resorption data with an added safety margin. The efficacy variables are essential when evaluating response or safety of maintenance treatment. In a recently published study, over 16 weeks of 120 older people, with mild vitamin B_{12} deficiency, 500 µg of oral cyanocobalamin was the lowest dose associated with a maximum reduction in plasma MMA and maximum increase in holo-TC concentrations. The proportional reductions in plasma MMA concentrations did not differ significantly between oral dose of 500 µg or 1000µg, but the dose calculated to achieve an optimal MMA response was calculated to be somewhat higher (Eussen *et al.* 2005). However, apart from excluding subjects with serum creatinine above 120 µmol/L, no adjustment for renal function was made.

In a meta analysis, supplementation of 650µg folic acid/day was enough to reduce tHcy (de Bree *et al.* 1997) and there were no differences in response between doses of 0.5 mg to 5mg. Vitamin B_6 did not have any significant additional effect (Clarke 1998).

In conclusion, elevated plasma tHcy and serum MMA levels were common, suboptimal vitamin status was considered an important factor. Four months of oral treatment with vitamins B_{12}, folic acid and B_6 produced significant declines in metabolite concentrations, leading to normalisation of the distributions in the healthy subgroup.

PAPER II

Results

The aim of the study was to explore the significance of GFR on plasma tHcy and serum MMA and the consequences for the diagnosis of B-vitamin deficiency.

Serum cystatin C and serum creatinine were used as GFR markers. In TSG at start of the study, elevated serum cystatin C (>1.55 mg/L) was found 31.3% of the men and 13% of the women. Corresponding values for serum creatinine (≥120 µmol/L for men, ≥110 µmol/L for women) was seen in 29.4% and 7.3%. Mean values (SD) in TSG of serum cystatin C and serum creatinine were 1.33 (0.4) mg/L and 101 (20.6) µmol/L, respectively. The distribution of serum cystatin C in TSG is shown in Figure 7. Reference intervals comprising the central 0.95 interfractile interval for serum creatinine and serum cystatin C in TSG are shown in Table 3.
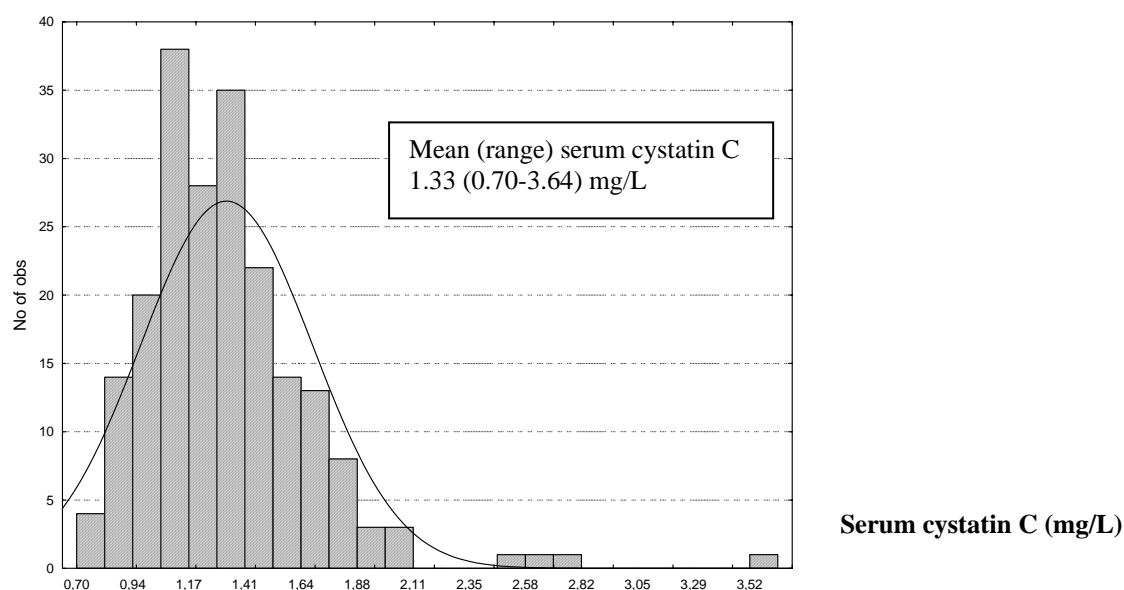

Mean (range) serum cystatin C
1.33 (0.70-3.64) mg/L

Serum cystatin C (mg/L)

Fig 7.
Distribution of serum cystatin C (mg/L) in the total study group

Table 3.
Reference intervals, mean (SD) and range at baseline for the total study group (n=209)

	0.025	**M (SD)**	**Median**	**0.975**	**Range**
Serum creatinine (umol/L)	72.7	101 (20.6)	96.1	158	70-218
Serum cystatin C (mg/L)	0.86	1.33 (0.36)	1.28	2.1	0.70-3.64

Serum creatinine correlated positively with serum cystatin C (r= 0.61, p<0.001) (Figure 8). We did not find any correlations between vitamin B_{12}/folate and cystatin C. Plasma tHcy correlated with serum creatinine (r =0.49, p<0.001) and with serum cystatin C (r = 0.45, P<0.001) (Figure 9). Serum MMA correlated with serum creatinine (r = 0.30, P< 0.001) and with serum Cystatin C (r = 0.28, P< 0.001) (Figure 10).
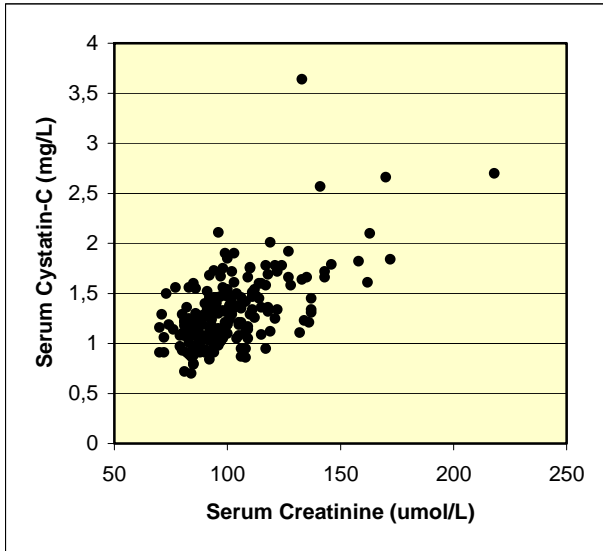

Fig 8. Bivariate scattergram of serum cystatin C and serum creatinine concentrations in the total study group (n=209) at start of the study (r = 0.61 P<0.001).
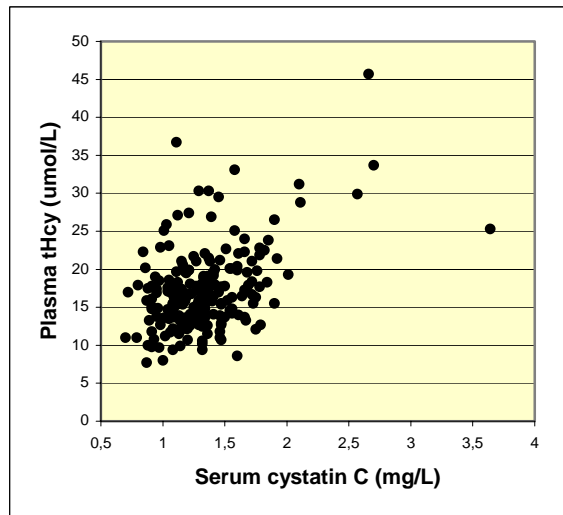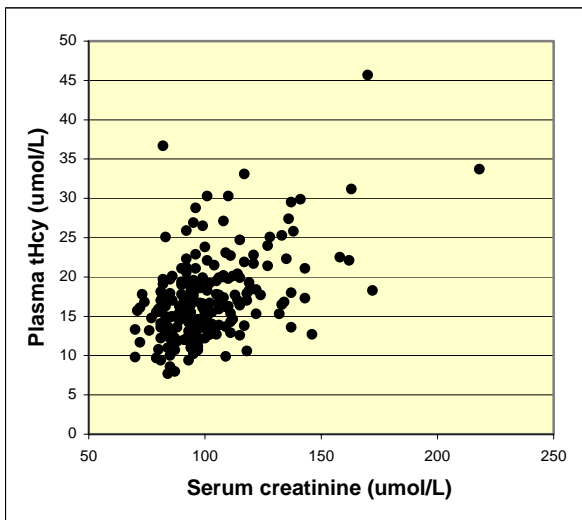

Fig 9.
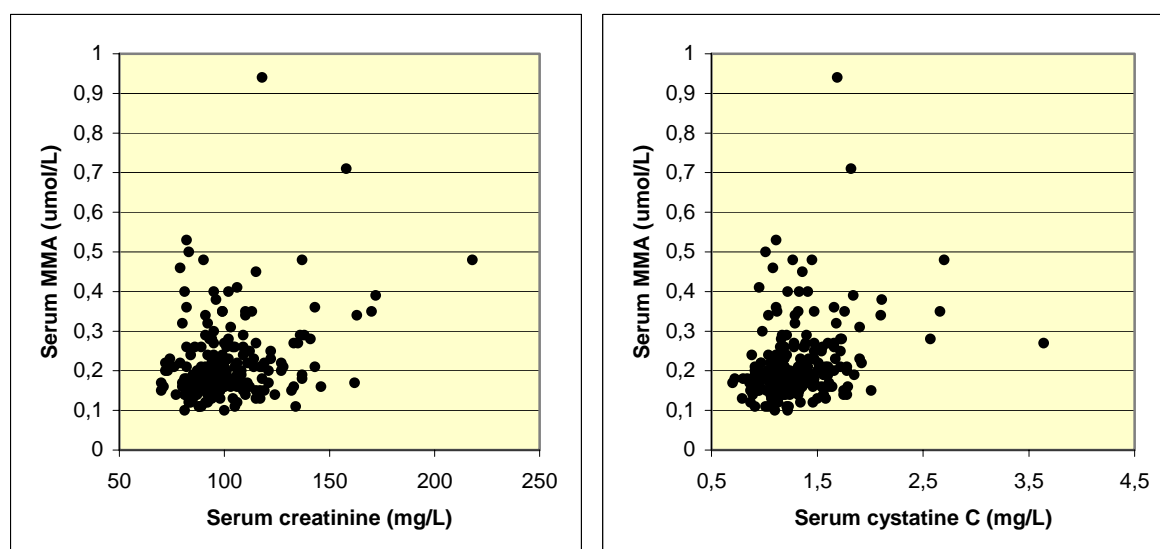**Correlations in TSG at start of the study between plasma tHcy and renal function**

Fig 10.
Correlations in TSG at start of the study between serum MMA and renal function

Thereafter, possibly vitamin deficient subjects were excluded (n=36). In the remaining 173 subjects, plasma tHcy and serum MMA correlated with serum cystatin C (r=0.42 and r=0.35, respectively, p<0.001, n=170) and serum creatinine (r=0.52 and r=0.43, p<0,001, n=173). We then used the confidence intervals from this regression analysis, as depicted in Figure 11, to identify subjects, in the total study group, with seemingly higher plasma tHcy/serum MMA than expected in relation to their GFR.

When using the statistical relations between plasma tHcy/serum MMA and serum cystatin C in non-vitamin deficient subjects, instead of fixed decision limits as criteria for elevated plasma tHcy, the proportion of subjects with elevated plasma tHcy in the total study group declined from 64% to 9% in the men and from 45% to 10% in the women, whereas for serum MMA the proportions remained unchanged. Similar results were found when serum creatinine was used instead of serum cystatin C. The group identified as having elevated values of plasma tHcy (n=20) and serum MMA (n=23) in relation to GFR did not differ in vitamin concentrations from the rest of the subjects (n=72) with elevated plasma tHcy according to fixed upper reference limits.
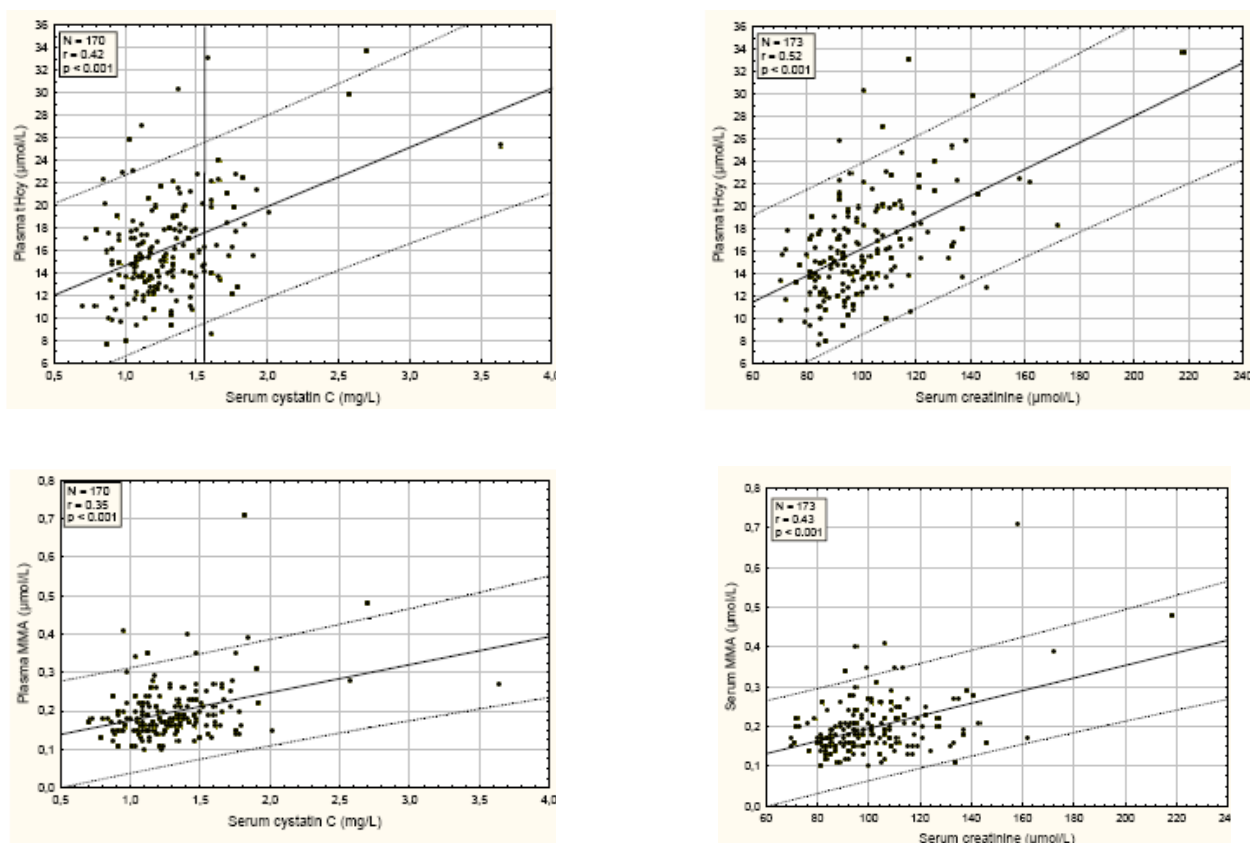
Fig 11. Nomograms for tHcy and MMA in relation to serum cystatin C/serum creatinine in non-vitamin-deficient subjects (n=170). Regression line and confidence intervals (dotted lines) corresponding to $\pm$ 2 SD are given.

To further elucidate the impact of renal function on the metabolites, multiple regression models were performed with plasma tHcy and serum MMA as dependent variables. The base model (age, sex smoking habits, height and weight) explained 9% and 8% of the variation in plasma tHcy and serum MMA, respectively. The base model *and* serum cystatin C explained 20% of the variation in plasma tHcy, by exchanging serum cystatin C against serum creatinine the explanatory value increased to 22%. The corresponding values for serum MMA were 16% and 18% respectively. After vitamin treatment (n=115) serum cystatin C together with the base model explained 26% and 27% of the variation in plasma tHcy and serum MMA, respectively.

Comments

Plasma/serum concentrations of tHcy and MMA are influenced of other factors than B-vitamin deficiency, e.g. renal function, which is known to decrease in elderly subjects. We investigated the importance of GFR, as measured by both serum creatinine and cystatin C, for the levels of plasma tHcy and serum MMA. Serum cystatin C has been considered a better marker for GFR than serum creatinine (Newman *et al.* 1995) and the age-dependent continuous decrease in GFR in elderly populations may be underestimated by serum creatinine (Fliser *et al.* 2001). Mean serum creatinine in the present study was 101μmol/L, as compared to a Swedish study with mean age 76 years old (n=161) and mean serum creatinine 90 μmol/L (Björkegren *et al.* 2001). We found, like other investigators, GFR to correlate to plasma tHcy and serum MMA concentrations within the whole range from normal to decreased GFR (Hultberg *et al.* 1993; Arnadottir *et al.* 1996; Hvas *et al.* 2000).

The correlation between GFR and the metabolites in the subgroup without vitamin deficiency was the basis for calculation of a nomogram depicting a "normal" range of the metabolites for each increment in serum cystatin C or creatinine. Applying this nomogram to the total study group, the fraction of "high" plasma tHcy decreased from 53% to 10%, whereas for serum MMA the proportions remained unchanged. Thus, renal function seemed to be more important for plasma tHcy than serum MMA. One obvious limitation of this nomogram was the relatively small number of subjects used for the calculations. Further, as in all situations where reference limits are used, some subjects may fall outside the interval without being abnormal, and other subjects may have individually elevated metabolites within the borders of the nomogram.

Serum creatinine had a higher explanatory variable for variations in plasma tHcy and serum MMA than serum cystatin C. This might be attributed to the good health status and normal BMI of the study population. In vitamin repleted subjects, renal function explained as expected even more of the variations in plasma tHcy and serum MMA. This shows the importance of GFR when assessing plasma tHcy and serum MMA also in vitamin repleted subjects.

In conclusion, renal function, also within the normal range, must be taken into account when interpreting plasma tHcy and serum MMA values in elderly subjects. Failure to do so might lead to an overdiagnosis of "abnormal" levels in both untreated and vitamin treated subjects.

In future studies on the prognostic significance of elevated plasma tHcy and serum MMA, the cause(s) of the elevations should be taken into account, be it vitamin depletion, renal (dys)function, or both.

PAPER III

The aim was to explore any association between vitamin status and movement and cognitive performance, and if vitamin treatment would lead to a clinical improvement in these respects.

Results

At study start, all the 209 subjects participated in the cognitive tests, 195 in the PLM test. After the intervention period, 171 subjects participated in both tests (Figure 12). The 48 subjects who dropped out during the study were slightly older, and performed worse in some of the initial tests.
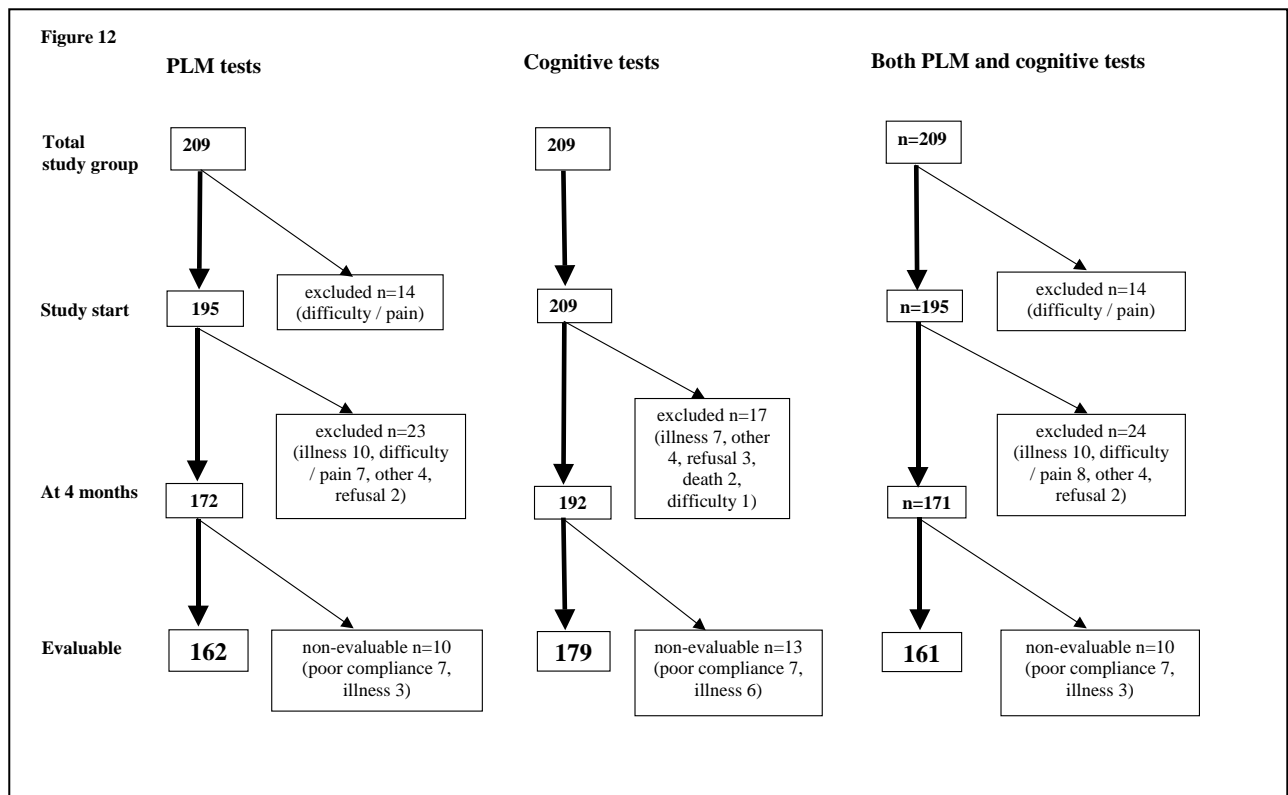

Fig 12. The number of subjects undergoing the Postural-Locomotor-Manual (PLM) test, the cognitive test, and both tests at different times of the study

Serum MMA, plasma folate, whole blood folate and serum B_{12} correlated significantly with plasma tHcy but serum B_{12} did not correlate with serum MMA (Table 4). Four out of five Postural-Locomotor-Manual (PLM) components correlated with plasma tHcy and two with serum MMA. Seven out of nine components of the cognitive performance tests correlated with plasma tHcy and three with serum MMA. There were no significant correlations across plasma/blood folate and PLM/cognitive variables. Serum B_{12} correlated only with one cognitive variable and with none of the PLM variables. A majority of the PLM variables correlated with the cognitive variables. Subjects defined as vitamin B_{12} deficient (7.2%) (Lewerin *et al.* 2003) showed inferior movement time, simultaneity index, visual reproduction and block design as compared to non-deficient subjects, whereas subjects defined as folate deficient (11%) did not demonstrate such differences.

Table 4. Laboratory measures, PLM and cognitive tests and correlations at start of the study

				Correlations[1] with			
				Plasma tHcy		**Serum MMA**	
	n	Mean	SD	*r*	*p*	*r*	*p*
Serum MMA (μmol/L)	208	0.22	0.10	0.35	<0.001	-	-
Plasma tHcy (μmol/L)	209	17.2	5.41	-	-	-	-
Plasma folate (nmo/L)	209	16.0	6.61	-0.33	<0.001	-0.04	NS
Whole blood folate (nmo/L)	209	351	134.9	-0.16	0.020	-0.05	NS
Serum B_{12} (pmol/L)	209	325	159.3	-0.15	0.029	-0.11	0.10
PLM test (s)							
Movement time	195	2.10	0.58	0.27	<0.001	0.16	0.023
Postural phase	195	0.91	0.15	0.11	0.13	0.002	NS
Locomotor phase	195	1.54	0.36	0.21	<0.01	0.08	NS
Manual phase	195	1.37	0.41	0.23	<0.01	0.11	0.12
Simultaneity index	195	1.85	0.16	-0.16	0.026	-0.20	<0.01
Cognitive tests (score)							
Digit span forward	209	5.80	1.12	-0.14	0.039	-0.05	NS
Digit span backward	209	4.46	1.14	-0.16	0.018	-0.12	0.1
Identical forms	206	23.49	8.05	-0.21	<0.01	-0.08	NS
Visual reproduction	205	6.88	2.99	-0.18	<0.01	-0.21	<0.01
Synonyms	202	22.42	4.89	-0.14	0.045	-0.01	NS
Block design	207	18.74	6.89	-0.28	<0.001	-0.20	<0.01
Digit symbol	204	35.83	10.78	-0.20	<0.01	-0.11	0.12
Thurstone´s Picture Memory test	205	20.57	4.54	-0.06	>0.2	-0.08	NS

[1] **Partial (adjusted for age and sex) correlation coefficients (*r*)**

For further analyses of the relation between the PLM/cognitive variables and plasma tHcy/serum MMA, a multiple regression analysis was performed with adjustment for age, sex and serum creatinine. Two PLM and two cognitive variables were selected which were thought to be most suitable for comparison with the metabolites. Movement time correlated independently with plasma tHcy, simultaneity index with serum MMA, digit symbol with plasma tHcy and block design with plasma tHcy and serum MMA.

After four months of treatment with 500 µg cyanocobalamin, 800 µg folic acid and 3 mg pyridoxine hydrochloride the mean time span for the PLM variables became shorter but there were no significant differences between the vitamin treated and the placebo group. There were improvements in the cognitive tests in both groups, in two cognitive variables; identical forms and synonyms, the mean scores of the placebo group improved significant compared to the placebo group. The univariate correlations seen before treatment between the PLM/cognitive variables and the metabolites remained significant after vitamin treatment both in the vitamin and placebo groups.

Comments

Deficiency of vitamin B_{12} was observed in 7.2% and of folic acid in 11%, whereas elevated plasma tHcy and serum MMA was much more common as shown in paper I. There were, at study start, strong correlations between cognitive and movement performance. Further, these two functional tests correlated to plasma tHcy/serum MMA, but the correlations to the vitamin concentrations were less impressive. Correlations between low B-vitamin status and cognitive decline function was first demonstrated by Goodwin et al (Goodwin *et al.* 1983) and has then been shown in an numerous of studies of community-dwelling elderly, both in cross sectional (Duthie *et al.* 2002; Miller *et al.* 2003a) and longitudinal (Wang *et al.* 2001; Seshadri *et al.* 2002) surveys. However, the inverse association between plasma tHcy and serum/plasma concentrations of both vitamin B_{12} and folate makes it difficult to distinguish the independent effect of each variable. Low B-vitamin concentrations could also be a result of impaired cognitive function (Clarke *et al.* 1998).

The present study population performed better in single cognitive tests than a representative population (Kauppinen *et al.* 2002). Nevertheless, we found inverse correlations both between plasma tHcy/serum MMA and cognitive function/ PLM. These correlations were different for

plasma tHcy and serum MMA. Thus different patho-physiologic mechanisms might be at hand. Movement performance is associated with vascular disease, brain atrophy and cerebral white matter lesions (Guo *et al.* 2000, 2002; Guo 2001). Elevated plasma tHcy have been found to be associated with brain infarcts and white matter lesions and to hippocampal with on magnetic resonance imaging (Vermeer *et al.* 2002; Williams 2002).

The lack of significant clinical response to B-vitamin treatment in this study might have different reasons. First, the study was powered to detect estimated differences in plasma tHcy and serum MMA as a result of the vitamin treatment; the corresponding calculations for the neurocognitive tests were in practise virtually impossible. The vitamin doses used were sufficient for a biochemical response, and later published data point to the difficulties to ascertain beneficial clinical effects of vitamin treatment. In a double-blind placebo-controlled study, 1 mg B_{12} alone or in combination with 0.4 mg folic acid during 6 months of treatment, no improvement in cognitive function in free-living elderly subjects with mild B_{12} deficiency (Eussen *et al.* 2006) was found. The duration of symptoms (Martin *et al.* 1992) and treatment may be of importance. In a recently published placebo controlled study over 2 years, 276 healthy elderly people > 65 years, with elevated plasma tHcy, (>13mmol/l) were treated with 0.5 mg B_{12}, 1mg folate and 10 mg B_6, no improvement in cognitive function were seen (McMahon *et al.* 2006) but even this study might have been to short and under powered (Clarke 2006). Possibly, prevention of cognitive decline with B-vitamins has to start earlier (Martin *et al.* 1992) in younger cohorts (Duthie *et al.* 2002). Second, our results point to the necessity of investigating vitamin effects in large randomised placebo controlled studies, since repeated testing results in a training effect, with improvements also in the placebo group. However, accumulating evidence point to vitamin-independent or vitamin deficiency associated but irreversible cognitive decline.

In our study we did not take possible mutation of the MTHFR gene, intelligence, educational status or cardiovascular disease into consideration but adjusted for renal function.

There are few studies addressing the association between movement performance and B-vitamin status. A decline in physical function, measured as time needed to sign the name, walk 3 meters, do chair stands and foot taps and balance, during a three year interval was associated with plasma tHcy at baseline in 499 highly functioning elderly in a community-based study (Kado *et al.* 2002) and were not explained by increasing age, B-vitamin status, cardiovascular disease or impaired renal function. Gait abnormalities were shown to predict non-Alzheimer dementia in a prospective study of elderly (Verghese *et al.* 2002).

In patient's with Parkinson's disease, a motor disorder which is the second most common neurodegenerative disease of the ageing, elevated levels of plasma tHcy concentrations are seen, especially in patients treated with L-dopa and most markedly in patient with polymorphism in the MTHFR (Yasui *et al.* 2000) gene. The extent of plasma tHcy elevation in patients treated with L-dopa is influenced by B-vitamin status (Brattström 2001; Miller *et al.* 2003b) and B-vitamin supplements may be considered. It has been demonstrated that patients with Parkinson's disease, treated with L-dopa have reduced levels of SAM in blood (Cheng *et al.* 1997) and L-dopa increases the demand for methyl groups.

In the present study we measured movement performance with the PLM test (Matousek *et al.* 1994), which objectively and precisely measures the subject's mobility of lower and upper limbs and movement coordination, a method primary developed for patients with Parkinson's disease. The PLM test reflects an elderly subject's mobility status in a daily situation. In the present study, we found significant correlations within a majority of movement and cognitive variables, like in other studies (Soumaré *et al.* 2006). In a recent cross-sectional study of 3609 elderly community-dwelling subjects, elevated concentrations of plasma tHcy was associated with worse motor performance measured as gait and balance (Soumaré *et al.* 2006). Neither cognitive function, nor depression or fractures was associated with plasma tHcy. The authors discussed various hypotheses explaining the association between plasma tHcy and motor function. First, the direct vascular effect of plasma tHcy on the brain and increased risk of arteriosclerosis leading to white matter lesions but also the direct neurotoxic effect of plasma tHcy. Plasma tHcy concentrations are also influenced by lifestyle factors that could be associated with nutritional status and physical functioning (Stampfer *et al.* 2002).

There was an inverse relationship between plasma tHcy/serum MMA and cognitive and movement performance. Since these correlations were independent of serum cystatin C (paper II), they might be related to a suboptimal vitamin status.

Thus, the connections between B-vitamin status, the B-vitamin-dependent intermediate metabolites plasma tHcy and serum MMA and movement/cognitive function is complex and not completely understood. So far, there is no evidence on the level of randomised trials that treatment with B-vitamins improve cognitive and/or movement performance. It seems, however, questionable whether such studies will be realistic to perform.

PAPER IV

The aims were to explore the significance of AG with respect to prevalence, relation to HPAb, deficiency of cobalamin, folate, iron, to cognitive and movement performance and response to oral vitamin treatment. Subjects on gastric acid inhibitory medication (n=19) were excluded. Thus, the population in this part of the study consisted of 190 individuals.

Results

Atrophic gastritis (AG), defined as a pepsinogen I/II ratio of <2.9 was present in 14% (n=26) of whom 22 were HPAb+. Antrum-sparing AG, defined as gastrin $\geq$75 and pepsinogen I $\leq$30 occurred in 4% (n=8), of whom 4 subjects were HPAb+. In the total study group, 102 subjects (54%) were HPAb+. Of these, 22 had AG. Subjects with AG and/or HPAb+ were older, but no sex difference was found. The study group (n=190) was divided into four subgroups according to occurrence/absence of AG and HPAb, respectively. Subjects with AG but no HPAb (n=4) showed higher serum MMA, plasma tHcy, also after adjustment for serum cystatin C. They also showed higher MCV, vitamin B_{12} deficiency was more common than in subjects negative for both AG and HPAb. Subjects with both AG and HPAb (n=22) did not show any significant differences as compared to subjects negative for both AG and HPAb except for lower blood hemoglobin. There were no correlations between AG and markers for movement and cognitive performance.

To further elucidate the independent influence of the variables correlated to plasma tHcy, serum MMA and serum B_{12}, a multiple stepwise regression analysis was performed. Age, sex, renal function, plasma folate and serum B_{12} explained 34% of the variation in plasma tHcy, but the presence of AG or HPAb did not influence plasma tHcy. Age, sex, renal function and antrum sparing gastritis explained 23% of the variation in serum MMA and 7% of the variation in serum B_{12}. After vitamin treatment, subjects with AG according to different sets of criteria for the pepsinogens and gastrin showed larger declines in serum MMA, plasma tHcy and larger increases in serum B_{12} and whole blood folate. These differences reached statistical significance in a number of the groups.

	Mean (SD)	Median	Range
Plasma holo-TC men (pmol/L)	72.5 (25.8)	68.8	25.4-139.3
Plasma holo-TC women (pmol/L)	84.1(38.1)	74.4	25.9-205.0
Plasma holo-TC total (pmol/L)	79.1(33.7)	71.2	25.4-205.0

Table 5. Mean median and range of plasma holo-TC in the placebo group.

Holo-TC

Plasma holo-TC was, due to limited supply of frozen serum, measured in a subpopulation only, n=66, and the distribution is seen in Table 5.

The central 0.95 interfractile interval was 25.7-176.7 pmol/L (non-parametric). There were no significant associations between holo-TC and age and sex, respectively. Subjects with elevated plasma tHcy ($\geq$16µmol/L) showed lower mean holo-TC compared to subjects with normal plasma tHcy, such differences in were not seen in subjects with elevated vs normal serum MMA. After adjustment for age and sex, holo-TC correlated significantly with serum B_{12} (r=0.63, p<0.001), but there were no correlations with serum MMA, plasma tHcy, renal function, AG, occurrence of H. pylori or movement/cognitive performance. The independent explanatory value of holo-TC vs serum B_{12} for plasma tHcy and serum MMA was tested in multiple stepwise regression analysis (Table 6). Age, sex, serum creatinine, plasma folate and holo-TC explained 58% of the variation in plasma tHcy. Age, sex, serum creatinine and holo-TC explained 30% of the variation in serum MMA. By exchanging holo-TC with serum B_{12}, the predictive capacity of the model decreased from 58 to 55% and from 30 to 23% for plasma tHcy and serum MMA, respectively.

Table 6.
Multiple stepwise regression analysis in the placebo group (n=66), of the correlation between plasma tHcy/serum MMA and laboratory variables at the start of the study. Adjusted for age and sex. Variables included in the model were serum creatinine, serum B_{12}, holo-TC and further for tHcy plasma folate.

Dependent variable	n	Explanatory variable	Regression coefficient (β)	R^2	p
Plasma tHcy	66	Serum creatinine	1.06	0.33	<0.001
		Plasma folate	-0.29	0.51	<0.001
		Holo-TC	-0.18	0.58	<0.01
Serum MMA	65	Serum creatinine	0.95	0.22	<0.001
		Holo-TC	-0.26	0.30	<0.01

Comments

Atrophic gastritis was not uncommon in this population, as judged by serological markers. Serum pepsinogen I and pepsinogen I/II ratio have high specificity but lower sensitivity for the histological diagnosis of AG (Nardone *et al.* 2005) making the prevalence of AG thus defined of 14% reliable as a minimum estimation. The prevalence of AG varies between populations (Krasinski *et al.* 1986; Sipponen *et al.* 2003; Weck *et al.* 2006), presumably due to both genetic (autoimmune) factors and H. pylori prevalence. H. pylori infection is proposed to trigger and aggravate the autoimmune process (Sakaki *et al.* 2002). The prevalence of HPAb in half of the subjects is in accordance with a previous study on a representative population in this region (Gause-Nilsson *et al.* 1998). The substantial overlap of subjects with AG and HPAb makes the relative influence of these abnormalities hard to calculate, not least since their impact might differ along the course of the development of AG. Although the numbers were small, AG without HPAb resulted in higher serum MMA, plasma tHcy and prevalence of B_{12} deficiency. Why the absence of HPAb in these cases was associated with an inferior vitamin B_{12} status could be explained by a "burnt out" long-standing H. pylori infection or as a predominantly autoimmune aetiology of the atrophy. H. pylori positivity has been shown to decline as the atrophic gastritis progresses (Sande *et al.* 2001). This is presumably due to the progressive achlorhydria, with subsequent failure to thrive for H. pylori. Thus, the antigenic stimulation will gradually disappear and the HPAb concentrations may normalize. In the present study, presence of HPAb in subjects with AG correlated with lower haemoglobin, but without corresponding MCV changes, making iron deficiency anaemia (Annibale *et al.* 2001; Hershko *et al.* 2006) less likely. In multiple stepwise regression analysis, the influence of antrum sparing gastritis was more prominent on serum MMA as compared to serum B_{12}, probably due to the inferior sensitivity of serum B_{12} as compared to MMA for vitamin B_{12} deficiency. AG subjects responded bio chemically just as well as non-AG subjects, consistent with early absorption studies (Berlin *et al.* 1968), in which 1.2% of an oral B_{12} dose was absorbed also in patients with severe B_{12} malabsorption.

Measurement of vitamin B_{12} bound to transcobalamin, holo-TC, has emerged as a potentially better marker for vitamin B_{12} deficiency, the methods have developed and this assay might soon be incorporated into clinical routine. We had the opportunity to analyse holo-TC in a subset of individuals in this study. Holo-TC correlated with serum B_{12} and was a stronger explanatory variable for plasma tHcy and serum MMA than serum B_{12}.

We did not find any correlations between holo-TC and renal function, both measured as serum creatinine and serum cystatin C as found in some (Hvas *et al.* 2005b; Refsum *et al.* 2006) but not other studies (Miller *et al.* 2006). Holo-TC did not, in this rather small subgroup analysed, correlate to cognitive function like in another study (Hin *et al.* 2006).

In conclusion, AG and HPAb were common in this population without obvious clinical vitamin deficiency. The presence of HPAb correlated strongly to AG. AG affected vitamin B_{12} status, for which holo-TC is a promising measure, an alternative to serum B_{12}. AG subjects responded just as well as non-AG subjects to oral vitamin treatment with regard to vitamin, plasma tHcy and serum MMA changes. Indirect assessment of gastric mucosal function provides means to identify a subgroup of elderly at risk for vitamin depletion, which in those cases is easy to correct.

CONCLUDING REMARKS

GENERAL DISCUSSION

Subnormal cobalamin and folate status seems to be common in the elderly. This has gained increasing interest, mainly due to the notion that such vitamin deficiency would be easily treatable with efficient and non-toxic regimens. Thus, the possibility to treat and prevent irreversible nerve damage beyond megaloblastic anaemia has emerged, and the fraction of elderly subjects who might benefit from pharmacological vitamin treatment would thus be larger than the traditional 1-2% of the population suffering from traditional PA. Further, based on strong correlations between plasma tHcy and cardiovascular disease, the hypothesis of tHcy as a causal risk factor appeared, again with the assumption that this risk factor easily could be corrected. Thus, a non-toxic and easy way to significantly further reduce cardiovascular morbidity/mortality occurred, in addition to correcting proven causal risk factors like hypertension, high cholesterol and smoking. The present thesis has, however, not addressed the issue of cardiovascular morbidity in relation to elevated plasma tHcy.

Like in many previous studies of elderly, we found plasma tHcy and serum MMA above the reference limits to be very common. Even according to reference limits computed on this population after exclusion of non-healthy subjects, there was a high prevalence of elevated metabolite concentrations. However, when adjusting for renal function also within the normal range, our data indicate a reduction of the fraction of subjects with vitamin dependent elevations of plasma tHcy and serum MMA. On the other hand, in the vitamin treated and healthy subgroup, the upper reference limits for plasma tHcy and serum MMA were in accordance with those established for younger and healthy subjects. The vitamin concentrations were independent predictors of metabolite decline by treatment. An estimation of "biochemical" vitamin deficiency, based on criteria from the literature pointed to vitamin deficiency of up to 10%, and in conclusion, suboptimal vitamin status was considered an important factor for the metabolite elevations also in this seemingly healthy elderly population.

The clinical consequences of suboptimal vitamin status in this population was assessed with both haematological and neurocognitive investigations. There were no signs of macrocytic anaemia upfront or as revealed by vitamin therapy. In the neurocognitive investigations, we

utilized a comprehensive set of cognitive tests, and a test of motor and coordination ability, the PLM test. This test is validated, reproducible and the test situation resembles an activity in every-day life. There were independent correlations between the metabolites and cognitive and PLM performance. In spite of significant correlations between plasma tHcy and vitamin concentrations, the latter failed to show independent correlations to cognitive or PLM performance. Four months of vitamin therapy failed to improve neurocognitive performance, and this negative outcome may be explained by any combination of the following factors: irreversible or vitamin-independent neurocognitive decline, insufficient doses of vitamins or too short duration of treatment. In addition, the statistical power of the study was deemed too low to detect small changes in neurocognitive performance.

The inherent limitations of vitamin and metabolite assays are still there, as are the challenges to compute age- and gender specific sets of criteria for the laboratory diagnosis of vitamin deficiency. Measurement of holo-TC has emerged as a potentially better marker for vitamin B_{12} deficiency, the methods have developed and the present assay might, after further validation, be suitable for clinical routine use. In a subset of subjects in the present study, holo-TC correlated with serum B_{12} and was a better predictor for plasma tHcy and serum MMA.

TREATMENT OPTIONS

Clinical B-vitamin deficiency

In the minority of patients who present with overt clinical cobalamin and folate deficiency, treatment is obligatory and unquestionable. Failure to do so has since decades been associated with a fear that any vitamin dependent neurocognitive decline would become irreversible. Thus, not least since vitamin treatment is simple and non-toxic, numbers of subjects in this country, not fulfilling criteria of PA or not investigated for malabsorption have, during the last decades, been put on often life long oral or parenteral vitamin treatment. However, treatment in suspect subclinical cases, e.g. borderline laboratory findings and no or unspecific clinical findings, might also reveal clinical deficiency by a haematological response or improvement in epithelial dysfunction (including infertility). When the diagnosis thus is made *ex juvantibus,* co-existing deficiency states of iron or folate, common in e.g. atrophic gastritis and celiac disease, may hamper the efficacy of the vitamin(s). The above clinical responses are obvious within one-three months, and are accompanied by rapid declines in serum MMA and plasma tHcy. However, normalization of the latter should *per se* not be regarded as proof of clinical deficiency, merely as a reflection of a normalization of the intracellular metabolism of methionine and adenosyl-CoA. Improvement of vitamin-dependent neurocognitive dysfunction may take one year or more to obtain, but a second and equally important goal outside clinical trials would be to prevent further decline in risk groups.

Subclinical B-vitamin deficiency

Whether subjects with subclinical B-vitamin depletion, defined as laboratory deviations (n b with respect to renal function), and with unspecific or no clinical symptoms should be treated with vitamins is somewhat controversial. Important lessons from the past regarding the ignorance of the late effects of partial gastrectomy has been told, and this concept might be extrapolated into other risk groups, preferably in the context of prospective clinical trials. In clinical practice today, the most relevant way seems to make an individual evaluation, taking anamnestic information into consideration e.g. dietary habits, hereditary (autoimmune) diseases, medication, previous gastrointestinal surgery etc. to evaluate predisposing factors for vitamin deficiency. A thorough physical examination noting the general state of nutrition, moth and tongue, vitiligo and neurological examination should be performed. Hopefully,

holo-TC will turn out to be a better tool than total serum B_{12} for the laboratory evaluation. After this diagnostic work-up, a decision on whether or not to treat the individual patient with vitamins or resort to clinical follow up has to be made. Since biochemical evidence of AG was correlated to inferior B-vitamin status also in this seemingly healthy population, it might be argued that these subjects should have vitamin treatment, the alternative would be monitoring for developing clinical deficiency. Concern relates to the fear that the B vitamins might not be as innoxiously as previously believed, and treatment with supra physiological doses of vitamins B_{12} and folic acid for years seem doubtful. Clinical monitoring of the patient and laboratory testing (plasma tHcy, blood haemoglobin, MCV, serum iron and vitamin concentrations) at regular intervals can still be recommended. The data from this thesis might pave the way for avoiding unnecessary vitamin treatment in the elderly, as well as identifying risk groups who according to current knowledge have a high chance to benefit from an appropriate vitamin therapy.
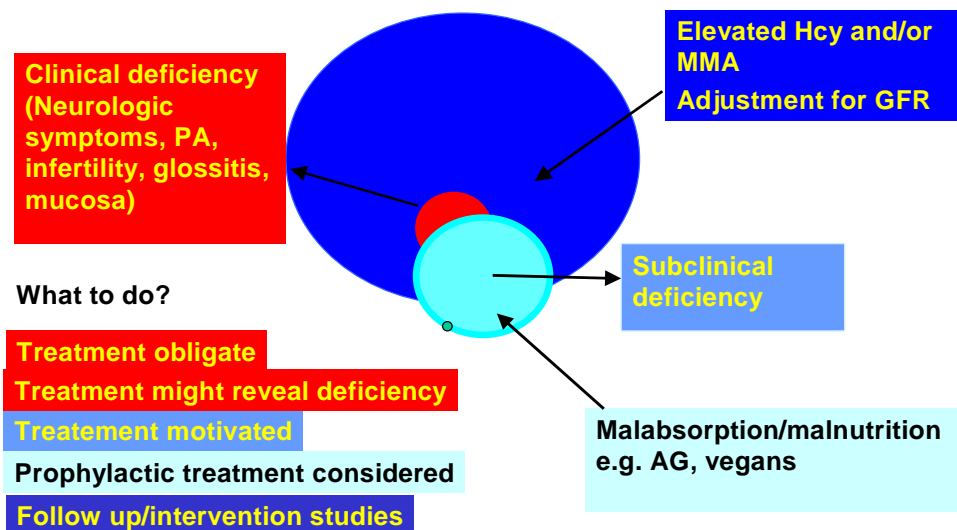
B-vitamin deficiency and risk groups

Fig 13. Combinations of clinical and subclinical deficiency and malabsorption/malnutrition

FUTURE PROSPECTS

Prospective randomised trials, with clinical endpoints similar to the ones we have used, on the long term effects of treatment of vitamin depleted subjects, at best stratified for e.g. malabsorption/malnutrition, gene polymorphisms and cardiovascular disease would be desirable, although not plausible. Food folate and cobalamin fortification and an increasing public awareness of the potential risks with vitamin deficiency are serious challenges for any such study. Holo-TC, markers for gastric mucosal status and reliable measurements of renal function appear necessary in the light of our data. On the population level, there is more to be learnt from the correlations between vitamin status and other body compartments, e.g. bone tissue. For selected groups of patients, further knowledge on the significance of vitamins B_{12} and folic acid might be gained. One example would be earlier intervention than in old age in subjects with atrophic gastritis. Further, the impact of vitamin status on cancer-related anaemia and recovery after treatment for haematological malignant diseases, e.g. in haematology/oncology, needs to be explored, not least in the light of increasing use of haematopoietic growth factors.

CONCLUSIONS

- Elevated concentrations of plasma tHcys and serum MMA were common in this healthy community-dwelling population with a median age of 76 years. Four months of daily substitution with a combination tablet containing 0.5 mg vitamin B_{12}, 0.8 mg folic acid and 3 mg of vitamin B_6 produced significant declines in plasma tHcy and serum MMA. Suboptimal vitamin status is an important factor behind abnormal metabolite concentrations in healthy elderly subjects.

- Glomerular filtration rate is independent of vitamin B_{12} and folate status and is a significant determinant of plasma tHcy and serum MMA in the elderly. Using "high" plasma tHcy and serum MMA levels without adjustment for reliable measures of glomerular filtration rate may lead to an overdiagnosis of vitamin deficiency.

- Plasma tHcy and serum MMA correlated independently and differentially with movement and cognitive performance. Four months of oral vitamin treatment normalized plasma tHcy and serum MMA but failed to improve movement and cognitive performance. This might be attributable to irreversible or vitamin-independent neurocognitive decline, or to an insufficient dosage of vitamins or duration of treatment.

- Atrophic gastritis was prevalent in this elderly community-dwelling population and affected B-vitamin status negatively. Antibodies to Helicobacter pylori correlated to the presence of atrophic gastritis. Subjects with atrophic gastritis responded to B-vitamin treatment just as well as non-AG subjects.

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