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VITAMIN B_{12} DEFICIENCY IN THE AGED:

Laboratory Diagnosis, Prevalence and Clinical Profile

by

Saila Loikas

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From the Departments of Family Medicine and Clinical Chemistry, University of Turku, Turku, Finland

Supervised by

Docent Tarja-Terttu Pelliniemi
Department of Clinical Chemistry
University of Turku
Turku, Finland

Docent Pertti Koskinen
Department of Clinical Chemistry
University of Turku
Turku, Finland

Reviewed by

Professor Raimo Sulkava
School of Public Health
and Clinical Nutrition
Department of Geriatrics
University of Kuopio
Kuopio, Finland

Docent Kari Pulkki
HUSLAB
Helsinki, Finland

Dissertation opponent

Professor Juhani Vilpo
Yhtyneet Laboratoriot Oy
Helsinki, Finland

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Things are only impossible until they're not.
Captain Jean-Luc Picard

ABSTRACT

Saila Loikas

Vitamin B_{12} deficiency in the aged: laboratory diagnosis, prevalence and clinical profile

Departments of Family Medicine and Clinical Chemistry, University of Turku, Turku, Finland
Annales Universitatis Turkuensis, Medica-Odontologica, 2007, Turku, Finland

Background: Vitamin B_{12} deficiency is common in the aged, and early recognition is essential to prevent irreversible damage. There is uncertainty as to whether certain risk groups or the entire aged population should be screened and which laboratory tests should be used.
Aims: The aims of this study were to evaluate the new HoloTC RIA method and to establish reference values, to assess the prevalence of vitamin B_{12} deficiency in the Finnish aged population, to examine risk factors and clinical aspects of vitamin B_{12} deficiency in the aged and to assess the impact of renal function on vitamin B_{12}-related laboratory variables. The goal was to create an algorithm for laboratory tests when diagnosing vitamin B_{12} deficiency.
Subjects and methods: 1260 subjects ≥65 years participated in the Lieto study, a population-based health study on unselected aged population. Data on lifestyle factors and clinical conditions were collected, physical examinations were conducted and laboratory variables related to vitamin B_{12} were measured. 84 healthy adults were recruited for reference value assessment and 107 hospital patients for method evaluation.
Results: The precision of the HoloTC RIA was adequate. The 95% central reference interval for holotranscobalamin was 37-171 pmol/l. All subjects with probable vitamin B_{12} deficiency had holotranscobalamin below the reference limit. Impaired renal function as indicated by increased cystatin C correlated strongly with total homocysteine (r_s=0.53, $p<0.001$) and methylmalonic acid (r_s=0.27, $p<0.001$) but not with total vitamin B_{12} (r_s=-0.04, p=0.227) or holotranscobalamin (r_s=-0.01, p=0.817). The prevalence of vitamin B_{12} deficiency was 12% among the Finnish aged population. The proportion of subjects with low total vitamin B_{12} (<150 pmol/l) was 6%. Male gender (OR 1.9, 95% CI 1.2-2.9), age ≥75 (OR 2.2, 95% CI 1.4-3.4) and refraining from milk products (OR 2.3, 95% CI 1.2-4.4) increased the probability for vitamin B_{12} deficiency. Anemia (OR 1.3, 95% CI 0.7-2.3) or macrocytosis (OR 1.2, 95% CI 0.6-2.7) did not predict vitamin B_{12} deficiency.
Conclusions: Undiagnosed vitamin B_{12} deficiency is common in the aged, but no clinically relevant specific risk group was identified. Since absence of anemia and macrocytosis does not rule out vitamin B_{12} deficiency and renal impairment compromises the use of metabolic markers, the measurement of total vitamin B_{12} is the appropriate first-line test. Further testing of total homocysteine and holotranscobalamin are in order for subjects with borderline total vitamin B_{12} concentrations in their serum for an appropriate diagnosis.

Keywords: vitamin B_{12}, holotranscobalamin, reference values, vitamin B_{12} deficiency, aged

TIIVISTELMÄ

Saila Loikas

B_{12}-vitamiinin puute iäkkäillä: laboratoriodiagnostiikka, yleisyys ja yhteys sairastavuuteen

Turun yliopisto, yleislääketieteen laitos ja kliinisen kemian laitos, Turku
Annales Universitatis Turkuensis, Medica-Odontologica, 2007, Turku, Suomi

Tausta: B_{12}-vitamiinin puute on yleistä iäkkäillä ja se tulisi todeta riittävän varhaisessa vaiheessa palautumattomien vaurioiden estämiseksi. On epäselvää pitäisikö diagnostiikka kohdistaa tiettyihin riskiryhmiin vai mahdollisesti seuloa valikoimatonta vanhusväestöä. Myöskään yksimielisyyttä laboratoriotutkimusten valinnasta ei ole.
Tavoitteet: Tutkimuksen tarkoituksena oli evaluoida uutta HoloTC RIA menetelmää ja tuottaa viitearvot sille, selvittää B_{12}-vitamiinin puutteen yleisyys, yhteys sairastavuuteen ja mahdolliset riskitekijät suomalaisessa vanhusväestössä, arvioida munuaisfunktion vaikusta B_{12}-vitamiinin puutteen laboratoriotutkimuksiin ja näiden perusteella ehdottaa suomalaiseen terveydenhuoltoon sopivaa laboratoriotutkimusstrategiaa.
Aineisto ja menetelmät: Liedon iäkkäät -tutkimuksen vanhusaineisto on edustava otos yhden kunnan yli 65-vuotiaasta väestöstä, yhteensä 1260 henkilöä. Tutkittavat kävivät lääkärintarkastuksessa, ja heistä on käytettävissä runsaasti laboratoriotutkimuksia sekä tiedot sairauksista, ruokavaliosta, lääkkeiden ja vitamiinivalmisteiden käytöstä, dementiaseula ja depressiokysely. Viitearvoaineistoa varten kerättiin näytteet 84 vapaaehtoisesta terveestä aikuisesta ja menetelmäevaluaatiota varten 107 sairaalapotilaasta.
Tulokset: HoloTC RIA menetelmän toistettavuus oli hyvä manuaalimenetelmäksi. 95%:n viiteväli holotranskobalamiinille oli 37-171 pmol/l. Kaikilla tutkittavilla, joilla oli muilla laboratoriotutkimuksilla osoitettu todennäköinen B_{12}-vitamiinin puute, myös holo-transkobalamiini oli viitealueen alarajaa pienempi. Suurentuneella kystatiini C-pitoisuudella osoitettu munuaisten vajaatoiminta korreloi voimakkaasti homokysteiinin (r_s=0.53, p<0.001) ja metyylimalonihapon (r_s=0.27, p<0.001) pitoisuuksiin, mutta ei kokonais-B_{12}-vitamiinin (r_s=-0.04, p=0.227) tai holotranskobamiinin (r_s=-0.01, p=0.817) pitoisuuksiin. Suomalaisessa vanhusväestössä B_{12}-vitamiinin puutteen prevalenssi oli 12%. Kokonais- B_{12}-vitamiinin pitoisuus oli matala (<150 pmol/l) 6%:lla. Miessukupuoli (OR 1.9, 95% CI 1.2-2.9), ikä ≥75 (OR 2.2, 95% CI 1.4-3.4) ja maitotuotteiden välttäminen (OR 2.3, 95% CI 1.2-4.4) lisäsivät B_{12}-vitamiinin puutteen riskiä, mutta anemia (OR 1.3, 95% CI 0.7-2.3) tai makrosytoosi (OR 1.2, 95% CI 0.6-2.7) eivät.
Päätelmät: Diagnosoimaton B_{12}-vitamiinin puute on yleistä iäkkäillä, mutta kliinisesti merkityksellistä spesifistä riskiryhmää ei löydy. Koska anemian ja makrosytoosin puuttuminen ei poissulje B_{12}-vitamiinin puutetta ja munuaisten vajaatoiminta heikentää metabolisten merkkiaineiden käyttökelpoisuutta, kokonais-B_{12}-vitamiinia suositellaan ensisijaiseksi laboratoriotutkimukseksi epäiltäessä B_{12}-vitamiinin puutetta ja tarvittaessa varmentavina tutkimuksina käytetään homokysteiiniä ja holotranskobalamiinia.

Avainsanat: B_{12}-vitamiini, holotranskobalamiini, viitearvot, B_{12}-vitamiinin puute, iäkkäät

CONTENTS

ABBREVIATIONS

ADL	activities of daily living
CHD	coronary heart disease
CI	confidence interval
CV%	coefficient of variation (%) (SD/mean x 100%)
CysC	cystatin C
DSM-IV	diagnostic and statistical manual of mental disorders, fourth edition
ECG	electrocardiography
ELISA	enzyme-linked immunoabsorbent assay
ERC	erythrocyte
GFR	glomerular filtration rate
GP	general practitioner
HC	haptocorrin
holoTC	holotranscobalamin
IADL	instrumental activities of daily living
IF	intrinsic factor
MCV	mean cell volume
MMA	methylmalonic acid
OR	odds ratio
PPI	proton pump inhibitor
RIA	radioimmunoassay
SD	standard deviation
TC	transcobalamin
tHcy	total homocysteine

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by their Roman numerals (I-VI) in the text.

I Loikas S, Löppönen M, Suominen P, Møller J, Irjala K, Isoaho R, Kivelä SL, Koskinen P, Pelliniemi TT. RIA for serum holo-transcobalamin: method evaluation in the clinical laboratory and reference interval. Clin Chem 2003;49:455-62.

II Loikas S, Pelliniemi TT, Koskinen P. No bias between the first and the new version of radioimmunoassay for serum holo-transcobalamin by Axis-Shield. Clin Chem Lab Med 2004;42:569-70.

III Loikas S, Koskinen P, Irjala K, Löppönen M, Isoaho R, Kivelä SL, Pelliniemi TT. Renal impairment compromises the use of total homocysteine and methylmalonic acid but not total vitamin B_{12} and holotranscobalamin in screening for vitamin B_{12} deficiency in the aged. Clin Chem Lab Med 2007;45:197-201.

IV Loikas S, Koskinen P, Irjala K, Löppönen M, Isoaho R, Kivelä SL, Pelliniemi TT. Vitamin B_{12} deficiency in the aged: a population-based study. Age Ageing 2007;36:177-83. Epub 2006 Dec 21.

V Loikas S, Koskinen P, Irjala K, Löppönen M, Isoaho R, Kivelä SL, Pelliniemi TT. Clinical signs and symptoms in the aged with low total vitamin B_{12} concentrations. Submitted.

VI Loikas S, Koskinen P, Irjala K, Löppönen M, Isoaho R, Kivelä SL, Pelliniemi TT. Laboratory diagnosis of vitamin B_{12} deficiency. Submitted.

1. INTRODUCTION

Vitamin B_{12} deficiency is common and its prevalence increases with age. It is estimated to affect 5-40% of the aged, depending on the diagnostic criteria used (1-5). However, data about vitamin B_{12}-related factors in unselected aged populations are scarce and many previous studies have been based on small and selective samples.

The diagnosis of vitamin B_{12} deficiency is not straightforward. There are no uniform diagnostic criteria and no single laboratory test constitutes a gold standard. Advanced stages of vitamin B_{12} deficiency seldom cause diagnostic problems, but early diagnosis is complicated by limitations of the laboratory methods and by the subtle, non-specific symptoms of vitamin B_{12} deficiency (6, 7). The typical hematological changes may be absent or develop late, and neurological and cognitive symptoms are often the first indication of vitamin B_{12} deficiency (8). However, early recognition is essential, because the damage is progressive and may be reversible if treated early enough (1, 3, 9). Therefore, even screening for asymptomatic aged people or subjects with possible risk factors for vitamin B_{12} deficiency has been suggested (5, 7, 10).

Measurement of total vitamin B_{12} is simple, inexpensive and widely available, but it lacks both sensitivity and specificity (11). Elevated plasma concentrations of total homocysteine (tHcy) or methylmalonic acid (MMA) are sensitive metabolic markers for vitamin B_{12} deficiency. However, neither of them is specific for vitamin B_{12} deficiency and they are also increased in renal impairment (12, 13) and other causes for increased tHcy are also common (13).

Measurement of holotranscobalamin (holoTC), the biologically active fraction of vitamin B_{12} has been suggested as a sensitive marker of tissue vitamin B_{12} deficiency but clinical experience is still sparse (14-19). The evaluation of the clinical usefulness of holoTC measurement has been difficult, mainly because no suitable methods for routine use have been available. Recently, the measurement of holoTC has become a real option with the introduction of sensitive immunological methods which replace the labor-intensive physicochemical methods (16, 20).

The present study was conducted to evaluate the new commercially available HoloTC RIA method, and to assess the prevalence, the risk factors and clinical correlates of vitamin B_{12} deficiency among the Finnish aged population. In addition, an algorithm for the optimal use of laboratory tests to diagnose vitamin B_{12} deficiency is presented.

2. REVIEW OF THE LITERATURE

2.1. Vitamin B_{12}

2.1.1. Structure

Vitamin B_{12} was first identified in the 1920's by Minot and Murphy as being the extrinsic factor present in the liver which reverses the symptoms of pernicious anemia, as reviewed by Markle and Okuda (21, 22). The substance was isolated and named vitamin B_{12} in 1948 (23, 24). Hodgkin and co-workers determined the crystalline structure of vitamin B_{12} molecule in the 1950's (25). Vitamin B_{12} is a term used for a group of physiologically active substances, which are chemically classified as cobalamins (Figure 2.1). The basic structure is a tetrapyrrole ring, a corrin, with a central cobalt atom and a purine nucleotide (5,6-dimethylbenzimidazole) linked to it. The various cobalamins differ in the other side chain attached to the cobalt and are named by the organic ligand. The biologically active compounds methylcobalamin and deoxyadenosylcobalamin are labile, whereas hydroxocobalamin and cyanocobalamin, which are used therapeutically, are more stable, but must be converted to the former two to function as enzyme cofactors. The labile forms are converted to cyanocobalamin when the serum concentration of vitamin B_{12} is quantified.

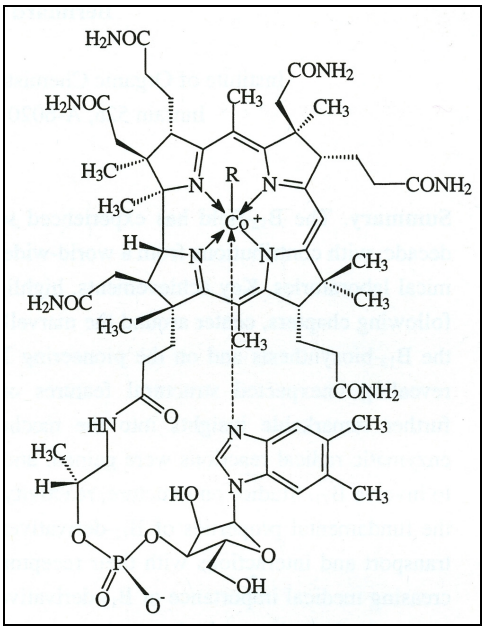

Figure 2.1. Structural formula of vitamin B_{12}. The side chain R is CN in cyanocobalamin, 5'-deoxy-5'-adenosyl in adenosylcobalamin, methyl in methylcobalamin, and HO in hydroxycobalamin.

2.1.2. Sources

Vitamin B_{12} is an essential vitamin for man. It is synthesized only by bacteria. Unlike man, many animals can utilize vitamin B_{12} synthesized by their own colonic flora. Therefore, man must obtain vitamin B_{12} from food, and food of animal-origin is the only natural source of vitamin B_{12} in man. In the Finnish diet, the most important sources of vitamin B_{12} are meat, dairy products and fish (26). An ordinary mixed food diet provides 5-30 μg of vitamin B_{12} daily, an amount at least double of the recommended daily intake of 2 μg (26, 27).

2.1.3. Function

Vitamin B_{12} is essential for two enzymatic reactions in humans and other mammals (22). Firstly, in the methionine synthase reaction occurring in the cytoplasm, methylcobalamin serves as an intermediate: it transfers a methyl group from 5-methyl-tetrahydrofolate to homocysteine to form methionine and tetrahydrofolate (Figure 2.2). In vitamin B_{12} deficiency, the formation of thymidylic acid, and thus of DNA, is impaired due to shortage of this functional form of folate. This is called the methyl-folate trap, since conversion of 5,10-methylene-tetrahydrofolate to 5-methyl-tetrahydrofolate is irreversible. At the same time, homocysteine accumulates due to impaired methionine synthesis.

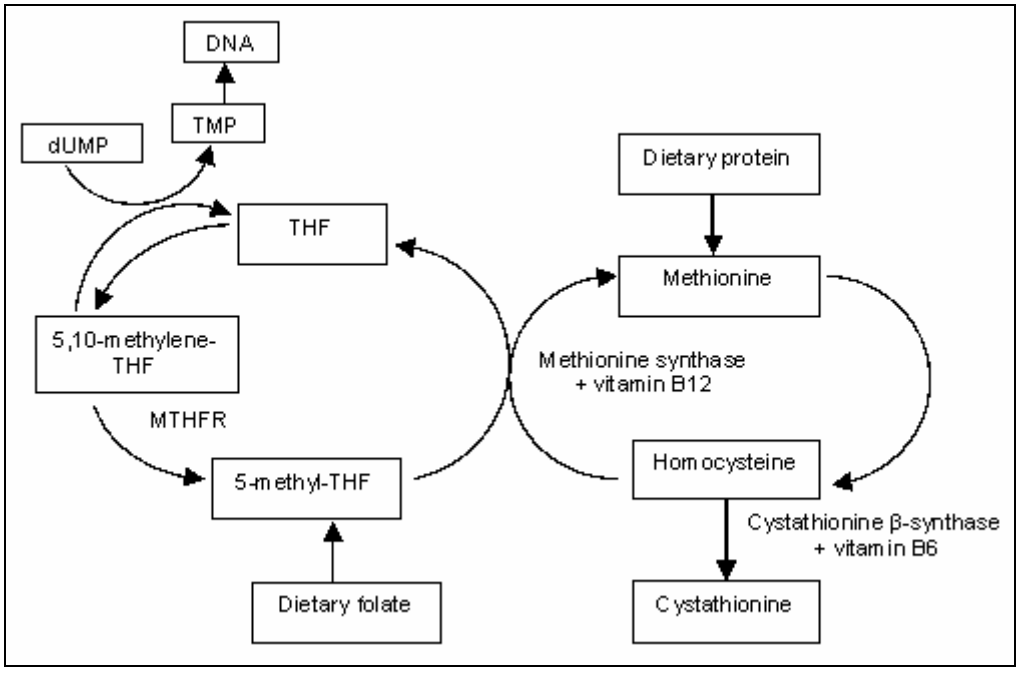

Figure 2.2. Demethylation catalyzed by methionine synthase: methylcobalamin acts as an intermediate that transfers a methyl group.

dUMP, deoxyuridne monophosphate; TMP, thymidine monophosphate; 5-methyl-THF, 5-methyl-tetrahydrofolate; THF, tetrahydrofolate; 5,10-methylene-THF, 5,10-methylene-tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase

Secondly, in the methylmalonyl-CoA mutase reaction which occurs in mitochondria, adenosylcobalamin is required as a coenzyme (Figure 2.3). Methylmalonyl-CoA, a derivative of propionic acid which is synthesized by intestinal bacteria, is converted to succinyl-CoA, which then enters the citric acid cycle. This reaction is essential for the degradation of fatty acids with an odd number of carbons in the chain. Vitamin B_{12} deficiency causes accumulation of methylmalonic acid.

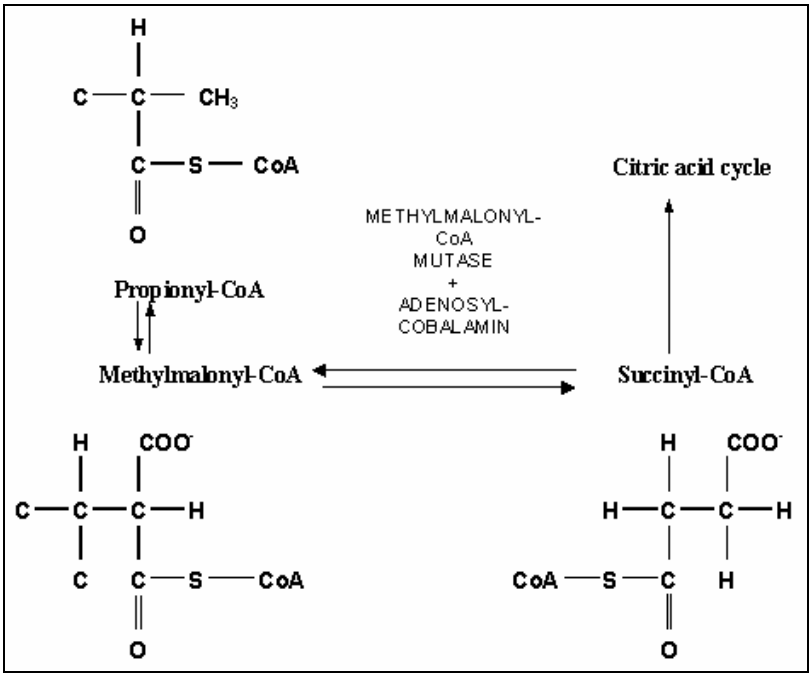

Figure 2.3. Isomerization reaction catalyzed by methylmalonyl-CoA mutase: adenosylcobalamin acts as a coenzyme.

2.2. Vitamin B_{12} binding proteins and their receptors

2.2.1. Nomenclature

Three binding proteins, intrinsic factor (IF), haptocorrin (HC) and transcobalamin (TC), are involved in the uptake and transport of vitamin B_{12} (21, 22). There is a considerable similarity in the gene structure among the three proteins. They are found either saturated with vitamin B_{12} as holoproteins or unsaturated as apoproteins.

The name intrinsic factor, as distinguished from extrinsic factor, was given by Castle in 1929 (22). Later, the name S binder was used for IF indicating its slower mobility in electrophoresis when compared to the R binders, which are more rapidly moving vitamin B_{12} binding proteins (28). R binders have also been called cobalophilin and most recently HC, to express their ability

to bind not only cobalamins but also biologically inactive corrins.

The names transcobalamin I, II and III were originally used for the vitamin B_{12} binding proteins in plasma (22, 29). Transcobalamins I and III proved to be two forms of haptocorrin with an identical primary structure, although their content and structure of carbohydrates were different. The name transcobalamin has been proposed for transcobalamin II because it transports vitamin B_{12} from the plasma into the cells. Unfortunately, terminology is not consistent, although the names IF, HC and TC are preferred.

2.2.2. Intrinsic factor

Intrinsic factor is an approximately 45 kDa glycoprotein (30) synthesized by the parietal cells of the gastric mucosa. It is mainly present in the gastric juice and in only very low quantities in the plasma. Its secretion is stimulated by gastrin. The gene for IF is located in chromosome 11 (31). IF is essential for the transport of vitamin B12 from food into intestinal cells. The IF-vitamin B12 complex has a specific receptor in the terminal ileum (32). The receptor is a complex of two epithelial proteins, cubilin and amnionless, recently named cubam (33). The receptor is present in the apical membrane of epithelial cells of intestine and kidney. In addition to vitamin B12, it recognizes several other low-molecular-weight proteins for endocytosis. Previously, the LDL receptor family receptor megalin was considered as a requirement for cubilin to function, but recent data suggests that the cubilin/amnionless complex can function independently of megalin (33).

2.2.3. Haptocorrin

Haptocorrin is a 58 kDa glycoprotein (34) abundant in plasma, blood cells and most exocrine secretions (34). It is synthesized in neutrophils and numerous glands, including salivary glands. The gene for HC is located in chromosome 11 (35), like the gene for IF. Almost 80% of the vitamin B_{12} in the plasma is carried by HC, although a lack of HC is not related to vitamin B_{12} deficiency findings (36).

The role of HC in the metabolism and transport of vitamin B_{12} is still unclear. It may function as a circulating storage molecule for vitamin $B_{12,}$ like ferritin in the iron metabolism (22). Interestingly, the vitamin B_{12} attached to HC is mainly methylcobalamin, which is the active coenzyme for methionine synthetase. The ability of HC to bind corrins other than vitamin B_{12} and its presence as an apoprotein in white blood cells and in exocrine secretions has given rise to two hypotheses. First, it may transport inactive or even toxic corrins to the liver for excretion via the bile or (37), second, it may protect against infections caused by microbes dependent on vitamin B_{12} or corrins for survival (38).

HC present in the saliva and gastric juice binds vitamin B_{12} that enters the digestive tract with food. HC is degraded in duodenum allowing IF to bind vitamin B_{12} and to transport it to the intestinal cells. There, most of the vitamin B_{12} is transferred to HC and less to TC which carries it in the plasma. Cell surface HC receptors are present only in the liver. The receptor is a multifunctional asialoglycoprotein receptor that recognizes glycoproteins containing terminal galactose residues (21, 39).

2.2.4. Transcobalamin

Transcobalamin (40) unlike IF and HC, not glycosylated. The gene for TC is located in chromosome 22. All three vitamin B_{12} binding proteins seem to be derived from the same gene, but TC has diverged from the common ancestry before HC and IF (41, 42). TC is synthesized by various tissues including the intestinal cells, liver, kidney, central nervous system cells, bone marrow and fibroblasts (43).

Only approximately 20% of the circulating vitamin B_{12} is carried by TC (36). However, TC is the transport protein required for the cellular uptake of vitamin B_{12} (44). Two different cell membrane receptors for TC have been identified. Firstly, the receptor, which is responsible for transport of vitamin B_{12}-TC complex from plasma to cells has been identified in several tissues (45, 46). Though its functional properties are well established, its primary structure is still unconfirmed; a 124-kD dimeric protein and a 58 kD monomeric protein have been presented (45-47). Secondly, the multifunctional megareceptor megalin, a 600 kD protein, is present in the proximal tubules of kidney and is responsible for the renal uptake of vitamin B_{12} (48).

2.3. Vitamin B_{12} homeostasis

2.3.1. Absorption

Absorption of vitamin B_{12} is a complex process involving several organs of gastrointestinal tract (1, 49-51). An outline of the active absorption of vitamin B_{12} is presented in Figure 2.4. In the 1950's and 1960's several investigators showed that there is another pathway for cobalamin absorption that does not require intrinsic factor or the presence of an intact ileum. Approximately 1% of the ingested vitamin B_{12} can be absorbed by passive diffusion. Thus, if large doses of vitamin B_{12} are administered to patients with pernicious anemia, enough can be absorbed by passive diffusion to meet the daily requirement.

2.3.2. Excretion

Vitamin B_{12} is a highly conserved vitamin and its daily loss is approximately only 0.1-0.2% of its total body content (1). Excretion is mainly fecal. The sources of fecal vitamin B_{12} are unabsorbed vitamin B_{12} from food or bile and secretions from the gastrointestinal tract. Also, vitamin B_{12} synthesized by colonic bacteria is lost directly with the feces. Some loss of vitamin B_{12} occurs also in sweat and other body secretions, and in desquamated cells. Normally, urinary excretion of vitamin B_{12} is low, but it increases in excess of vitamin B_{12}. Recent data suggest that the kidneys have a more important role in the vitamin B_{12} homeostasis than previously recognized, as explained in the next section.

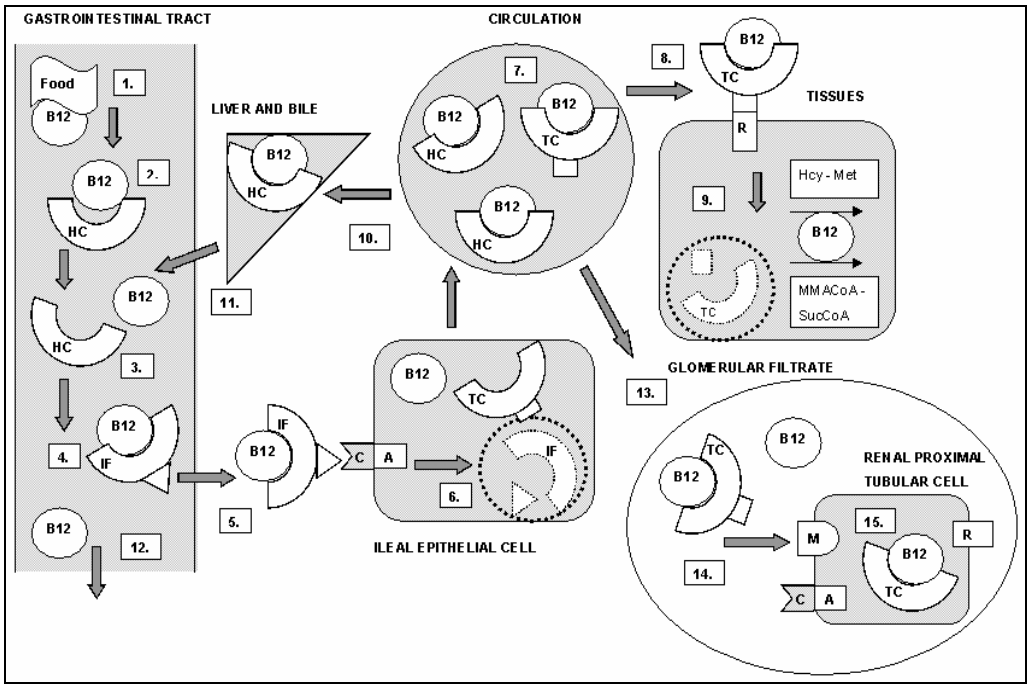

Figure 2.4. Absorption and excretion of vitamin B_{12}. **1.** Dietary vitamin B_{12} is protein bound. It is released by pepsin and gastric acid in the upper gastrointestinal tract. **2.** Free vitamin B_{12} is attached to HC produced by salivary glands. **3.** HC has high affinity to vitamin B_{12} at the acidic pH of the stomach. HC is degraded by pancreatic proteases in the duodenum. **4.** IF secreted by parietal cells of gastric mucosa binds to vitamin B_{12} in the neutral or alkaline pH of duodenum. **5.** The IF-vitamin B_{12}-complex is endocytosed by the cubilin-amnionless receptor in the terminal ileum. **6.** IF is degraded in lysosomes. Vitamin B_{12} is bound to HC and TC and transported to plasma **7.** In plasma vitamin B_{12} is carried by HC and TC. **8.** The TC-bound, biologically active fraction of vitamin B_{12} is internalized in the cells by the TC receptor. **9.** TC is degraded in lysosomes. Vitamin B_{12} is enzymatically converted into two active forms, methylcobalamin and adenosylcobalamin. **10.** Most of the circulating vitamin B_{12} is bound to HC and delivered mainly to the liver. **11.** The enterohepatic circulation recirculates vitamin B_{12}. Vitamin B_{12} is secreted into the bile and two-thirds of it is then reabsorbed in the terminal ileum. **12.** Vitamin B_{12} is mainly excreted through feces. **13.** Unbound vitamin B_{12}, TC and TC- vitamin B_{12}-complex are filtered in the glomeruli. **14.** TC-B_{12} complex is reabsorbed by the megalin receptor in the renal proximal tubule. **15.** In the proximal tubular cells vitamin B_{12} is metabolized, stored or released.

B12, vitamin B_{12}; HC, haptocorrin; IF, intrinsic factor; TC, transcobalamin; C, cubilin; A, amnionless, R, TC receptor; M, megalin; Hcy, homocysteine; Met, methionine; MMACoA, methylmalonyl-CoA; SucCoA, succinyl-CoA.

2.3.3. Role of the kidneys

Vitamin B_{12} is filtered in the glomeruli and reabsorbed in the renal tubular system to prevent its urinary loss (52). Tubular uptake may result in renal accumulation and metabolism of vitamin B_{12}. The glomerular filtration of vitamin B_{12} depends on its concentration and the amount of binding proteins in the plasma. Unbound vitamin B_{12} is freely filtered. Also, the molecular mass

of TC suggests that TC as well as the TC-B_{12} complex are filtered whereas glycosylated HC is not (53, 54). At physiological serum concentrations the amount of filtered vitamin B_{12} in the glomeruli is approximately equivalent to the daily intake. However, the efficient receptor-mediated uptake in proximal tubuli conserves most of the filtered vitamin B_{12}, and the excreted amount is minimal (52).

Studies on the renal tubular uptake of vitamin B_{12} are largely based on experiments in animals (52). The both receptors megalin and cubilin are heavily expressed in the proximal renal tubular cells (48, 52). Also, the other TC-receptor has been identified in the kidney, but in contrast to megalin, which is expressed in the apical brush border membranes, this additional receptor is located mainly in the basolateral membranes (55).

Megalin is essential for the reabsorption of filtered TC-B_{12}-complex in the proximal tubule (53). Megalin and cubilin may interact in the binding and uptake of their ligands, but recently it has been shown that endocytic function of cubilin is dependent on amnionless protein (33). Although cubilin is essential for the absorption of the vitamin B_{12}-IF complex in the terminal ileum its significance in the renal uptake of vitamin B_{12} is probably small, since only minute amounts of IF or B_{12}-IF complex are present in serum or filtered in the glomeruli. Because cubilin is important for the uptake of several proteins that are filtered in the glomeruli, mutations in the cubilin or amnionless gene (which is clinically seen as the Imerslund-Gräsbeck-syndrome) lead to proteinuria in addition to vitamin B_{12} malabsorption (56, 57).

After endocytosis into the proximal tubular cells, vitamin B_{12} may be metabolized, stored or released. Significant amounts of free vitamin B_{12} accumulate in lysosomes of the proximal tubular cells, suggesting that the vitamin is stored in the kidney. The amount of accumulated vitamin B_{12} seems to be larger in states of vitamin overload than vitamin depletion (55), which indicates that the release of vitamin B_{12} from the kidneys is negatively regulated by the vitamin B_{12} status. The mechanisms by which reabsorbed vitamin B_{12} is further transported, processed and released in proximal tubular cells are largely unknown (58).

2.4. Causes of vitamin B_{12} deficiency

Since vitamin B_{12} metabolism is a complex process, there are many check points that may function pathologically (3, 5, 22, 49, 50). Roughly speaking, the etiology of vitamin B_{12} deficiency is either inadequate intake or impaired absorption, the latter due to chronic atrophic gastritis or some other cause. An increased requirement for vitamin B_{12} or functional "resistance" might also cause deficiency symptoms (9). Nevertheless, a rule of the thumb is that there is some gastroenterological disorder behind vitamin B_{12} deficiency until proven otherwise. The most common cause of vitamin B_{12} deficiency is food vitamin B_{12} malabsorption.

2.4.1. Impaired absorption

2.4.1.1 Pernicious anemia

The first descriptions of patients with symptoms compatible with pernicious anemia originate in the middle of the 19th century by Combe and Addison (reviewed in 21, 22, 59). A few years later Flint associated this anemic disease to the stomach. In 1872 Biemer named this disease pernicious anemia, because it would inevitably lead to death and no cure was known. Then, in the 1920's, it was shown that pernicious anemia was alleviated by ingestion of extrinsic factor present in liver. This substance was later purified and named vitamin B_{12}. Later on, Castle laid the foundation to the etiology and pathogenesis of pernicious anemia by showing that insufficient absorption of this extrinsic factor was caused by lack of substance called IF in gastric juice. An immunological basis of the gastritis that causes lack of IF and thus pernicious anemia was suggested when autoantibodies to IF and parietal cells were found in the early 1960's. The pathogenesis was further clarified in the late 1980's by the demonstration that the antigen recognized by parietal cell antibodies is gastric H+/K+-ATPase. Recently, it has been suggested that *Helicobacter pylori* may be involved in the development of pernicious anemia (60).

Pernicious anemia is the classic cause of vitamin B_{12} deficiency. It is often diagnosed in the aged, but it may be the reason for vitamin B_{12} deficiency only in about 15-20% of subjects aged over 60 years (49). Pernicious anemia is a consequence of end stage chronic atrophic autoimmune gastritis, which is characterized by atrophy of the gastric mucosa in the fundus and the corpus of the stomach (59, 61). Parietal cell antibodies directed toward H+/K+-ATPase destroy of gastric parietal cells, impair IF production and lead to vitamin B_{12} malabsorption.

There are also other mechanisms that contribute to the impaired absorption of vitamin B_{12} in pernicious anemia. Loss of parietal cells causes loss of gastric acid secretion. Also, there is loss of zymogenic cells and impaired pepsinogen I production. Due to hypochlorhydria and loss of pepsinogen I production, protein bound vitamin B_{12} cannot be released and absorbed. Approximately 50% of subjects with pernicious anemia have anti-IF antibodies, which may prevent the formation of the vitamin B_{12}-IF complex.

Pernicious anemia is associated with other autoimmune disorders: primary hypoparathyreoidism, chronic autoimmune thyreoiditis, Graves' disease, Addison's disease, primary ovarian failure, type I diabetes, vitiligo, myasthenia gravis and the Lambert-Eaton syndrome (59).

2.4.1.2 Food-vitamin B_{12} malabsorption

In the early 1970's Doscherholmen and Swaim showed that many patients with atrophic gastritis or partial gastrectomy could not absorb food-bound vitamin B_{12} despite a normal absorption of free vitamin B_{12} (62). Later on, many studies confirmed the basis of the disorder: vitamin B_{12} could not be released from food or gastrointestinal transport proteins (HC), and consequently binding to IF was blocked (63, 64). The primary cause for food-vitamin B_{12} malabsorption is chronic gastritis. Usually it is due to *Helicobacter pylori* infection, which involves the antrum of the stomach and causes impaired gastric acid and pepsin secretion, while

IF production, and thus the absorption of unbound vitamin $B_{12,}$ remains unaffected. However, hypochlorhydria and food-vitamin B_{12} malabsorption occur also in autoimmune atrophic gastritis as presented in the previous chapter.

Food-vitamin B_{12} malabsorption can also be caused by other conditions than chronic gastritis. An apparent reason is hypochlorhydria caused by gastric surgery (partial gastrectomy, vagotomy, gastric bypass surgery for treatment of obesity). Several studies have suggested that vitamin B_{12} malabsorption can occur with long-term use of acid-suppressing drugs such as H_2-receptor antagonists and proton pump inhibitors (reviewed in 65).

2.4.1.3 The role of Helicobacter pylori

Helicobacter pylori infection is strongly associated with chronic gastritis of the antrum of the stomach, which causes impairment in gastric acid and pepsin secretion, and is thus linked to malabsorption of food-vitamin B_{12}. Initially, a negative association between *Helicobacter pylori* and pernicious anemia was stated in a study in which the prevalence of *Helicobacter pylori* (by biopsy) was significantly lower in patients with pernicious anemia than in controls (11% vs. 71%) (66). Later, this was explained by anassociation between *Helicobacter pylori* and gastric autoimmunity: in a retrospective follow-up study of 102 patients for 32 years (67), the appearance of parietal cell antibodies led to progression of corpus atrophy and disappearance of *Helicobacter pylori*, whereas patients with duodenal ulcer disease remained positive for *Helicobacter pylori*. Also several other studies (reviewed in 60) have suggested that *Helicobacter pylori* is involved in the pathogenesis of pernicious anemia by causing atrophy of the corpus of the stomach by inducing parietal cell antibodies. The autoantigen in *Helicobacter pylori*-associated corpus atrophy is H+/K+-ATPase – the same as in classic pernicious anemia (68).

2.4.1.4 Other causes for vitamin B_{12} malabsorption

Total and partial gastrectomy eliminates the production of IF and gastric acid, which inevitably causes vitamin B_{12} malabsorption. Exocrine pancreatic insufficiency following chronic pancreatitis or pancreatomy may impair vitamin B_{12} absorption due to the reduced production of pancreatic proteases which are needed for degradation of HC from vitamin B_{12} in the duodenum (64). The oral antidiabetic drug metformin impairs endocytosis of the IF-vitamin B_{12} complex by chelating calcium ions essential for receptor binding (69-71). Because absorption of vitamin B_{12} takes place in the terminal ileum, surgical resection of the terminal ileum and diseases like Crohn's disease and celiac disease that damage the mucosa of the terminal ileum cause malabsorption of vitamin B_{12}. Also, folate deficiency may damage the ileal epithelium and impair vitamin B_{12} absorption.

2.4.2. Dietary insufficiency

Inadequate intake is an extremely rare cause of vitamin B_{12} deficiency in industrialized countries because ordinary mixed food diet provides enough vitamin B_{12} (49). A recent review summarizes that several studies have shown that the intake exceeds the recommended daily intake in healthy adults and aged in the United States, Canada and Europe (72). Poor intake of

vitamin B_{12} and other nutrients may rarely occur in malnourished aged persons in institutions or persons with eating disorders.

Vitamin B_{12} intake is certainly insufficient in vegetarian diets, especially in strict vegan diets, which contain no food of animal origin at all. Most Western people start vegetarian diet in adult life, when they have adequate hepatic stores of vitamin B_{12}. Thus, because of efficient enterohepatic circulation, it may take several years before overt vitamin B_{12} deficiency develops. The need for vitamin B_{12} supplementation is well recognized among vegetarians. On the other hand, if a vegetarian diet is consumed from infancy, vitamin B_{12} deficiency is more likely to develop. Also, breast-fed infants of vegetarian mothers are at risk of inadequate vitamin B_{12} gain, because the vitamin B_{12} content in breast milk may be low (3, 72-75).

2.4.3. Other causes for vitamin B_{12} deficiency

2.4.3.1 Biological competition

Although hypochlorhydria causes impaired absorption of vitamin B_{12} by preventing its release from proteins, hypochlorhydria may also facilitate intestinal bacterial overgrowth and biological competition for vitamin B_{12}. Long-term treatment with antimicrobial agents may also induce intestinal bacterial overgrowth (5). Fish tapeworm (*Diphyllobothrium latum*) infection, which results from consumption of raw or poorly cooked freshwater fish, also causes vitamin B_{12} deficiency by biological competition (76). However, although *Diphyllobothrium* infections are still occasionally detected, tapeworm infestation causes vitamin B_{12} deficiency very seldom, because of an abundant nutritional supply.

2.4.3.2 Hereditary disorders

Inborn errors of cobalamin metabolism are rare and should be diagnosed in infancy. The disorders that affect vitamin B_{12} absorption are congenital intrinsic factor deficiency and the Imerslund-Gräsbeck syndrome. In the Imerslund-Gräsbeck syndrome the receptor for the IF-vitamin B_{12} complex is defective due to mutations in the cubilin or amnionless gene (56, 57). Also, IF may have an abnormal structure, and thus be unable to bind vitamin B_{12} or its receptor. TC deficiency cause impairment in the transport of vitamin B_{12} into the cells (77). Defects in HC do not cause vitamin B_{12} deficiency (78). Also, the intracellular metabolism of vitamin B_{12} may be affected. Impairment of adenosylcobalamin synthesis (cblA and cblB), methionine synthase function (cblE and cblG) or both (cblC, cblD, cblF and cblH) are possible (79, 80).

2.5. Symptoms and signs

Vitamin B_{12} deficiency can cause a wide range of signs and symptoms (Table 2.1). Traditionally, the deficiency has been considered to present with severe megaloblastic anemia and progress to a neurological condition called subacute combined degeneration. However, this represents only the end stage of the slowly progressive process. At earlier stages clinical manifestations are subtle and highly variable, and neurological and cognitive defects may occur in the absence of hematological signs (8). Consequently, the diagnostic value of these unspecific symptoms and signs is low (6, 7).

Table 2.1. Symptoms and signs related to vitamin B_{12} deficiency.

Hematological	
	Anemia (palpitations, short of breath, fatigue, pallor)
	Macrocytosis
	Hemolytic anemia, schistocytes, elevated lactate dehydrogenase and bilirubin, jaundice
	Neutrophil hypersegmentation
	Leucopenia (susceptibility to infections)
	Thrombocytopenia (bruising, bleeding complications)
Neurological	(peripheral polyneuropathy and/or myelopathy)
	Paresthesias
	Impaired vibration sensation
	Impaired cutaneous sensation
	Muscle weakness
	Impaired tendon reflexes
	Impaired position sense and balance (unsteadiness in walking)
	Ataxia
	Optic neuritis
	Autonomic neuropathy (erectile impotence, urine and fecal incontinence)
Cognitive and affective	
	Memory impairment
	Dementia
	Depression
	Delirium, psychosis
Gastroenterological	
	Lingual atrophy, stomatitis or sore tongue
	Reduced appetite, weight loss
	Gastrointestinal pain or disorders

2.5.1. Hematological symptoms and signs

Classic vitamin B_{12} deficiency is characterized by macrocytic anemia and megaloblastic finding at bone marrow examination (22, 81). Hypersegmented neutrophils are present in the peripheral blood, and thrombocytopenia and leucopenia may occur. Also, intramedullar hemolysis may be present and cause increased lactate dehydrogenase and bilirubin and jaundice. The

hematological symptoms are mainly caused by anemia (pallor, fatigue, short of breath and palpitation). The underlying mechanism is impaired DNA synthesis in rapidly dividing cells of the bone marrow. DNA synthesis is decreased due to inadequate formation of thymidine triphosphate in lack of tetrahydrofolate because of impaired methionine synthase reaction, which needs vitamin B_{12} as a cofactor (Figure 2.2). Since DNA synthesis is impaired, but RNA synthesis remains unaffected, there is a dysbalance in the maturation of cellular nuclei and their cytoplasm. Inhibited maturation of the nucleus leads to megaloblastic cells in the bone marrow, macrocytosis, and cytopenias. Because tetrahydrofolate is a crucial factor in DNA synthesis, both vitamin B_{12} and folate deficiency lead to similar hematological consequences.

2.5.2. Neurological and cognitive symptoms and signs

Against the classic view that neurological disorders occur only at the late stage of vitamin B_{12} deficiency, it is currently obvious that neurological findings may be an early manifestation of the vitamin deficiency and – importantly – that neurological disorders often occur in the absence of hematological changes (8, 82). In addition, it has been shown that the occurrence of neurological signs is inversely correlated to the degree of anemia (82, 83). The damage to the nervous system is progressive, but may be reversible if treated early enough.

The classic neurological manifestation of vitamin B_{12} deficiency is subacute combined degeneration, a specific spinal cord lesion in the posterior and lateral columns, involving the corticospinal and spinocerebellar tracts (82-84). It causes sensory disturbances and pyramidal motor disturbances resulting in ataxia. Vitamin B_{12} deficiency causes also peripheral polyneuropathy. Common symptoms include paresthesias and numbness, loss of sense of vibration and position, muscle weakness and, in advanced stages, symptoms of autonomic neuropathy. Optic neuritis and optic atrophy have been reported. Cerebral disorders due to vitamin B_{12} deficiency include cognitive impairment ranging from mild concentration loss to florid dementia (5, 22, 85, 86). Also, hallucinations, psychosis and depression have been linked to vitamin B_{12} deficiency (8, 87, 88).

The neurological and cognitive manifestations vary and the underlying pathogenesis is poorly understood. Several mechanisms have been suggested (84, 85, 89). Firstly, impaired activity of methylmalonyl CoA mutase causes a lack of succinyl CoA, which may lead to decreased myelin production and incorporation of abnormal fatty acids into neuronal lipids. Secondly, impaired activity of methionine synthase, which decreases the production of S-adenosylmethionine, an important methyl group donor in many transmethylation reactions in nervous system, may lead to myelin damage and disturbed neurotransmitter metabolism. Thirdly, a direct toxic effect of increased homocysteine to the brain has been suggested. Fourthly, cytokines have been linked to the neuropathology in vitamin B_{12} deficiency: the myelinolytic tumor necrosis factor α is increased and neurotropic cytokines epidermal growth factor and interleukin 6 are decreased. Observations in animals and humans with inborn errors of vitamin B_{12} metabolism do not consistently support the sole role of any of these mechanisms. Also, variations in folate metabolism might interact to promote the nerve injury by inhibition of glycine N-methyltransferase.

2.5.3. Other symptoms and signs

Among the classic manifestations of vitamin B_{12} deficiency is Hunter's glossitis, which is characterized by atrophy of the lingual papillae, causing a smooth and sore tongue. Gastrointestinal symptoms probably related to the underlying gastric disorder include reduced appetite, weight loss, diarrhea and constipation and occur frequently. Vitamin B_{12} deficiency has also been reported to affect immune function by reducing the total lymphocyte count and the CD8+ cell count and by impairing natural killer cell activity (90). Vitamin B_{12} may be required for normal osteoblastic activity and this may increase the risk for osteoporosis. The adverse impact on skeletal health may be augmented by the osteoclast stimulating activity of tumor necrosis factor α which is increased in vitamin B_{12} deficiency (91, 92).

2.6. Epidemiology of vitamin B_{12} deficiency

Vitamin B_{12} deficiency was once considered a rare disease easy to diagnose because of the dramatic signs of megaloblastic anemia. At present it is known that vitamin B_{12} deficiency is common, but frequently presents with subtle manifestations, and its prevalence increases with age (1-5, 8, 93). The diagnosis is complicated by limitations of the laboratory methods and the lack of consensus regarding the diagnostic criteria.

Several studies have estimated the prevalence of vitamin B_{12} deficiency in the aged populations in the industrialized countries, but the results vary considerably depending on the diagnostic criteria used and the populations studied. The prevalence of subnormal vitamin B_{12} concentration ranges from 3.0% to 40.5% in studies with cut off limits for the serum concentration of vitamin B_{12} ranging from 103 pmol/l to 258 pmol/l (1). The limit of 150 pmol/l is frequently considered as the standard. In some studies increased concentrations of the metabolic markers (tHcy and MMA) were used singly, together, or in combination with a decreased total vitamin B_{12} concentration in the serum. The cut off limits for these markers differed also among studies which often focused on small and selected populations (e.g., hospital patients, patients with a particular disease or patients referred for vitamin B_{12} analysis) and this may have overestimated the prevalence of vitamin B_{12} deficieny in the general population.

Data from properly designed studies on the unselected general aged population is sparse. Clarke and co-workers (4) have estimated the age-specific prevalence of vitamin B_{12} deficiency in a population-based cross sectional analysis of 3511 subjects aged 65 years or older in three different studies. They defined a low vitamin B_{12} concentration as being below 150 pmol/l and metabolically significant vitamin B_{12} deficiency as a total vitamin B_{12} concentration of less than 200 pmol/l and total homocysteine of more than 20 μmol/l. Low vitamin B_{12} or metabolically significant vitamin B_{12} deficiency was found in about 5% of subjects aged 65-74 and in about 10% of subjects aged 75 or older. In an Australian study of 2901 subjects over 50 years, the serum vitamin B_{12} concentration was low (<185 pmol/l) in 22.9% of participants (94).

2.7. Laboratory diagnosis of vitamin B_{12} deficiency

The diagnostic approach to vitamin B_{12} deficiency includes, firstly, the demonstration that the deficiency exists and, secondly, the identification of the cause of the deficiency. The diagnosis of vitamin B_{12} deficiency has become complex since the discovery of the clinical impact of subtle deficiency (3, 8, 50, 93). Regardless of the long history of vitamin B_{12} research, there is still neither a generally accepted definition for vitamin B_{12} deficiency nor are there clear guidelines on the application of diagnostic tests. No single laboratory test is the gold standard of diagnosis. Impaired renal function impedes the use of many tests in the aged population. Furthermore, the development of the new, more sensitive tests has made it possible to diagnose vitamin B_{12} deficiency early, even before clinical manifestations. The diagnosis of vitamin B_{12} deficiency is an art of balancing between the risks of underdiagnosis and the inconvenience of overtreatment in case of overdiagnosis.

2.7.1. Hematology

Traditionally, vitamin B_{12} deficiency is suspected in subjects with macrocytic anemia, and therefore measuring hemoglobin concentration and mean cell volume (MCV) are considered as appropriate screening test for vitamin B_{12} deficiency. However, neurological and cognitive defects may occur in the absence of hematological signs. Many studies have shown that anemia and macrocytosis are often not present in subjects with vitamin B_{12} deficiency (8, 93, 95-98). Of note, all these studies included only subjects with suspected vitamin B_{12} deficiency, which may have increased the frequency of pathologic results compared to unselected populations. In a systematic review on the diagnostic value of increased MCV-values 23-84% of vitamin B_{12} deficiency patients would have been missed if only elevated MCV had been used for selecting subjects for further testing (98). The review included data from 37 studies, which were very heterogenic in their populations. Also definitions for vitamin B_{12} deficiency varied, but in all studies a combination of low serum total vitamin B_{12} and some other pathological laboratory finding was required. Most studies were biased due to selected population, which will lead to overestimation of the diagnostic value of MCV. Clearly, the automated complete blood cell count is not sufficient for screening for vitamin B_{12} deficiency.

The importance of examining the peripheral blood smear is often emphasized, but it is seldom used in clinical routine, probably because the procedure is not very familiar to clinicians generally. In vitamin B_{12} deficiciency, the erythrocytes are oval and enlarged. Schistocytosis (red cell fragmentation) and basophilic stippling may be present as signs of disturbed erythropoiesis. Hypersegmented neutrophils with five or more nuclear lobes are common due to disturbed granulopoiesis. In fact, neutrophil hypersegementation may be a more sensitive hematological sign of vitamin B_{12} deficiency than macrocytosis (99). However, neutrophil hypersegmentation is rarely assessed because the procedure is time-consuming and the assessment is subjective. The diagnostic value of neutrophil hypersegmentation has also been criticized. In a study of 169 patients who were examined for possible vitamin B_{12} deficiency only 6 had neutrophil hypersegmentation and there was no statistically significant difference in the frequency of neutrophil hypersegmentation between vitamin B_{12} deficient and non-deficient subjects (100).

Bone marrow examination may be necessary for differential diagnosis between conditions producing macrocytosis, but it is not always essential in the diagnosis of vitamin B_{12} deficiency (81, 101). Vitamin B_{12} deficiency causes a megaloblastic bone marrow, characterized by significant hypercellularity due to erythroid hyperplasia. Because of impaired DNA synthesis nuclear division and maturation is delayed, which leads to undeveloped nuclei with open, loose, chromatin, whereas the cytoplasmic maturation dependent on RNA synthesis is unaffected. Hence, there is asynchrony between maturation of the nucleus and cytoplasm leading to megaloblastic erythroblasts, giant metamyelocytes and hyperlobulated megakaryocytes. Intramedullary hemolysis is reflected by increased concentrations of lactate dehydrogenase and bilirubin in the plasma. Hemolysis is due to the destruction of defective erythroid cells.

2.7.2. Total vitamin B_{12}

Measurement of serum total vitamin B_{12} concentration is still the basis for diagnosing vitamin B_{12} deficiency. This variable has a long history, is widely available and is inexpensive (3, 22, 101-103). The original assays for measuring serum vitamin B_{12} were introduced in the 1950's and were based on growth requirements of certain microorganisms (e.g. *Euglena gracilis*) (22, 101). The growth of the microorganisms is directly proportional to the concentration of vitamin B_{12} in the test serum. These methods are time-consuming and labor intensive and have largely been abandoned and replaced by radiodilution assays and, more recently, by non-isotopic competitive protein binding assays. Vitamin B_{12} in plasma is bound to two different proteins (HC and TC) and is present in four different forms (methyl-, adenosyl-, hydroxyl-, and cyanocobalamin). These assays require extraction of the vitamin B_{12} from the proteins in an alkaline pH and conversion to cyanocobalamin, which is stable and used as the calibration standard. The binding protein that is used in modern assays is IF; HC has been abandoned because it binds also other corrins than vitamin B_{12}.

Several studies have questioned the sensitivity and specificity of the serum total vitamin B_{12} concentration for the diagnosis of vitamin B_{12} deficiency (3, 11, 81, 104-106). The interpretation of the assay results is affected by many circumstances. Serum total vitamin B_{12} does not directly reflect the vitamin B_{12} status in tissues: the major fraction of vitamin B_{12} is attached to HC and only about 20% is bound to TC and thus biologically active (36) and the vitamin B_{12} status in tissues may be inadequate even though the serum total vitamin B_{12} concentration is in the reference range. The cut off limit of 150 pmol/l is frequently considered as the standard and is derived from several studies, in which vitamin B_{12} deficient subjects defined by clinical and hematological criteria have been compared to healthy subjects. Since the discovery of the entity of subtle vitamin B_{12} deficiency, recommendations have been put forward to raise the cut off limit to 200-250 pmol/l or even to 258 pmol/l (11, 50, 105, 107-109).

Vitamin B_{12} deficiency may be masked by a normal or even an increased total vitamin B_{12} concentration in conditions that raise the HC concentration (81). In chronic myeloproliferative diseases the synthesis of HC is enhanced due to a high granulocyte count. Liver disease may cause increased HC concentrations and thus increase HC-bound vitamin B_{12} levels. Impaired renal function may also raise the total vitamin B_{12} concentrations in the serum (110) due to reduced filtration or increased release from renal stores. Patients with impaired renal function

might also require higher plasma concentration of vitamin B_{12} for metabolic demands (111).

On the other hand, a low serum total vitamin B_{12} concentration does not always indicate vitamin B_{12} deficiency. Vitamin B_{12} malabsorption may explain only about half of the measured low total vitamin B_{12} values. Metabolic markers of DNA-synthesis have been unaffected in 15-40% of subjects with low total vitamin B_{12} values (3, 106). This may be due to changes in the concentrations of vitamin B_{12} binding proteins and is the cause for low vitamin B_{12} values in pregnancy and multiple myeloma. Hereditary, mild haptocorrin deficiency may explain as much as 15% of the low total vitamin B_{12} concentrations (106) and subjects with folate deficiency have often low total vitamin B_{12} values (81). The mechanism of this phenomenon is not known, but the vitamin B_{12} concentration is restored with folate substitution. True vitamin B_{12} malabsorption due to damaged ileal epithelium in folate deficiency is possible.

2.7.3. Deoxyuridine suppression test

The deoxyuridine suppression test is a sensitive and specific indicator of both vitamin B_{12} and folate deficiency (3, 22, 112, 113). It is based on the requirement of tetrahydrofolate for the formation of thymidylic acid and DNA. In vitamin B_{12} and folate deficiencies there is lack of tetrahydrofolate because of impaired methionine synthase activity. In the test, a bone marrow sample from subject suspected for vitamin B_{12} is incubated with deoxyuridine and radioactively labeled thymidine. In the presence of vitamin B_{12} and folate, deoxyuridine is metabolized to thymidine and the incorporation of radio-labeled thymidine to DNA is suppressed. In vitamin B_{12} or folate deficiency suppression is reduced. Whether there is vitamin B_{12} or folate deficiency is tested by adding vitamin B_{12} or folate (112, 113). Although sensitive, the deoxyuridine suppression test is too labor intensive for routine laboratories and not in clinical use. Subtle vitamin B_{12} deficiency was established as a clinical entity with the deoxyuridine suppression test (114).

2.7.4. Homocysteine

Vitamin B_{12} and 5-methyl-tetrahydrofolate are required for conversion of homocysteine to methionine in the methionine synthase reaction. Homocysteine accumulates both in vitamin B_{12} and folate deficiency. Homocysteine is present in the plasma in several unstable forms (13). Over 80% is bound to albumin, about 15% is in disulphide form, and only about 1% is free homocysteine. Therefore, total homocysteine (tHcy) is measured after conversion of all species to the reduced form. Methods for tHcy measurement were introduced in the 1980's (115). There are two kinds of methods for tHcy measurement: chromatographic and enzyme-immunoassays (13, 116, 117).

Preanalytical factors may cause considerable variation in the tHcy concentrations (13, 115, 116). Fasting is not required for blood collection, but a large protein-rich meal may increase tHcy concentrations significantly as excess methionine is converted to homocysteine. As the major proportion of homocysteine is bound to albumin, tHcy concentrations are lower if the sample is taken when the subject lies supine. After blood collection, homocysteine is released from the erythrocytes. This can be prevented by keeping the samples cooled on the ice until centrifuged, which should be done within one hour. Consequently, tHcy should be measured in

plasma instead of serum. After removal of blood cells, tHcy in plasma is stable even in room temperature and tolerates well freezing and thawing.

Elevation of tHcy is a sensitive indicator of vitamin B_{12} deficiency (13, 104, 118). However, the specificity is poor because the concentration is increased by numerous other factors (13). Firstly, not only vitamin B_{12} deficiency but also folate and vitamin B_6 deficiencies raise tHcy (119). In renal impairment, increased tHcy is most likely attributable to decreased renal clearance, although an additional influence of extrarenal metabolic changes have been suggested (120, 121). Several studies (summarized in 13) have demonstrated that hyperhomocysteinemia occurs often in conditions like diabetes, hypothyroidism and several forms of cancer. Some drugs like methotrexate, anticonvulsants and biguanides also increase the tHcy concentration, mainly by affecting vitamin B_{12} and folate metabolism. Lifestyle factors like smoking, alcohol intake, and coffee consumption also affect the tHcy concentration (122, 123). There is an association between age and gender and tHcy: tHcy increases throughout the life and is higher in males than in females. The gender difference is approximately 2 µmol/l, but decreases in the aged (124). The most common genetic factor causing elevated tHcy is homozygosity (TT genotype) for the methylene tetrahydrofolate reductase (C677T) polymorphism (125, 126) whereas inborn errors affecting homocysteine metabolism are rare.

2.7.5. Methylmalonic acid

In vitamin B_{12} deficiency, methylmalonic acid (MMA) accumulates due to impaired methylmalonyl-CoA mutase activity. Increased serum MMA concentration is a sensitive and very specific marker of vitamin B_{12} deficiency (11, 101, 103, 109, 127). MMA may be increased by impaired renal function (12) and intestinal bacterial overgrowth (due to production of propionic acid, which is the precursor of MMA).

In 1962, Cox and White reported that high urinary excretion of MMA is a marker of vitamin B_{12} deficiency (128). However, the methods of measuring MMA (colorimetry and thin-layer or gas chromatography) were unreliable for diagnosing vitamin B_{12} deficiency. The first method for measuring the concentration of MMA in the serum was published by Marcell and co-workers in 1985 (129) who had developed a gas chromatography-mass spectrometry assay. This method is laborious and subsequently more accurate and convenient methods have been developed; still the methods are not suitable for routine laboratory use (130-133). MMA is stable in blood or plasma, and thus less vulnerable to preanalytical variations than tHcy (101), which would favor the development of easy laboratory testing of MMA.

2.7.6. Holotranscobalamin

Only the TC-bound fraction of vitamin B_{12}, holoTC, enters the cells and is biologically active (44). HoloTC reflects the immediate changes in Cbl status (134). The half-life of holoTC in the plasma is short, only about 60 minutes (135, 136). At least theoretically, the holoTC concentration could provide a reliable measure of the amount of vitamin B_{12} availability to tissues. Indeed, a decreased holoTC concentration has been promoted as the most sensitive and specific indicator of early vitamin B_{12} deficiency (14, 15, 19). Since holoTC is also the form in which absorbed vitamin B_{12} enters the circulation, holoTC has been proposed as a marker of

vitamin B_{12} absorption (137). Reconciling these two phenomena is problematic because, although frequently coexisting, they are not identical. The metabolic vitamin B_{12} status is the major determinant of the serum holoTC concentration, and absorption impacts it only slightly (138). Nevertheless, the metabolism of holoTC and the pathologic processes influencing it are not fully understood, and several mechanisms seem to be involved. The holoTC concentrations may be influenced by the uptake and release of holoTC by the liver (139) and kidneys (55, 58) and by increased turnover of holoTC due to increased tissue requirements of vitamin B_{12}.

Although several methods to quantify holoTC have been described since the discovery of TC in 1960's, unresolved methodological problems still remain. HoloTC measurement involves two stages: firstly, separation of total TC from total HC, and, secondly, quantification of vitamin B_{12} bound to TC. Both steps present a challenge for analytical chemistry.

Separation of TC from other proteins is seldom complete, since this step in the analysis of holoTC has usually been based on the differential absorption of the proteins on absorbents like microfine silica or glass particles (15, 19, 140, 141). Immunoabsorption (14, 142) and liquid chromatography (139) have also been used. The reliable quantification of the amount of vitamin B_{12} bound to TC provided another problem. Usually, holoTC has been measured indirectly as the difference in the vitamin B_{12} concentration before and after the removal of TC. This measurement has been unreliable due to imprecision of the vitamin B_{12} assay and to variable absorption of TC and HC by the different absorbents. Two direct methods for measurement of holoTC have been described (19, 143), but also these methods rely on the differential physicochemical absorption properties of TC and HC. In these methods, the holoTC concentration is measured directly in the precipitate and not as a numerical difference between two large values, total vitamin B_{12} and vitamin B_{12} bound to HC.

The availability of recombinant human transcobalamin and monoclonal antibodies has promoted the development of immunological methods for assessing holoTC. The first commercial method was based on solid phase absorption of TC to magnetic beads covered with anti-human TC antibodies and subsequent determination of vitamin B_{12} released from sequestered TC by RIA (20). The advantages of this method are that human recombinant holoTC is used as the standard and the concentration of the final sample which allows the use of conventional vitamin B_{12} assays. This assay requires a substantial amount of sample (400 µl of serum). A modification of this method to reduce the sample volume needed has been described; the method uses a microbiological assay for vitamin B_{12} determination (144). The other method, which employs anti-human TC antibodies, has a reverse approach (16). The apoproteins (TC and HC) are absorbed onto a solid phase covered with vitamin B_{12} and then the remaining holoTC is quantified with ELISA (145), which uses anti-human TC antibodies as the detecting antibody and the problematic measurement of vitamin B_{12} is entirely avoided.

2.7.7. Schilling test and protein-bound absorption test

The free vitamin B_{12} absorption test was introduced by Schilling in 1952 and has since been the cornerstone of diagnosing pernicious anemia (146). Unfortunately, it has also been used incorrectly to rule out vitamin B_{12} deficiency in subjects with low vitamin B_{12} concentrations due to other reasons. In the first stage of Schilling test, a dose of free vitamin B_{12} labeled with

radioactive cobalt is administered orally followed by a flushing dose (a parenteral injection of unlabelled vitamin B_{12} to saturate endogenous vitamin B_{12} binding proteins). If the subject is able to absorb free vitamin B_{12} the radioactive vitamin B_{12} is excreted in urine. In the Schilling test, urine is collected for 24 hours and the percentage of labeled vitamin B_{12} excreted is measured. In patients with pernicious anemia or impaired absorption of vitamin B_{12} from the gut the excretion of labeled vitamin is reduced. In the second stage of the Schilling test, labeled vitamin B_{12} is administered together with IF. In pernicious anemia absorption is increased whereas in intestinal malabsorption it remains reduced.

The Schilling test, despite its theoretical attraction, has several limitations, especially if used to study aged patients (81). Firstly, it is crucial to understand that the Schilling test measures only the absorption of free vitamin B_{12}. Therefore, the result is normal in food-vitamin B_{12} malabsorption, which is the most common cause of vitamin B_{12} deficiency. Inadequate urine collection is a frequent reason for unreliable results and the same is true, if the urine is contaminated with feces. Also, impaired renal function may delay excretion of the labeled vitamin B_{12}. The radioactive cobalt dose is negligible and does not limit the use of the test. However, the Schilling test is no longer readily available because labeled vitamin B_{12} and IF are difficult to obtain.

A modification of the traditional Schilling test has been developed to investigate food-vitamin B_{12} malabsorption (62). The protein-bound cobalamin was obtained by feeding chicken with labeled vitamin B_{12}. The eggs laid by these chicken were then used for the test that was otherwise similar to traditional Schilling test. Later on, several variations of the test have been introduced, but their use and utility are of minor importance.

2.7.8. Other tests

There is a variety of tests for assessment of autoantibodies and of gastrointestinal function that may be used to identify the cause of vitamin B_{12} deficiency, although they are not suitable for making a proper diagnose of vitamin B_{12} deficiency. Anti-parietal cell antibodies are present in about 85% of subjects with pernicious anemia (59, 81). However, they are nonspecific because they are frequently present also in healthy persons. IF antibodies are far more specific but are present only in about 50% of subjects with pernicious anemia. Serum gastrin and pepsinogen I concentrations or assessing the pepsinogen I – pepsinogen II ratio may be useful for diagnosing gastric atrophy and evaluating its location (22, 50, 81).

3. AIMS OF THE STUDY

The present study was undertaken to:

I evaluate the commercially available HoloTC RIA method (I, II) and determine the reference interval for the method (I)

II assess the prevalence of vitamin B_{12} deficiency in a Finnish aged population (IV)

III examine the risk factors and clinical correlates of vitamin B_{12} deficiency in the aged (IV, V)

IV assess the effect of renal function on vitamin B_{12}-related laboratory parameters (III)

V present an algorithm for optimal use of laboratory tests for diagnosing vitamin B_{12} deficiency (I, III, IV, V, VI)

4. SUBJECTS AND METHODS

4.1. Subjects

4.1.1. Lieto study population

Lieto is a rural district in the southwestern part of Finland, near the city of Turku. It has a population of 13000, with 12% aged (≥65 years). The Lieto Study is a population-based health study on unselected aged population living in this area (147). The data were collected in collaboration among the Department of Family Medicine of the University of Turku, the Department of Clinical Chemistry of Turku University Central Hospital and the municipal health center of Lieto. This study is based on the second phase of the Lieto Study, which was conducted from March 1998 to December of 1999. All residents born in 1933 or earlier living in Lieto on February 16th 1998 (n=1596, 666 men and 930 women, 42% and 58%, respectively) were invited to participate in the study. Of them, 63 died before data collection, 69 did not respond to the invitation, 190 refused, 4 had moved, and 10 could not be traced. A total of 1260 subjects (533 men and 727 women, 42% and 58%, respectively) participated in the interview. The participation rate was 82% (Figure 4.1). Because 5 subjects did not participate in the physical examinations and 6 neither in physical examinations nor laboratory testing, the total study population in this study was 1249 subjects aged 65-100 years (526 men and 723 women, 42% and 58%, respectively). The selection of different study populations from Lieto study population is summarized in Figure 4.1. A more detailed description about selection criteria is presented in Figure 1 in paper I and in Figure 1 in paper IV. The study designs are summarized in Table 4.3.

4.1.2. Assessment of the holoTC reference values for the aged

The healthy aged subjects were selected among the Lieto Study population (Figure 1 in paper I). The exclusion criteria are presented in Table 4.1. The remaining 226 participants (18% of the total Lieto Study population) constituted the healthy aged reference sample group (93 men and 133 women, 41% and 59%, respectively). They had a mean age of 71 years (range 65-88). The average number of drugs used was 2 (range 0-9) and the average number of previously diagnosed diseases in the medical records was 4 (range 0-12), (Table 4.2). The laboratory characteristics of the subjects in the reference sample group are summarized in Table 2 in paper I.

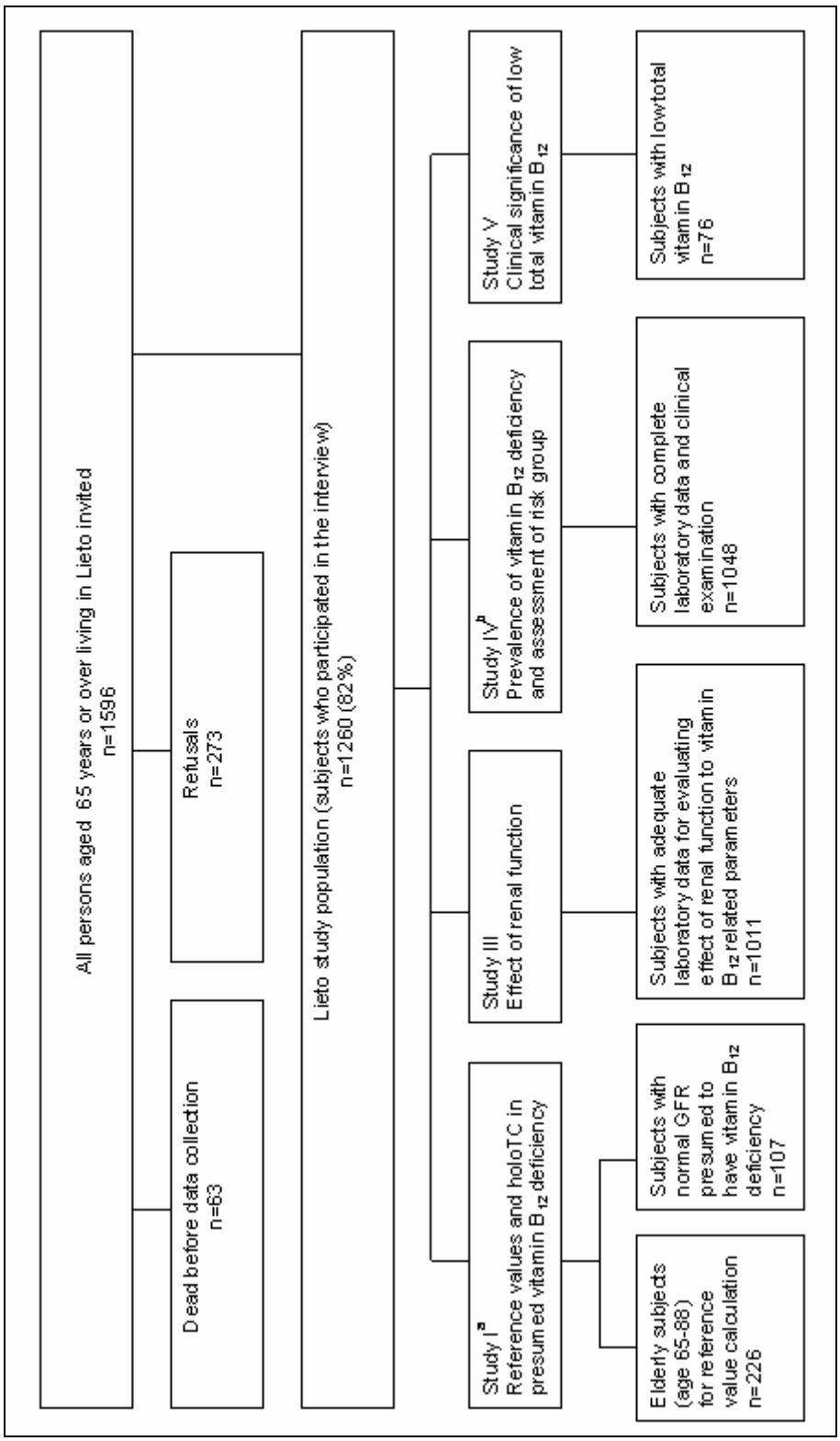

Figure 4.1. Selection of study populations.
[a] more detailed description in Figure 1 in paper I; [b] more detailed description in Figure 1 in paper IV; GFR, glomerular filtration rate.

Table 4.1. Exclusion criteria for the healthy aged reference sample group.

Previously diagnosed diseases (according the medical records)	
	Anemia
	Vitamin B_{12} or folate deficiency
	Cardiovascular disease
	Dementia
	Renal failure
	Gastric or intestinal diseases that may cause malabsorption
	Hepatic or pancreatic disorders
	Any autoimmune disease (except treated hypothyroidism)
	Type I diabetes
	Type II diabetes treated with oral medication or insulin
	Parkinson's disease
	Polyneuropathy
	Lymphoproliferative diseases
Medication affecting vitamin B_{12} or homocysteine metabolism	
	Proton-pump inhibitors (PPI)
	Antiepileptic drugs
	Peroral estrogen replacement therapy
	Methotrexate, triamterene, trimethoprim, sulfasalazine
Life style factors affecting vitamin B_{12} or homocysteine metabolism	
	Alcoholism
	Smoking
	Excessive coffee consumption (9 cups or more per day)
Laboratory markers of potential vitamin B_{12} deficiency	
	Hyperhomocysteinemia (plasma tHcy ≥15 μmol/l)
	Increased serum MMA (>0.28 μmol/l)
	No adequate samples for plasma tHcy measurement available

tHcy, total homocysteine; MMA, methylmalonic acid.

Table 4.2. Most common diagnoses and medications used among the aged who were included in reference sample group.

Diagnoses in the medical records	n (%)
Degenerative diseases of spinal column	88 (39%)
Arthrosis	73 (32%)
Essential hypertension	53 (23%)
Hypercholesterolemia (mainly treated with diet)	39 (17%)
Hearing impairment	38 (17%)
Depression	36 (16%)
No previous diseases	14 (6%)
Medication	
Non-steroidal anti-inflammatory drugs	75 (33%)
Antihypertensive drugs	44 (19%)
Sedatives	24 (11%)
No medication	51 (23%)

4.1.3. Assessment of reference values for adults

The subjects were healthy Finnish adult volunteers recruited among the laboratory personnel and their spouses in the Turku area. The participants had to meet the following criteria: regular Finnish diet, no vitamin B_{12} or folate-containing vitamin supplementation, no chronic diseases and no medications influencing vitamin B_{12}, folate or Hcy metabolism. Blood samples for serum total vitamin B_{12}, serum holoTC and the complete blood counts were collected in March and April 2001. Of the 84 volunteers enrolled, 1 subject was excluded because of vegetarian diet and 3 because of missing hematological laboratory values. The remaining 80 participants constituted the adult reference sample group (22 men and 58 women, 28% and 72%, respectively) who had a mean age of 45 years (range 22-62 years). Their laboratory characteristics are summarized in Table 2 in paper I.

4.1.4. Evaluation of holoTC in presumptive vitamin B_{12} deficiency

Presumptively, subjects with a markedly increased plasma tHcy concentration (≥19 μmol/l) or a low serum total vitamin B_{12} concentration (≤150 pmol/l) were considered most likely to have vitamin B_{12} deficiency among the Lieto study population (Figure 1 in paper I). There were 183 (15%) subjects who fulfilled one or both criteria. The serum MMA concentration was determined in these subjects to confirm vitamin B_{12} deficiency. To avoid the influence of impaired renal function on vitamin B_{12} related biochemical markers, 76 (42%) subjects with increased serum cystatin C concentration (≥1.3 mg/L) were excluded. There were finally 107 subjects in the study group.

4.1.5. Comparability of two versions of HoloTC RIA

A total of 107 hospital patients were recruited to give an additional serum sample for holoTC measurements in connection with routine morning blood sampling. The samples were collected in four days during May 2003. No other data were recorded.

4.1.6. Prevalence of vitamin B_{12} deficiency in the Finnish aged and risk groups for vitamin B_{12} deficiency

Of the total Lieto study population of 1249 subjects, 198 firstly examined were excluded because appropriate samples were not available for tHcy measurements. Because the examination date was randomized, the result is unlikely to be biased by the exclusion. In addition, 3 subjects were excluded because they did not have adequate serum samples for holoTC measurements. The study population consisted of 1048 subjects (age 65-100 years), which is 66% of the total aged population of the area (Figure 1 in paper IV). There was a slight predominance of women (438 men and 610 women, 42% and 58%, respectively), which is the same proportion as that of the total aged population in the area. The laboratory characteristics of the subjects are summarized in Table 1 in paper IV.

4.1.7. Clinical significance of low total vitamin B_{12}

Of the Lieto study population, all 76 subjects (age 65-95 years) with low serum total vitamin B_{12} (≤150 pmol/l) were included (Figure 1 in paper V). They were invited to a follow-up laboratory control in March 2000 after 9-24 months had passed from the baseline

measurements. By that time, 4 subjects had died, 9 did not attend and 63 (83%) participated. The laboratory control was considered as a treatment follow-up control for 16 subjects with parenteral vitamin B_{12} supplementation already started by a GP. The remaining 47 subjects were invited to attend for an additional clinical examination. Of them, 2 refused, 45 participated in a symptom interview, 40 attended for cognitive assessment and 36 for a clinical neurological examination. Also, 10 subjects with a low total vitamin B_{12} concentration (≤150 pmol/l) and anemia or macrocytosis (MCV ≥97 fl) in their laboratory control results were invited for a bone marrow aspiration and free vitamin B_{12} absorption test (the first stage of Schilling test).

4.1.8. Effect of impaired renal function on vitamin B_{12}-related laboratory values

Of the Lieto study population, 198 subjects were excluded because of missing plasma tHcy results, 3 because of missing serum holoTC results and 1 because of missing plasma creatinine result. MMA results were available only in subjects with increased tHcy or low or borderline holoTC (<54 pmol/l). This group included all subjects with a low total vitamin B_{12} concentration in their serum. 27 subjects receiving parenteral vitamin B_{12} substitution and 9 subjects with total vitamin B_{12} or holoTC values higher than the concentration of the highest calibrator (>1200 pmol/l for total vitamin B_{12} and >320 pmol/l for holoTC) were excluded. The study population thus consisted of 1011 subjects (418 men and 593 women, 41% and 59%, respectively) with a median age of 73 years (65-100 years).

4.2. Ethics

The Ethics Committee of the Hospital District of Southwest Finland approved the Lieto Study. All participants or their representatives gave written informed consent. Healthy volunteers in the non-aged adult reference group and hospital patients in the method comparison study gave oral consent; their samples were coded and processed anonymously throughout the study.

Table 4.3. Summary of study designs.

Objective of study	Population	N	Laboratory measurements	Other data
Study I				
Reference values for aged	Lieto study population	226	total B_{12}, holoTC, tHcy, MMA, Hb, MCV, creatinine, cysC	Lifestyle factors, medications, diagnoses in medical records
Reference values for adults	Healthy volunteers	80	total B_{12}, holoTC, blood count	Dietary habits, medications, vitamin supplementation
HoloTC in presumed vitamin B_{12} deficiency	Lieto study population	107	total B_{12}, holoTC, tHcy, MMA, cysC	None
Study II				
Comparability of two versions of HoloTC RIA	Hospital patients	107	holoTC assessed with both versions of HoloTC RIA	None
Study III				
Effect of renal function on vitamin B_{12} related laboratory values	Lieto study population	1011	total B_{12}, holoTC, tHcy, MMA, cysC, creatinine	None
Study IV				
Prevalence of vitamin B_{12} deficiency and risk group for vitamin B_{12} deficiency	Lieto study population	1048	total B_{12}, holoTC, ERC folate, tHcy, MMA, blood count, creatinine, cysC	Sociodemographic data, lifestyle factors, subjective health status, items on functional abilities, MMSE, medications, vitamin supplementation, diagnoses in medical records, physical examination
Study V				
Clinical significance of low total vitamin B_{12}	Lieto study population	76	Baseline: total B_{12}, holoTC, MMA, tHcy, ERC, folate, cystatin C, blood count Follow-up up: total B_{12}, tHcy, blood count, neutrophil hypersegmentation, bone marrow aspiration, free vitamin B_{12} absorption test for 10 subjects	Baseline: cognitive assessment (MMSE) Follow-up: symptom interview, cognitive assessment (MMSE), clinical examination with neurological assessment

total B_{12}, total vitamin B_{12}; holoTC, holotranscobalamin; tHcy, total homocysteine; MMA, methylmalonic acid; cysC, cystatin C; Hb, hemoglobin; MCV, mean cell volume; ERC, erythrocyte; MMSE, Mini Mental State Examination

4.3. Methods

4.3.1. Laboratory methods

Venous blood samples were drawn after the subject had fasted overnight, with light proximal compression of the vein and the subject in the sitting position. Sample types, handling, storage and laboratory methods are summarized in Table 4.4.

4.3.2. Serum holoTC

Serum holoTC was measured directly with a commercial radioimmunoassay (HoloTC RIA kit, Axis Shield ASA, Oslo, Norway). For the assay, 400 µl of serum was diluted with 400 µl of 0.1 mol/l phosphate-buffered saline, and magnetic microspheres coated with anti-transcobalamin antibodies were added. Samples were incubated for one hour on a roller mixer at room temperature. Then, holoTC absorbed to solid phase was precipitated on a magnetic rack, the supernatant was discarded, and the precipitate was washed with phosphate-buffered saline. The holoTC concentration was determined with a radioimmunoassay standardized with recombinant human holoTC using intrinsic factor as a binder and $^{57}CoB_{12}$ as a tracer. The calibrators that were used to construct the standard curve were processed identically to the samples. All samples were analyzed in duplicate.

During this study, the company made some changes in the method to increase its sensitivity and precision at low concentrations. In the new version of the of HoloTC RIA kit, the amount of solution with magnetic microspheres coated with anti-transcobalamin was increased from 40 µl in the first version (20) to 50 µl. The anti-transcobalamin antibody was identical to that in the first version. The coupling procedure (to the magnetic microspheres) was performed by Axis-Shield, which was not done with the first version. A new calibrator with a concentration of 10 pmol/l was added. The highest calibrator with a concentration of 320 pmol/l was left out. The volumes of sample and calibrator and the incubation times were not changed.

4.3.3. Criteria for abnormal laboratory test results

The criteria of abnormal laboratory test results are presented in Table 4.5.

Table 4.4. Laboratory methods.

Analyte	Method	CV%	Mean concentration	Storage temperature and duration
Blood count	Advia 120 hematology analyzer, Bayer Corporation, New York, USA			Room temperature, maximum 4 hours
Serum[a] total vitamin B_{12}	Competitive protein binding assay, AutoDelfia, Wallac, Turku, Finland	2%	238 pmol/l	Lieto study -20°C, 1.5 years Lieto follow-up samples +4°C, 1-5 days Adult reference group +4°C, 1-5 days
Serum[a] holoTC	Radioimmunoassay, HoloTC RIA, Axis Shield ASA, Oslo, Norway			Lieto study -70°C, 2-3 years
First version		6%	38 pmol/l	Adult reference group -20°C, 1-3 months Hospital patients +4°C, maximum 4 hours
New version		7%	34 pmol/l	
Plasma[b] tHcy	Fluorescence polarization assay, IMx system, Abbott Laboratories, Abbot Park, IL, USA	2%	13 μmol/l	Lieto study -70°C, 1-1.5 years Lieto follow-up samples -70°C, 2 years
Serum[a] MMA	Stable-isotope-dilution capillary gas chromatography-mass spectrometry	6%	0.38 μmol/l	-20°C, 2-3 years
ERC folate	Competitive protein binding assay, AutoDelfia, Wallac, Turku, Finland			+4°C, 1 week,
Plasma[c] creatinine	Jaffe's method, Hitachi 917 Automatic Analyzer, Roche Diagnostics GmbH, Mannheim, Germany	2%	118 μmol/l	Room temperature, maximum 4 hours,
Serum[a] cysC	Immunonephelometric assay, Behring nephelometer, Dade Behring, Marburg, Germany	3%	1.7 mg/l	-20°C, 2-3 years

[a] Allowed to clot at room temperature, centrifuged 2110 x g, 10 min, within one hour; [b] heparin anticoagulated sample, placed instantly on ice, centrifuged 2110 x g, 10 min, +4°C, within 1 hour; [c]heparin anticoagulated sample, centrifuged 2110 x g, 10 min, +4°C, within 1 hour; holoTC, holotranscobalamin; tHcy, total homocysteine; MMA, methylmalonic acid; ERC, erythrocyte; cysC, cystatin C; CV% coefficient of variation.

Table 4.5. Criteria for abnormal laboratory test results

Variable	Definition
Low total vitamin B_{12}	total vitamin B_{12} <150 pmol/l
Borderline total vitamin B_{12}	total vitamin B_{12} 150-250 pmol/l
Low holoTC	holoTC ≤37 pmol/l
Marginally increased tHcy	≥15 μmol/l
Hyperhomocysteinemia	tHcy 20 μmol/l
Elevated MMA	>0.28 μmol/l
Low ERC folate	ERC folate <320 nmol/l
Potential vitamin B_{12} deficiency	total vitamin B_{12} ≤150 pmol/l or tHcy ≥19 μmol/l
Possible vitamin B_{12} deficiency	total vitamin B_{12} ≤150 pmol/l and either tHcy ≥19 μmol/l or MMA ≥0.45 μmol/l
Probable vitamin B_{12} deficiency	tHcy ≥19 μmol/l and MMA ≥0.45 μmol/l
Laboratory diagnosis of vitamin B_{12} deficiency in Study IV	total vitamin B_{12} <150 pmol/l or total vitamin B_{12} 150-250 pmol/l together with holoTC ≤37 pmol/l and tHcy ≥15 μmol/l
Anemia	Men Hb <134 g/l; women Hb <117 g/l
Macrocytosis	MCV >98 fl
Neutrophil hypersegmentation	five or more neutrophils with six or more lobes per 100
Pathological neutrophil lobularity index (total number of nuclear lobes per 100 neutrophils divided by 100)	>3.25
Increased plasma creatinine	Men >135 μmol/l Women >125 μmol/l
Increased serum cysC, impaired GFR	>1.3 mg/l for age 65-74, >1.45 mg/l for age 75-84 and >1.62 mg/l for age ≥85
Impaired GFR in study I	cysC >1.3 mg/l
Impaired GFR in study III	cysC in the highest quartile (>1.16 mg/l)
Pathological free vitamin B_{12} absorption test	Urinary excretion of <10% of the administered dose of $^{57}CoB_{12}$

holoTC, holotranscobalamin; tHcy, total homocysteine; MMA, methylmalonic acid; ERC, erythrocyte; Hb, hemoglobin; MCV, mean cell volume; cysC, cystatin C; GFR, glomerular filtration rate.

4.3.4. Interviews in the Lieto study

Two trained nurses interviewed all subjects in the second phase of the Lieto study in the health center of Lieto. Demographic data, subjective health status, items on functional abilities, lifestyle factors, medication and vitamin supplementation were recorded. The Mini-Mental State Examination (MMSE) was administered and the Zung Self-Rating Depression Scale was applied. During the follow-up visit for subjects who had a low serum total vitamin B_{12} concentration, information on symptoms was obtained by a short structured interview and the MMSE was administered.

4.3.5. Physical examinations in Lieto study

The laboratory personnel in the health center of Lieto recorded a 12-lead resting

electrocardiography (ECG) and draw the venous blood samples. Trained nurses measured the height and weight and screened the vision and hearing of the subjects. One of the two research physicians, both experienced GPs, performed the physical examination and registered the subject's diseases as stated in the medical records. At the follow-up visit, the clinical examination focused on the presence of peripheral neuropathy.

4.3.6. Clinical outcome measures

The Activities of Daily Living (ADL) were assessed using a 5-item questionnaire (dressing, eating, bathing, going to bed and toileting) and the Instrumental Activities of Daily Living (IADL) with a 9-item questionnaire (using public transportation, using a phone, shopping, handling finances, cooking, managing medication, cutting toe nails, light and heavy housework). Mobility was evaluated in terms of the capability to walk outdoors, between rooms, up and down stairs or at least 400 m. Each item was scored as either 0 (unable to do or help needed) or 1 (with difficulty, but no help needed or no limitations). Sum scores of less than 5 in ADL, less than 9 in IADL and less than 4 in the mobility index were considered to indicate independence. Cut-off limits of 23 or less for dementia in the MMSE and 45 or above for depression in the Zung Self-Rating Depression Scale were used. ECG findings were interpreted using the Minnesota Code.

For dementia and depression, the DSM-IV criteria were used. Coronary heart disease (CHD) was diagnosed if the person had a diagnosis of ischemic heart disease in the medical records, and/or had a history of coronary by-pass surgery or angioplasty, and/or was entitled to reimbursements from the Finnish National Health Insurance for CHD medication, and/or had ischemia or infarction according to an ECG (Minnesota codes 1.1-1.3, 4.1-4.4, 5.1-5.3 or 7.1). Stroke was diagnosed if the person had the diagnosis in the medical records, and/or a subjective history of stroke with neurological symptoms persisting for more than 24 hours, verified by the clinical examination. The criteria for hypothyroidism were a diagnosis of hypothyroidism in the medical records, and/or treatment with thyroxin, and/or a serum free thyroxin level of less than 9.6 pmol/l.

Symptoms from the interview in the follow-up control were classified as anemic (palpitations, short of breath), gastrointestinal (sore mouth or tongue, reduced appetite, weight loss, gastrointestinal pain or disorders, presence of tongue atrophy), neurological (muscle weakness, unsteadiness in walking, paresthesias, sensory loss), cognitive or affective (memory loss, sleeping disorders, depressive symptoms) and unspecific (tinnitus, visual disturbances, muscle cramps, increased susceptibility to infections or bruising and tremor). Assessment of peripheral neuropathy is outlined in Table 4.6.

Table 4.6. Peripheral neuropathy assessment at follow-up control.

Variable	Test	Interpretation
Cutaneous sensation	Semmes-Weinstein monofilaments	Decreased sensation if inability to feel a monofilament 5.07 (10 g)
Vibration sensation	Portable vibrameter	Impaired vibration sensation if threshold ≥15V
Balance	Romberg test	Impaired if compensatory movements or inability to finish the test
Balance	Tandem walk	Impaired if stepping off the line or taking steps with the heel and toe visibly separated
Position sense	Three positions at first toe	Impaired if any of the three positions were not correct
Muscle force	Dorsiflexion of foot, extension of knee joint, flexion of elbow and scissor movement of fingers	Rated as normal or decreased
Tendon reflexes	Finger flexor, brachioradialis, patellar and Achilles tendon reflexes	Rated as normal if normal or increased and impaired if absent or decreased

4.4. Statistical analysis

Imprecision results are presented as within-run and between-run coefficients of variation (CV), mean concentration and standard deviation (SD). Differences between groups were tested with Kruskal-Wallis's nonparametric test for quantitative data and with the χ^2 test for categorical data. The reference intervals for holoTC were calculated after log-transformation because the holoTC values were positively skewed, using a parametric method, and presented as the 95% central interval with 95% confidence limits. Spearman's rank-order correlation coefficients were calculated. Because there was a significant deviation from linearity ($p<0.05$), non-parametric Passing-Bablok regression was used for comparison between the two versions of HoloTC RIA. Logistic regression was used to estimate odds ratios (OR) and their 95% confidence limits (CI) for the associations between vitamin B_{12} deficiency and selected clinical outcome measures. P values <0.05 were considered as significant. Data were analyzed using Medcalc software version 8.1 (MedCalc SoftWare, Mariakerke, Belgium) and the SAS software version 8.2 (SAS Institute Inc., Cary, NC, USA).

5. RESULTS

5.1. Method evaluation of HoloTC RIA

5.1.1. Precision

The within-assay imprecision was assessed by measurements of 10 replicates of low and high controls in the same assay. In addition, an intra-assay precision profile was produced based on 138 clinical samples analyzed in duplicates. The between-assay imprecision was calculated from 8-20 repeated measurements of high and low controls and 18-26 repeated measurements of two pooled patient samples. The within-assay imprecision (CV) for HoloTC RIA varied from 4% to 7% and the between-assay imprecision from 5% to 32%, and increased towards lower concentrations. The results are presented in detail in Table 5.1.

Table 5.1. HoloTC RIA imprecision.

Sample type	n	Mean ± SD,[a] pmol/l	CV%
Within-assay imprecision			
Low control	10	36 ± 2	4
High control	10	110 ± 8	7
Patient samples[b]	138	82 ± 40	8[c]
Between-assay imprecision			
Low control, first version, method evaluation study	20	38 ± 2	6
Low control, first version, comparison study	8	36 ± 2	7
Low control, new version, comparison study	8	34 ± 2	7
High control, first version, method evaluation study	20	107 ± 9	8
High control, first version, comparison study	8	94 ± 6	6
High control, new version, comparison study	8	88 ± 5	5
Low patient sample, first version	26	25 ± 4	14
Very low patient sample, first version	18	5 ± 2	32

[a] mean concentration ± standard deviation; [b] within reportable range 20-320 pmol/l; [c] calculated from duplicate analyses of patient samples, SD for duplicates was 7 pmol/l.

5.1.2. Comparability of two versions of HoloTC RIA

The method evaluation and reference value calculation were made with the first version of the HoloTC RIA. Later, the company increased the amount of solution with magnetic microspheres coated with anti-transcobalamin, started to perform the coating procedure and added a new

calibrator. Therefore, to study the comparability between the two version of the assay, holoTC concentrations in serum samples from 107 hospital patients were analyzed in four assay batches with both versions. After exclusion of 24 samples with concentration over 160 pmol/l to avoid the need for dilution and one sample as a between method outlier, a total of 82 samples were used for calculations. The two versions of the method presented with good agreement: r=0.974; slope 1.0131 (95% CI 0.9580, 1.0691); intercept 0.4683 (95% CI -3.0842, 4.2761), Figure 5.1. The slopes of the standard curves were similar, and shifts in binding were minimal between the new version and the first version: 0.4 pmol/l at 50% binding and 0.9 pmol/l and at 80% binding.

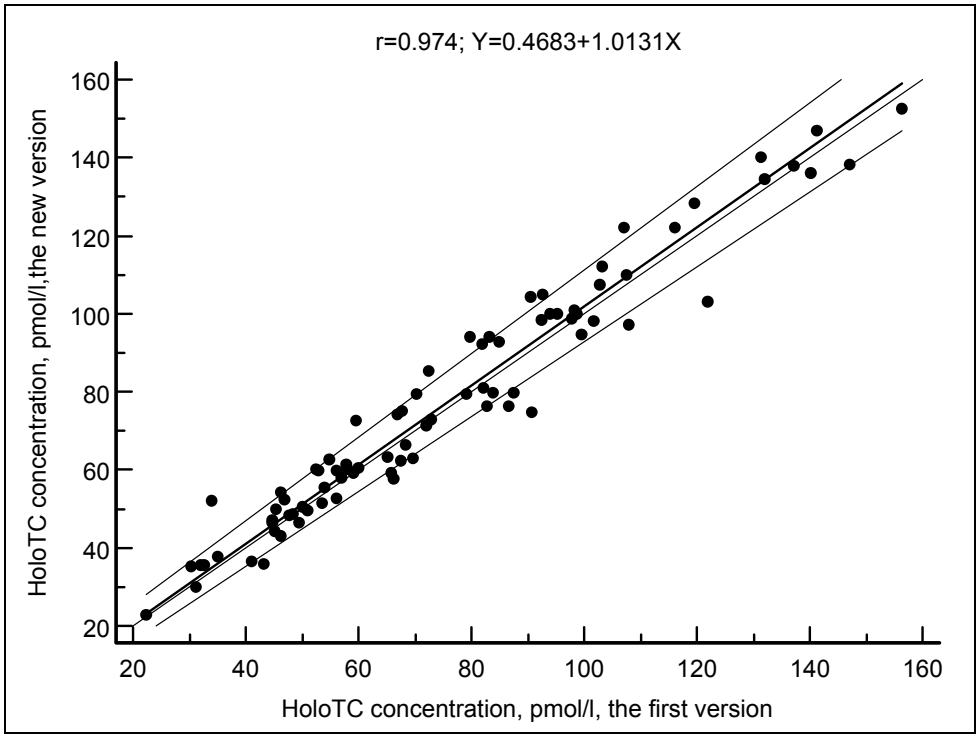

Figure 5.1. Passing-Bablok regression plot showing good agreement between the first and the new version. HoloTC concentration measured with the first version x-axis and of the new version on the y-axis.

5.1.3. Stability of HoloTC during storage

The stability of holoTC during storage was tested with 9 samples containing holoTC within the reportable range (range 36-210 pmol/l). The samples were stored at -70°C for 7-16 months. There was no statistically significant difference between the initial concentrations and those measured after storage at -70°C (mean of differences 4 pmol/l, p=0.56). 33 samples were stored for 4 months at -20°C. There was no statistically significant difference between the initial concentrations and those measured after storage (mean of differences 10 pmol/l, p=0.43).

5.1.4. Reference interval for HoloTC RIA for the Finnish population

There was no significant difference in holoTC concentrations between ages below and above 65 years (mean 84 pmol/l vs. 87 pmol/l, p=0.35) but holoTC concentrations were significantly higher in women than in men (mean 90 vs. 79 pmol/l p=0.02). However, at concentrations ≤100 pmol/l there was no significant difference between men and women. Therefore, the groups were combined for the determination of the reference interval instead of calculating age- or gender-specific reference values. The characteristics of the subjects in the whole reference sample group and in different subgroups are presented in paper I (Table 2). The 95% central reference interval calculated from the reference sample group of 303 subjects was 37-171 pmol/l. The 95% confidence interval for the lower reference limit was 35-38 and for the upper reference limit 164-179 pmol/l.

5.2. Epidemiology of vitamin B_{12} deficiency in the Finnish aged population

5.2.1. Prevalence of vitamin B_{12} deficiency

In the Lieto study population, 2.6% of the subjects were on parenteral vitamin B_{12} substitution therapy and were considered to have previous diagnosis of vitamin B_{12} deficiency. Low total vitamin B_{12} (<150 pmol/l) was found in 6% of the population. A new diagnosis of vitamin B_{12} deficiency was defined as low total vitamin B_{12} (<150 pmol/l) or borderline total vitamin B_{12} (150-250 pmol/l) and low holoTC (≤37 pmol/l) together with increased tHcy (≥15 μmol/l). This was present in 9.5% of subjects. Taken together, the prevalence of vitamin B_{12} deficiency was thus 12%. Vitamin B_{12} deficiency was more common in men than women (12% vs. 7.4%, p=0.018) and 78% of the subjects with vitamin B_{12} deficiency had not previously been diagnosed for the condition.

5.2.2. Distributions and correlations of vitamin B_{12} related laboratory variables

The median concentrations and range and frequencies of pathological results of vitamin B_{12} related laboratory parameters in the whole aged population and in men and women are presented in Table 1 in paper I and the correlations between vitamin B_{12} related laboratory parameters in Table 5.2.

Table 5.2. Correlations between vitamin B_{12}-related parameters. Spearman rank order correlation coefficients and p-values are presented.

	total vitamin B_{12}	holoTC	tHcy	MMA
holoTC[a]	0.78			
	<0.001*			
tHcy[a]	-0.32	-0.32		
	<0.001*	<0.001*		
MMA[b]	-0.28	-0.24	0.24	
	<0.001*	<0.001*	<0.001*	
ERC folate[a]	0.30	0.33	-0.39	-0.04
	<0.001*	<0.001*	<0.001*	0.401
Hb[a]	-0.003	-0.02	-0.10	-0.15
	0.914	0.584	0.001*	0.004*
MCV[a]	-0.06	-0.01	0.03	0.02
	0.053	0.694	0.294	0.741
Creatinine[c]	-0.05	-0.02	0.41	0.15
	0.117	0.622	<0.001*	0.006*
cysC[c]	-0.06	0.01	0.53	0.27
	0.082	0.656	<0.001*	0.001*

[a] n=1021; [b] n=359; [c] n=1011； holoTC, holotranscobalamin; tHcy, total homocysteine; MMA, methyl-malonic acid; Hb, hemoglobin; MCV, mean cell volume; ERC, erythrocyte; cysC, cystatin C. * p<0.05

5.3. Clinical signs and symptoms related to vitamin B_{12} deficiency

5.3.1. Hematological and biochemical signs

The frequencies of pathological results in vitamin B_{12} related laboratory variables in subjects with low or borderline total vitamin B_{12}, previously diagnosed vitamin B_{12} deficiency and new laboratory diagnosis of vitamin B_{12} deficiency are presented in Table 5.3.

Table 5.3. Frequencies of pathological results in vitamin B_{12} related laboratory variables.

	Low total B_{12} n=62	Borderline total B_{12} n=324	Previously diagnosed B_{12} deficiency n=27	New laboratory diagnosis of B_{12} deficiency n=97
Low holoTC	77%	17%	0%	86%
Marginally increased tHcy	74%	41%	37%	84%
Hyperhomocysteinemia	42%	18%	7%	48%
Elevated MMA	58%	.	.	54%
Low ERC folate	45%	30%	30%	47%
Anemia	18%	16%	26%	22%
Macrocytosis	11%	5.6%	11%	8%
Macrocytic anemia	1.6%	0.6%	4%	2%

holoTC, holotranscobalamin; tHcy, total homocysteine; MMA, methylmalonic acid; ERC, erythrocyte.

5.3.2. Neurological signs and reported symptoms

Some neurological finding was evident in 81% of the subjects with low total vitamin B_{12} who were investigated. Of the subjects who were interviewed, 76% reported unspecific, 73% cognitive, 62% neurological and 42% gastrointestinal symptoms. Only 2 subjects did not report any symptoms. Frequencies of the symptoms and signs are presented in detail in tables 1 and 2 in paper V.

5.3.3. Effect of vitamin B_{12} supplementation

Follow-up samples were drawn 9-24 months after baseline measurements of 63 subjects with low total vitamin B_{12}. Sixteen of these subjects had received parenteral vitamin B_{12} supplementation and 47 not. At follow-up, the tHcy concentrations of the subjects who had received vitamin B_{12} supplementation had significantly decreased ($p<0.001$) but were unchanged in subjects without supplementation ($p=0.923$). The hematological parameters had deteriorated in the majority of the subjects without vitamin B_{12} supplementation but in the group that had received supplementation there were no significant changes. The results of MMSE-testing at baseline and at follow-up were compared for 40 subjects who had not received vitamin B_{12} supplementation, and the result was poorer at control than at baseline in 21, unchanged in 13, and better in 6 subjects ($p=0.003$).

5.4. Assessment of risk groups for vitamin B_{12} deficiency

5.4.1. Sociodemographic variables and lifestyle factors

Subjects with a previous diagnosis of vitamin B_{12} deficiency made by a GP before the Lieto study were significantly older than the remaining study population (median age 81 vs. 73 years, $p<0.001$). After adjustment for age and gender, living in a long-term care institution, dependency regarding the capability of performing ADL and use of medications were more common among subjects with a previous diagnosis of vitamin B_{12} deficiency than among the remaining study population. When subjects with a new diagnosis of vitamin B_{12} deficiency (based on laboratory results) were compared with subjects with a normal vitamin B_{12} status, male gender (OR 1.9, 95% CI 1.2-2.9), age $\geq$75 (OR 2.2, 95% CI 1.4-3.4) and refraining from milk products (OR 2.3, 95% CI 1.2-4.4) increased the probability of vitamin B_{12} deficiency.

5.4.2. Predisposing diseases and clinical associations

Subjects with a previous diagnosis of vitamin B_{12} deficiency had more frequently a diagnosis of dementia (26% vs. 8.2%, $p=0.001$) and gastritis (19% vs. 5.8%, $p=0.006$) than those who did not have a previous diagnosis of vitamin B_{12} deficiency. Diverticulosis increased slightly but significantly the probability of a new diagnosis of vitamin B_{12} deficiency (OR 1.8, 95% CI 1.0-3.2). Anemia (OR 1.3, 95% CI 0.7-2.3) and macrocytosis (OR 1.2, 95% CI 0.6-2.7) did not predict vitamin B_{12} deficiency. Among subjects with low total vitamin B_{12} and normal renal function, there were no significant differences in the frequencies of reported symptoms or neurological or hematological signs between subjects with increased and normal MMA concentration in the serum.

5.5. Laboratory diagnosis of vitamin B_{12} deficiency

5.5.1. HoloTC in vitamin B_{12} deficiency

The holoTC concentration was low (≤37 pmol/l) in 77% of subjects with a low total vitamin B_{12}, in 22% of subjects with slightly increased tHcy (≥15 μmol/l), in 34% of subjects with markedly increased tHcy (≥20 μmol/l) and in 45% of subjects with increased MMA (>0.28 μmol/l). In 48 % of the subjects with normal renal function and either a tHcy concentration ≥19 μmol/l or a total vitamin B_{12} concentration ≤150 pmol/l, holoTC was low. When the likelihood of vitamin B_{12} deficiency among them was graded as potential, possible or probable, the frequencies of low holoTC in these groups were 23 of 77 (30%), 12 of 14 (86%) and 16 of 16 (100%), respectively (Figure 5.3, figure 3 in paper I). In the probable vitamin B_{12} deficiency group serum total vitamin B_{12} was low (≤150 pmol/l) in 81% of subjects and all had a low holoTC. The difference was not statistically significant ($p>0.05$). In the potential vitamin B_{12} deficiency group 10 subjects of 23 (43%) with normal total vitamin B_{12}, had both a low holoTC and a high tHcy.

5.5.2. Effect of renal impairment on vitamin B_{12} related biochemical variables

Both cystatin C and plasma creatinine correlated strongly with tHcy (0.53, $p<0.001$ and 0.41, $p<0.001$) and MMA (0.27, $p<0.001$, 0.15, $p=0.006$) but not with total vitamin B_{12} (-0.05, $p=0.117$ and -0.06, $p=0.082$) or holoTC (-0.02, $p=0.622$ and 0.01, $p=0.656$). The median concentrations of tHcy and MMA and the frequencies of pathological values were significantly higher in the highest quartile of cystatin C but there was no significant difference in total vitamin B_{12} or holoTC (Table 1 in paper II).

6. DISCUSSION

6.1. HoloTC measurement

6.1.1. HoloTC RIA method evaluation

A reduced concentration of holoTC in the serum has been offered as the earliest and most specific marker of tissue vitamin B_{12} deficiency (14, 15, 19), but methods suitable for the routine measurement of holoTC have been unavailable, until recently. In this study, the first commercial holoTC method, HoloTC RIA was evaluated. The method was found to be precise and not to require special equipment, which makes it suitable for routine laboratory use. This agrees with the report of Ulleland and co-workers (20) who published a thorough method evaluation.

The method is, nevertheless, cumbersome and requires multiple steps and incubations; the total turnaround time becomes long and one assay requires no less than one whole working day. The precision of the first version of HoloTC RIA is good for a manual method within the reference range, but decreases at lower concentrations; it is acceptable in the region of the lowest calibrator (20 pmol/l) but poor at lower concentrations. Therefore, duplicate measurement of holoTC in routine work is appropriate. To extend the reportable range to lower concentrations, technical improvements were made in the new version of the HoloTC RIA. Firstly, the amount of solution with magnetic microspheres coated with anti-transcobalamin was increased. Secondly, a new calibrator with concentration of 10 pmol/l was added. Thirdly, the company now coats the microspheres itself. The transferability between the first and the new version of HoloTC RIA is good and the lower limit of reference interval (37 pmol/l) of the first version of the HoloTC RIA is now validated for the new version, as well.

6.1.2. Reference interval for HoloTC RIA in the Finnish population

The central 95% reference interval for serum holoTC obtained in this study (37-171 pmol/l) is in agreement with the values previously obtained by methods separating TC from haptocorrin (14, 19), and also by a the ELISA method, in which the transcobalamin moiety is measured rather than the vitamin B_{12} moiety (16). Unexpectedly, however, Ulleland and co-workers report lower reference interval with the same method (24-157 pmol/l) than the present study (20). The discrepancy may arise from a different selection of reference sample group. Because Ulleland and co-workers described their reference sample group as being apparently healthy with no further specifications, subjects with undiagnosed vitamin B_{12} deficiency may have been included by them, which, naturally, would lower the lower reference limit. The reference sample group in the present study was selected carefully and individuals with any conditions or medications that might affect vitamin B_{12} status were carfully excluded, as were subjects that had potential vitamin B_{12} deficiency. This was done by assessment of tHcy and MMA – if the concentrations of these variables were elevated, the subjects were excluded as being potentially

vitamin B_{12} deficient. Furthermore, the reference sample group consisted of 303 subjects in the present study, in comparison to the 105 subjects in the study of Ulleland and co-workers – a circumstance that further confirms the reliability of the reference limits. Indeed, the narrow confidence interval (35-38 pmol/l) obtained for the lower reference limit (37 pmol/l) is a valid indicator of a reliable result. Recently, a similar reference interval (42-157 pmol/l) calculated from a reference sample group of 500 subjects aged 18-69 years was reported. The authors used a similar technique to separate holoTC with magnetic particles coated with anti-TC-antibody but a microbiological assay instead of RIA for measurement of vitamin B_{12} attached to TC (144).

6.2. Epidemiology of vitamin B_{12} deficiency in the Finnish aged population

6.2.1. Prevalence of vitamin B_{12} deficiency

The prevalence of vitamin B_{12} deficiency was 12% in this representative sample of an aged Finnish population. This frequency figure is in accordance with previously reported prevalence rates in other western countries (1, 4, 5). The proportion of previously undiagnosed vitamin B_{12} deficiency was surprisingly large, 78%. Only 2.6% of subjects in this study population were receiving vitamin B_{12} substitution. The proportion in similar aged populations is much greater, e.g., 15.6% in Sweden (2) and equals the prevalence of vitamin B_{12} deficiency. This suggests that vitamin B_{12} deficiency is much underdiagnosed in the Finnish aged population. Furthermore, subjects with previously diagnosed vitamin B_{12} deficiency were older, more often residents in institutions, demented and dependent on outside help in their daily living than other subjects. This indicates that diagnosis is delayed and irreversible damage occurs in spite of sufficient vitamin B_{12} substitution, which was demonstrated by normal tHcy values. Age did not explain the differences.

6.2.2. Associations between vitamin B_{12} status and demographic variables, life style factors and functional abilities

Aging is a substantial risk factor for vitamin B_{12} deficiency (3, 5, 7). Like in many studies (2, 5, 134), also in this study total vitamin B_{12} or holoTC concentrations did not correlate with age, whereas tHcy did. However, the risk of vitamin B_{12} deficiency was doubled in subjects aged $\geq$75 years compared to subjects aged <75 years. The same has been shown by others (4). Men had a twofold risk for vitamin B_{12} deficiency compared to women. Previous studies have reported similar findings: vitamin B_{12} concentrations have been higher in women than in men (2, 119) and vitamin B_{12} deficiency has been more common in men than in women (4, 119, 148).

After excluding subjects receiving vitamin B_{12} substitution, no associations were found with vitamin B_{12} deficiency and poor subjective health state, poor general sense of contentment or impaired functional abilities. Also certain demographic variables (being unmarried, divorced or

widowed, living alone, being resident in an institution) might have been expected to be related with vitamin B_{12} deficiency, but there was no association. Nor were smoking, alcoholism or consuming a vegetarian diet associated with vitamin B_{12} deficiency in this aged population. These factors have been linked with an increased risk of vitamin B_{12} deficiency in younger adults (5, 74), but these relationships have not been previously investigated in aged populations. Milk product use is the rule rather than an exception among the Finnish aged, and it correlated positively with total vitamin B_{12} concentrations. Refraining from milk products doubled the probability for vitamin B_{12} deficiency.

6.2.3. Associations between cognitive function and vitamin B_{12} status

Many previous cross-sectional studies have analyzed the associations between cognitive function and vitamin B_{12} status (6, 86, 149-153). The results have varied considerably and the relation remains still controversial, partly because various cognitive function tests and measures of vitamin B_{12} deficiency have been used. Some studies have reported a correlation between cognitive decline and low total vitamin B_{12}, holoTC and MMA, others not. However, a raised concentration of tHcy in the serum has constantly been linked to cognitive impairment. Causality is debated: vitamin B_{12} deficiency might be a consequence of cognitive impairment rather than vice cersa.

Only few randomized controlled trials have evaluated the effect of vitamin B_{12} supplementation on cognitive function (154) and there is a lack of evidence for a benefit of vitamin B_{12} supplementation on cognitive function. The trials have been of poor quality and short duration and included only small number of participants. Before making definitive conclusions regarding the association between a therapeutic benefit from vitamin B_{12} supplementation and improved cognitive function, large and well-conducted long-term trials are needed.

Rather insensitive and unspecific measures for cognitive function were used in this study. Subjective memory impairment, MMSE and dementia diagnosis are not sensitive enough to detect slight cognitive impairment, and independency in daily living may result from a variety of other reasons as well. As in several other studies, there was no association between the MMSE score and total vitamin B_{12} or holoTC, but tHcy did correlate inversely and significantly with the MMSE score. This is in contrast to a recent large study on people aged 75 years or more in the UK (150), in wich symptoms of memory impairment and a low MMSE score were associated with all measures of vitamin B_{12} status (holoTC, total vitamin B_{12}, tHcy and MMA).

Previously undiagnosed vitamin B_{12} deficiency was not more common in subjects with poor memory function or a low MMSE score. However, a diagnosis of dementia was markedly more common in the group of subjects on vitamin B_{12} substitution than among those not on substitution (26% vs. 8.2%, $p=0.001$). This is obvious because measurement of total vitamin B_{12} is recommended in evaluating the cause of dementia. During follow-up of 40 subjects, MMSE declined in 21 of the subjects. This might indicate progressive cognitive deterioration in untreated vitamin B_{12} deficiency.

6.2.4. Associations between other diseases or medications and vitamin B_{12} status

In this group of unselected aged people anemia was present in 21%, macrocytosis in 17% and frank macrocytic anemia in 6.3% of subjects with a low total vitamin B_{12} concentration in their serum. The frequencies were not significantly different between subjects with normal and increased MMA values. Anemia or macrocytosis did not predict vitamin B_{12} deficiency, which is in accordance with several previous studies that have shown that anemia and macrocytosis do not often coexist in subjects with vitamin B_{12} deficiency (8, 9, 93, 95, 96, 155).

Of the numerous diseases and medications previously reported to be associated with vitamin B_{12} deficiency only diverticulosis increased significantly the probability of vitamin B_{12} deficiency. This could be due to bacterial overgrowth resulting in mucosal damage and malabsorption of vitamin B_{12} (156). In addition to diverticulae, gastric acid suppressive drugs can cause vitamin B_{12} malabsorption by bacterial overgrowth but also by inducing achlorhydria (65). However, gastric acid suppressive drugs did not increase the possibility of vitamin B_{12} deficiency in this population. The greater occurrence of diagnosed gastritis among vitamin B_{12} substituted subjects could be explained by more frequent gastroscopies made for identifying the cause of vitamin B_{12} deficiency. Ubiquitous consumption of dairy products among Finnish aged might have decreased the effect of metformin by guaranteeing adequate calcium-intake. Only two subjects on metformin therapy had low total vitamin B_{12} and three had low holoTC. In other studies considerably greater percentages of subjects have had impaire vitamin B_{12} absorption (157-159).

Unexpectedly, the probability for vitamin B_{12} deficiency was slightly reduced in subjects with depression and CHD. Clearly, the need for screening for vitamin B_{12} deficiency in these subjects is not affected by this apparently fortuitous result. In fact, some studies have linked vitamin B_{12} deficiency and depression (87, 88, 160) and in a Finnish study vitamin B_{12} level and the probability of recovery from depression were positively associated (161) but this relation has not been confirmed in two large studies (150, 162). The role of vitamin B_{12} deficiency in depression of the aged might be causal, unlike folate deficiency, which is more sensitive to nutritional intake (87). Further study of these relations is required.

In the whole Lieto study, symptoms and signs of polyneuropathy were not recorded. Therefore, only pain and independency in daily living or mobility could be used as (poor and unspecific) indicators of polyneuropathy; neither was associated with vitamin B_{12} deficiency. Previously, pain has been reported more often in subjects with low vitamin B_{12} levels (163). At follow-up the subjects with low total vitamin B_{12} levels were tested for peripheral neuropathy test and neurological symptoms. As in previous studies, various symptoms and neurological signs were common (6, 95, 97, 164), but there was no difference in the occurrence of any sign or symptom by MMA level. Similarly, Hvas and co-workers reported that a high MMA level does not predict clinical manifestations of vitamin B_{12} deficiency (164) and Björkegren and co-workers found no association between the classical symptoms or clinical signs and total vitamin B_{12} or MMA in the serum (6). The lack of such associations is not surprising, considering that neurological signs and symptoms are very unspecific and may be due to numerous other clinical conditions than vitamin B_{12} deficiency.

The lack of significant associations other diseases or medications and vitamin B_{12} status in this study might be due to the fact that the study population was unselected and had incomplete medical history data, which allows misclassification of subjects with and without diagnoses. Many previous studies have used more selected populations and more specific diagnoses with a better control for confounding factors; in this study only age and gender were controlled for.

6.3. Laboratory diagnosis of vitamin B_{12} deficiency

6.3.1. Target group for vitamin B_{12} deficiency testing

The proportion of previously undiagnosed vitamin B_{12} deficiency was rather large in the Finnish aged population. This suggests that in present clinical practice only overt signs and symptoms trigger laboratory testing for vitamin B_{12} deficiency. Clinicians need to become much more aware of the need for vitamin B_{12} substitution if irreversible damage due to delayed diagnosis is to be prevented in this fragile population. Routine screening would provide earlier diagnosis and reduce disability (5, 10). Another option is to recognize the early clinical manifestations or possible risk factors. In an aged population, a high frequency of clinical conditions of various etiologies would be expected. According to this study, none of the numerous conditions that are known to predispose to or to be caused by vitamin B_{12} deficiency can be used to define a risk group in a large unselected aged population. Apparently, aging itself increases the probability of vitamin B_{12} deficiency. Therefore, any routine screening should be directed at least to persons aged 75 years and above. Obviously, long-term trials are required to establish the need for treatment of asymptomatic individuals.

6.3.2. Definitions of vitamin B_{12} deficiency

Currently, there are no consistent diagnostic criteria for vitamin B_{12} deficiency and no single laboratory test is the gold diagnostic standard. In this study, several cut off limits for vitamin B_{12}-related parameters were used. Vitamin B_{12} deficiency was defined as a low total vitamin B_{12} concentration or a combination of borderline total vitamin B_{12} together with low holoTC and an increased tHcy concentration. The cut-off limit 150 pmol/l of total vitamin B_{12} was the lower limit of the reference range for the method used. Despite some variation in the reference limits for different methods, this is the standard decision limit. Also, the upper limit of borderline total vitamin B_{12} set at 250 pmol/l was based on previous literature. Also the upper limit of borderline total vitamin B_{12} of 250 pmol/l was based on previous literature. Values in the range of 200-250 pmol/l have been suggested by using increased MMA concentration (with different cut off limits) or decrease in MMA concentration with vitamin B_{12} supplementation as the reference (50, 105, 107-109). In addition, a cost-benefit analysis indicated, that there is benefit in further measurement of MMA when total vitamin B_{12} concentration lower than 200-220 pmol/l (11).

The definitions of increased metabolite concentrations vary also substantially. A recent recommendation for determining tHcy (13) suggests laboratory and region specific reference values from a carefully selected healthy reference population. At present, there is no such

reference value available for the Finnish aged population. The recommendation presents upper reference limits for folate supplemented and non-supplemented individuals by age, and a cut off limit of 20 μmol/l for non-folate supplemented aged over 65 years is proposed. If, however, the folate status and lifestyle of the individual are optimal, the upper reference limit is set at 10-30% lower. Because of this, the cut-off limit was set at 15 μmol/l in this study to maximize sensitivity. Since tHcy is an unspecific measure of vitamin B_{12} status, also a concentration of holoTC below the reference range (37 pmol/l) was required in subjects with borderline total vitamin B_{12} for establishing vitamin B_{12} deficiency.

Unfortunately, MMA measurements were available only for subjects with increased tHcy or low total vitamin B_{12} or holoTC. Therefore, MMA could not be used for the diagnosis of vitamin B_{12} deficiency. However, it could be used to confirm vitamin B_{12} deficiency in subjects presumed to have vitamin B_{12} deficiency with other parameters, when evaluating the accuracy of holoTC. The likelihood of vitamin B_{12} deficiency was graded as potential (low total vitamin B_{12} or high tHcy), possible (low total vitamin B_{12} and either high tHcy or high MMA) or probable (high tHcy and high MMA). Subjects with impaired GFR were excluded and higher cut off limits for tHcy (≥19 μmol/l) and MMA (≥0.45 μmol/l) were used to maximize specificity. The cut off limit 0.45 μmol/l for serum MMA was calculated according to the within-person variation (13%) and the upper reference limit 0.28 μmol/l of the method (130, 165).

The definition of vitamin B_{12} deficiency used in the present study may have resulted in some overdiagnosis. Six subjects in this population had low total vitamin B_{12} although their MMA, tHcy and holoTC were in reference range. According to the definition of the reference range, few of them may represent the 2.5 % of normal subjects who have values below the lower reference range limits but some could have been regarded as having false low total vitamin B_{12}. This is considered justified since overdiagnosis of vitamin B_{12} deficiency is not as harmful as underdiagnosis because no toxic effects have been reported from vitamin B_{12} supplementation.

6.3.3. Hematological signs in diagnosing vitamin B_{12} deficiency

Traditionally, vitamin B_{12} deficiency is suspected in subjects with macrocytic anemia, but many previous studies have shown that anemia and macrocytosis are often not present in subjects with vitamin B_{12} deficiency (8, 93, 95-97, 155) and in a systematic review of the diagnostic value of an elevated MCV, Oosterhuis and co-workers concluded that up to 84% of patients would be missed if only elevated MCV was used for selecting subjects for further testing (98). In the present study based on unselected aged population, anemia was recorded in 21%, macrocytosis in 17% and macrocytic anemia in 6.3% of subjects with low total vitamin B_{12}. Overall, anemia or macrocytosis did not predict vitamin B_{12} deficiency. Indeed, if only subjects with macrocytic anemia were suspected for vitamin B_{12} deficiency only two subjects out of 97 would have been diagnosed.

Furthermore, neutrophil hypersegementation has been considered a more sensitive hematological sign of vitamin B_{12} deficiency than macrocytosis (99), but its diagnostic value has been criticized. In a systematic study, only 6 subjects out of 169 had neutrophil hypersegmentation and there was no difference between vitamin B_{12} deficient and non-deficient

subjects in this respect (100). In another study neutrophil hypersegmentation was present in 78% of subjects with a plasma total vitamin B_{12} concentration below 150 pmol/l (96). This is in agreement with the results in present study, but similar high frequency of neutrophil hypersegmentation was also present in treated subjects. In this study neutrophil hypersegmentation was estimated only at follow-up, and therefore the effect of vitamin supplementation cannot be evaluated. However, a significantly lower percentage of hypersegmentation after treatment would have been expected if hypersegmentation were due to vitamin B_{12} deficiency. Nor was there any difference in the frequency of neutrophil hypersegmentation between subjects with normal and increased MMA.

6.3.4. Effect of renal impairment on vitamin B_{12} related biochemical variables

Because vitamin B_{12} deficiency and renal impairment are both common in the aged, a screening test for vitamin B_{12} deficiency should not be affected by renal function. An inverse correlation between GFR and tHcy and MMA is well established (12, 13, 120, 121, 166). Some recent studies have reported a correlation between total vitamin B_{12} or holoTC and GFR (111, 167). Renal impairment is the most common cause for abnormally elevated total vitamin B_{12} and holoTC concentrations (110). The mechanisms of the increase in the vitamin concentrations in renal impairment are unknown and the regulatory function of kidney in vitamin B_{12} homeostasis has been mainly studied in animals (53, 58).

In previous studies on the relation between vitamin B_{12} and the kidneys, renal function has been mainly estimated by the plasma creatinine concentration (102, 110, 167, 168). However, its usefulness is limited especially in the aged because of large inter-individual variation caused by variable sizes of muscle mass and the fact that even a large reduction in GFR produces only a small increase in the plasma creatinine concentration. The serum cystatin C concentration reflects GFR better than creatinine (169-171) and creatinine-based formulae (172). In this study, cystatin C was used as a marker for GFR.

Increased serum concentrations of tHcy and MMA could be explained by renal impairment in over half of the cases. As expected, there was a strong correlation between tHcy and MMA and impaired GFR, and both cystatin C and creatinine were strongly associated with the metabolites. In contrast to some other studies (111, 167) there was no correlation between total vitamin B_{12} or holoTC and either cystatin C or creatinine. Also, Miller et al reported recently that there was no correlation between creatinine and holoTC in an aged population (102) and in a recent smaller study cystatin C did not correlation with vitamin B_{12}, but was strongly associated with tHcy and MMA, irrespective of vitamin B_{12} status (173).

6.3.5. Comparison of laboratory markers of vitamin B_{12} deficiency

In the absence of gold diagnostic standard, an objective comparison of clinical utility of laboratory tests for vitamin B_{12} deficiency is problematic. In cross-sectional studies, like the present study, circular reasoning should be bewared. Prospective follow-up studies and therapeutic trials are required for true assessment of the sensitivity and specificity of tests that assess vitamin B_{12} status. Shortcoming similar to previous studies may be identified in the present study. Firstly, MMA values were available only for subjects with increased tHcy or low

total vitamin B_{12} or holoTC, and therefore MMA could not be used as a reference for comparing other variables. At any rate, MMA and tHcy are both sensitive to renal impairment, and not suitable as references in aged populations. tHcy is also sensitive to several factors (e.g. preanalytical factors, lifestyle factors, folate deficiency etc.) which introduce uncertainty and cannot to be used alone as a reference for total vitamin B_{12} and holoTC. A comparison with these presumably less sensitive markers would not reveal whether holoTC is very sensitive or unspecific for vitamin B_{12} deficiency.

There are only a few studies that have properly compared the usefulness of total vitamin B_{12} and holoTC as measures of vitamin B_{12} deficiency (17, 102, 111, 167, 174). Some studies have reported that holoTC may compare favorably with total vitamin $B_{12,}$ as indicated by a slightly larger AUC for holoTC than for total vitamin B_{12} (111, 167, 174) but others did not identify any significant difference in the power of total vitamin B_{12} and holoTC to discriminate between subjects with metabolic vitamin B_{12} deficiency as indicated by increased tHcy and MMA concentrations in the serum (17, 102).

In the present study the holoTC concentrations were low in vitamin B_{12} deficiency and the frequency of low holoTC concentrations decreased with a decreased likelihood of vitamin B_{12} deficiency. Incongruence in pathological results of total vitamin B_{12} and holoTC was found, and thus differences in sensitivity and specificity can be speculated but not confirmed in this setting. tHcy and MMA, but not total vitamin B_{12} and holoTC, are very sensitive to even slight impairment in renal function and thus total vitamin B_{12} or holoTC are best suitable for first-line testing when screening an aged population, where renal impairment is not uncommon. Anemia and macrocytosis did not predict vitamin B_{12} deficiency, and thus the traditional hematological approach to vitamin B_{12} deficiency should be questioned.

6.3.6. Algorithm for laboratory diagnosis of vitamin B_{12} deficiency in Finland

When clinical judgment or patient-related risk factors generate a need for laboratory testing for vitamin B_{12} deficiency, guidelines for optimal use and interpretation of laboratory tests are essential. Several slightly different strategies for diagnosing vitamin B_{12} deficiency have been presented (7, 10, 50, 103, 174, 175). An adequate first-line test should be sensitive, specific, widely available and cost-effective. With these criteria in mind the measurement of total vitamin B_{12} is the best first-line test. When the concentration is below 150 pmol/l vitamin B_{12} deficiency is confirmed and when it is above 250 pmol/l it is excluded.

Additional measurements are required for the assessment of subjects with vitamin B_{12} concentrations in the range from 150 to 250 pmol/l. For this, tHcy and holoTC are suitable, because of their sensitivity. Both of these test should be used to overcome the unspecificity of tHcy and current uncertainty in clinical utility of holoTC. The effect of impaired renal function should be considered when the tHcy concentration is evaluated. MMA is poorly available in Finland and is thus not useful. In case of doubt, follow-up and control testing after a few months or a therapeutic trial are suitable procedures. Figure 6.1 is a proposition for an algorithm for laboratory testing of vitamin B_{12} deficiency suitable for the Finnish health care system. It is based on previous data (7, 10, 50, 103, 174, 175) added with results of the present study.

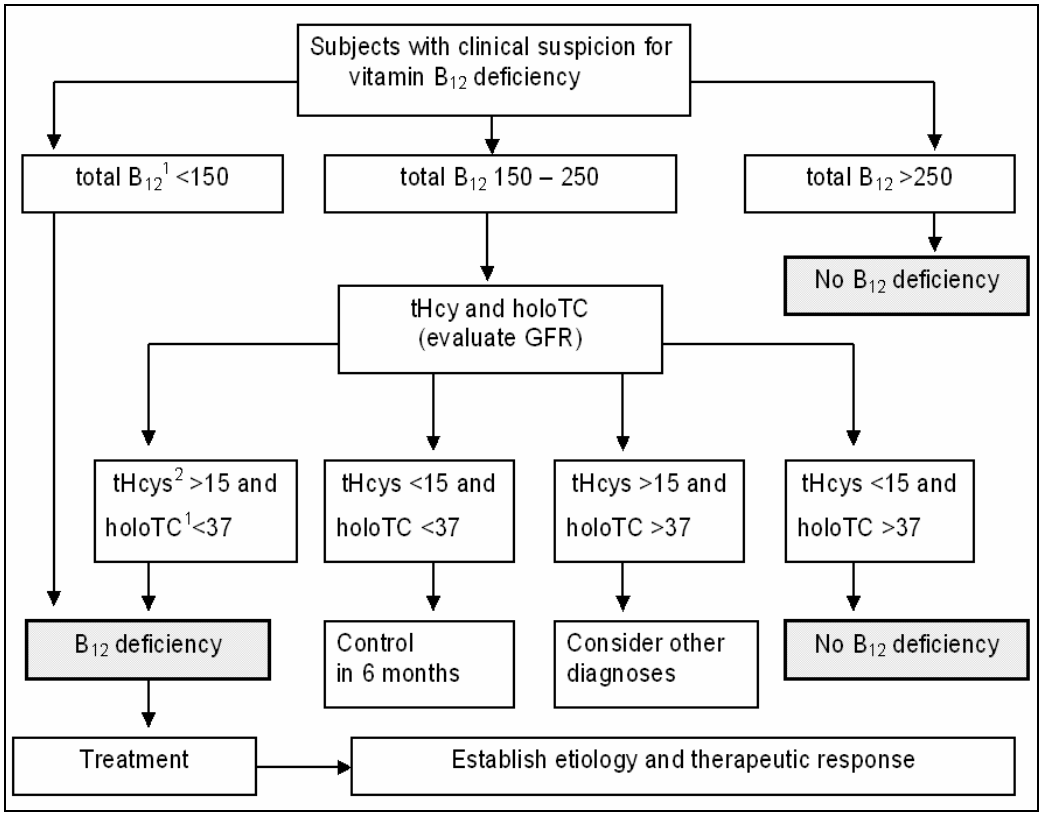

Figure 6.1. Algorithm for laboratory diagnosis for vitamin B_{12} deficiency.
total B_{12}, total vitamin B_{12}, tHcy, total homocysteine, holoTC, holotranscobalamin; GFR, glomerular filtration rate; [1] unit for total B_{12} and holoTC pmol/l; [2] unit for tHcy µmol/l.
Reprinted from Loikas S, Koskinen P, Irjala K, Löppönen M, Isoaho R, Kivelä SL, Pelliniemi TT. Laboratory diagnosis of vitamin B_{12} deficiency. Submitted.

6.4. Future of holoTC

6.4.1. Advantages of automated holoTC assay

The identification and characterization of a monoclonal antibody againt human holoTC has been reported (176). The antibody is highly specific for holoTC and the specificity arises from conformational changes occurring in transcobalamin upon binding to vitamin B_{12} and vitamin B_{12} is not a part of the epitope. The antibody has been used to develop an ELISA-based assay, which uses the anti-human holoTC antibody as the capture antibody and anti-human TC antibody as the detection antibody. An automated version of this assay has recently been launched. With automation, the availability of the holoTC test will improve and the cost decrease. This may lead to more liberal use of the holoTC assay at the expense of tHcy or even total vitamin B_{12}. However, additional data on the clinical utility of holoTC is required before it

can become the first-line test for diagnosing vitamin B_{12} deficiency.

6.4.2. Vitamin B_{12} absorption test

Evaluation of the intestinal absorption of vitamin B_{12} is essential for defining the cause of vitamin B_{12} deficiency. The traditional Schilling test has several limitations and is no longer available in Finland. Evaluation of vitamin B_{12} absorption by the use of blood test have been studied for a long time, but the results have not been consistent. Major pitfalls have been the use of large doses of oral vitamin B_{12}, which enables significant passive absorption, and the measurement of serum total vitamin B_{12}, which is not good marker for sudden changes in vitamin B_{12} homeostasis (134).

With the availability of reliable assays for holoTC and with the demonstration that the serum holoTC concentration reflects recent vitamin B_{12} absorption better than total vitamin B_{12} (134, 137), a new vitamin B_{12} absorption test has been developed based on serum holoTC measurement before and after a physiological oral dose of vitamin B_{12}. So far, this method has been evaluated in only a few small series (137, 177, 178). In the test holoTC is measured at baseline, and if the concentration is lower than 75 pmol/l, three oral doses of 9 µg vitamin B_{12} are administered over two days. On day three, the absorption of vitamin B_{12} is evaluated by the increase in holoTC. When a change two times greater than day-day variation is considered significant, increase over 22% or 10 pmol/l in relation to baseline indicates normal absorption (177). The test is a promising candidate as a vitamin B_{12} absorption test, but further studies are needed before the test becomes clinically available.

7. CONCLUSIONS

The following findings and conclusions were made:

I The new commercially available HoloTC RIA method is suitable for routine laboratory work since it is precise and simple to perform, although time-consuming. The subjects with other biochemical signs of vitamin B_{12} deficiency had also low holoTC levels. The central 95% reference interval for serum holoTC for Finnish adults and aged subjects is 37-171 pmol/l. The reference interval obtained with the first version of HoloTC RIA can be used with the new version, as well.

II The prevalence of vitamin B_{12} deficiency was 12% in the Finnish aged population, which is in accordance with the previously reported prevalence rates in other countries. The proportion of previously undiagnosed vitamin B_{12} deficiency was remarkably large. This suggests that currently only overt signs and symptoms trigger laboratory testing for vitamin B_{12} deficiency and diagnostic guidelines should be revised.

III Male gender, age 75 years or above, and refraining from milk products doubled the probability for vitamin B_{12} deficiency. Still, no clinically meaningful risk group could be identified for screening. Anemia and macrocytosis did not predict vitamin B_{12} deficiency. In the subjects with low total vitamin B_{12} values, neurological symptoms and signs were frequent.

IV The metabolic markers tHcy and MMA were strongly affected by even slight impairment in renal function, whereas total vitamin B_{12} or holoTC were not. Therefore, either total vitamin B_{12} or holoTC rather than tHcy or MMA should be used for screening aged populations prone to renal impairment.

V Because absence of anemia and macrocytosis does not rule out vitamin B_{12} deficiency, because renal impairment compromises the use of tHcy and MMA, and because the utility of holoTC is uncertain, the measurement of total vitamin B_{12} is still the best first-line test for a laboratory diagnosis of vitamin B_{12} deficiency. Further testing of tHcy and holoTC are in order for subjects with borderline total vitamin B_{12} concentrations in their serum for an appropriate diagnosis.

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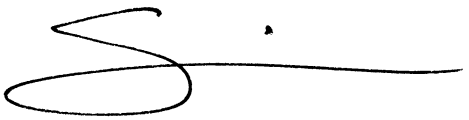

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10. ORIGINAL PUBLICATIONS