Approaches for setting micronutrient recommendations

a case study of vitamin B12 for adults and elderly people

Esmée Doets

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PROPOSITIONS

1. Harmonizing micronutrient recommendations requires harmonized definitions of requirements. (This thesis)

2. Vitamin B12 requirements can yet not be based on optimal status for cognitive performance. (This thesis)

3. Considering the "unquestionable positive effects of music on cognitive functioning", it could be considered to establish recommendations on daily music intake. (Inspired by Honing and Swaab, Volkskrant 18-06-2011)

4. Complying with micronutrient recommendations by a healthy varied diet is preferable over supplement use.

5. Evidence-based decision making in policy development requires expert judgement.

6. Going for a run during working hours is a distracting task that stimulates the unconscious mind to select the best ideas.

Propositions belonging to the thesis entitled 'Approaches for setting micronutrient recommendations-a case study of vitamin B12 for adults and elderly'

Esmée Doets
Wageningen, 15 June 2012

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Thesis

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There is no such thing as exact science

(after Heisenberg's uncertainty principle, 1927)

ABSTRACT

Background: Most countries in Europe provide recommendations on the micronutrient composition of diets to fulfil requirements of nearly all individuals in the general apparently healthy population. However as each country uses its own methods for deriving such recommendations, there is large variation between countries in the recommended micronutrient intakes. The objectives of this thesis are to signal key issues for harmonizing approaches for establishing micronutrient recommendations for adults and elderly across Europe and to illustrate standardized and transparent review methods that can be used to summarize and evaluate the evidence-base for setting recommendations, using vitamin B12 as a case micronutrient.

Methods: First the need for harmonization is substantiated by a descriptive study on the variation in published micronutrient recommendations across Europe. In addition methodological factors were identified that should be considered for alignment of recommended intakes across Europe. Secondly, the evidence-base for establishing recommended vitamin B12 intakes was summarized in two systematic reviews. One review focused on requirements for the compensation of daily obligatory losses (factorial approach) and the other review evaluated the relation of vitamin B12 intake and status with cognitive performance (dose-response approach). Whether interactions between folate and vitamin B12 on cognitive performance should be considered for establishing recommended vitamin B12 intakes was evaluated using data from 2203 Norwegian elderly from the Hordaland Homocysteine Study.

Results: For harmonizing approaches for establishing micronutrient recommendations, standard methods are needed to a)-select health indicators and define adequate biomarker concentrations, b)-make assumptions about inter-individual variation in requirements, c)-derive bioavailability factors, and d)-select and interpret evidence on requirements. The first systematic review showed that daily vitamin B12 losses in apparently healthy adults and elderly probably range between 2.6-3.9 µg and bioavailability from the usual diet may range between 29 and 37% rather than the generally assumed 50%.

Dose-response evidence from 2 randomized controlled trials and 19 prospective cohort studies showed no or inconsistent associations between vitamin B12 intake or status and dementia, Alzheimer's Disease, global cognitive function or domain-specific cognitive function in adults and elderly people. Cross-sectional analyses in the Norwegian cohort study showed that

low plasma vitamin B12 in combination with high folate was associated with better cognitive performance. However, these associations were not observed for sensitive markers of vitamin B12 status.

Conclusion: The main conclusion of this thesis is that evidence underlying current recommended vitamin B12 intakes is old and has large uncertainties**,** whereas the available evidence on the relation between vitamin B12 and cognitive performance is yet not convincing and thereby limits its use as an outcome for estimating vitamin B12 requirements.

The relation between vitamin B12 intake and markers of vitamin B12 status seems the best alternative, but sound statistical methods to define recommendations based on these dose-response data should be further developed.

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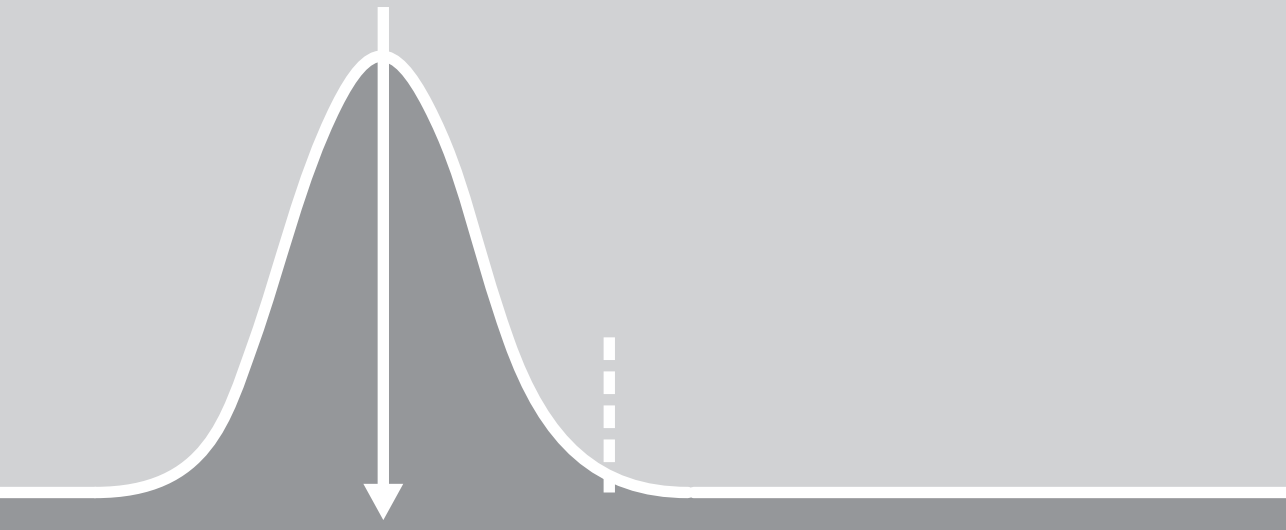
chapter 1



Introduction

Micronutrients, such as vitamins and minerals, are essential for proper growth and metabolism in humans. Although only small quantities of micronutrients are needed, their lack may have serious consequences for health and development of populations across the world (1). Most countries in Europe provide recommendations on the micronutrient composition of diets to avoid deficiency in the general apparently healthy population. These recommendations serve as a basis for national or regional nutrition policies, nutritional education programs and food regulation. However as each country uses its own methods for deriving recommendations, there is large variation between countries in the recommended micronutrient intakes (2-4). The EURRECA Network of Excellence was established in the beginning of 2007 with the aim to harmonize the process of setting micronutrient recommendations across Europe with special focus on vulnerable population groups, including elderly people (5, 6). This thesis was written in the context of the EURRECA network and includes a critical review of the variation in recommendations for adults and elderly across Europe to signal key issues for harmonizing the process for setting micronutrient recommendations. Furthermore, this thesis illustrates standardized, transparent and objective review methods that can be used to summarize and evaluate the evidence-base for setting recommendations, using vitamin B12 as a case micronutrient.

RECOMMENDATIONS

Although different terms are used by different national and international agencies to express micronutrient recommendations, e.g. Population Reference Intake, Recommended Intake, Recommended Daily Allowance, they all refer to the daily intake level that is sufficient to fulfil the requirements of nearly all healthy individuals in a defined population (7, 8). Requirements are here defined as the intake that is sufficient to fulfil micronutrient needs. These micronutrient needs are not fixed values, but depend on an a priori definition of adequate health using selected physiological, biochemical or clinical outcome measures and a cut-off level indicating the desired or acceptable level of the selected health outcome measures.

For an individual, the requirement for any micronutrient depends on a variety of factors such as age, gender, genotype, physical activity, health status and the efficiency with which an individual absorbs and metabolizes micronutrients. The requirement for a specific micronutrient can thus vary

both within and between individuals. For setting recommendations on intake of a specific micronutrient, one needs to estimate the distribution of requirements within a defined population. From this distribution, the average nutrient requirement (ANR) can be estimated, indicating the intake level that would be sufficient to fulfil the requirements of 50% of individuals in the population. Subsequently the recommended intake can be estimated as the ANR+2SD, to account for variation in needs between individuals. An intake equal to the recommended intake would thus be sufficient to fulfil the requirements of circa 97.5 % of the individuals in a population.
Often the distribution of requirements cannot be estimated and therefore no ANR can be derived. The recommended intake is then approximated based on observations of usual intakes in the specific population and estimated as the mean level of intake at which the prevalence of inadequacy is apparently low. A recommended intake derived in this way is referred to as the adequate intake (AI) (9). Thus, a recommended intake derived as ANR+2SD and the AI both target the intake that seems to cover requirements of nearly all individuals within a population, but methods used to derive them are different.

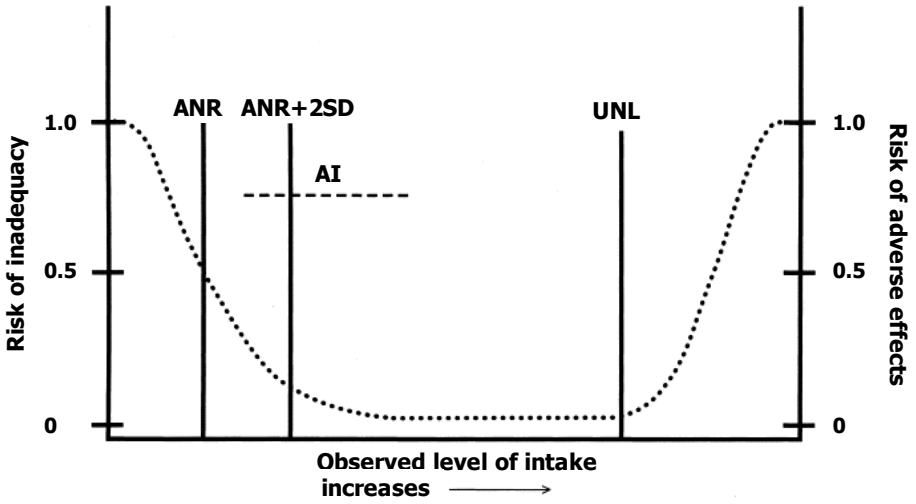


Figure 1 Overview of the different concepts related to micronutrient recommendations (10)

The upper nutrient level (UNL), also referred to as upper level, is defined as the highest level of usual intake that is likely to pose no risk of adverse

health effects in almost all individuals in the general population. Establishing UNLs requires a toxicological risk assessment method that is outside the scope of this thesis. Figure 1 gives an overview of all the concepts introduced above.

Application of recommendations

Two main applications of micronutrient recommendations can be distinghuished, namely evaluation and planning of intakes of population groups. These two application form the basic activities for developing nutrition policies by both national and international authorities (e.g. WHO/FAO)(11). Although recommendations are intended to be used for groups of individuals, they are in practice also used for evaluating and planning intakes of individuals (12-14). However in this thesis the focus is on applications for population groups only.

For evaluating intakes of population groups, the cut-point method can be used that, under some assumptions, estimates the proportion of individuals in the population that have an intake below their requirements by the proportion of the population with intakes below the ANR. In case the ANR cannot be estimated and therefore the recommended intake is derived as an AI instead of ANR+2SD, the prevalence of inadequate intakes in a population cannot be estimated quantitatively because the AI is higher than the requirements of most individuas within a population. It can be assumed that when the mean intake of a population approximates the AI, the prevalence of inadequacy is low. However when the mean intake of a population is below the AI, no such statements on the prevalence of inadequate intakes can be made (15).

For planning micronutrient intakes for a group/population, two methods have been proposed. The aim of the first method is to minimize the prevalence of intakes below the ANR following the cut-point method as described before (11, 16). Whereas the aim of the second and most commonly used method is to compose a diet with a micronutrient content equal to the recommended intake or AI (13, 14).

Current micronutrient recommendations vary largely between European countries which is likely due to differences in methods used by recommendation-setting bodies. Because variation in recommendations can be confusing for those involved in the evaluation and planning of micronutrient itnakes such as consumers, food producers and nutrition

policy makers, harmonization of the process for setting micronutrient recommendations is needed.

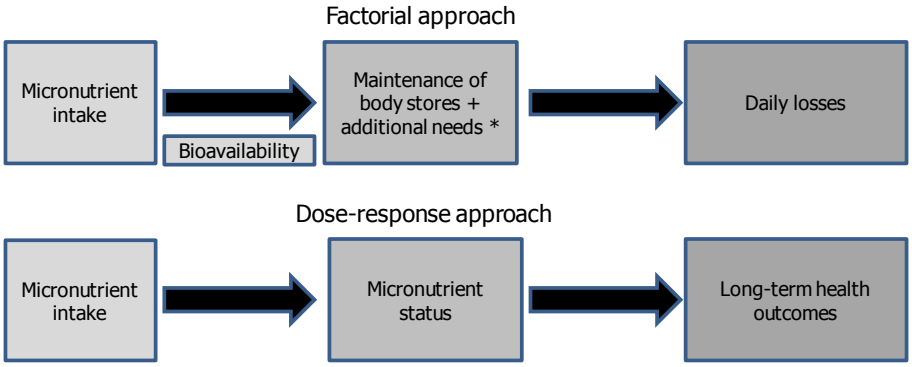
Network of Excellence EURRECA

The overall aim of the EURRECA network of excellence is to produce harmonized scientific guidelines for developing micronutrient recommendations to move towards a uniform, transparent and evidence-based process for deriving recommended micronutrient intakes (6).

A framework has been proposed that describes the process for deriving micronutrient recommendations in several steps. The first steps include the selection of micronutrients, population groups and health outcomes that require a revision of recommendations and the formation of an expert committee that has responsibility for deriving the specific micronutrient requirements and/or recommendations. Next, scientific data on requirements for the specific micronutrient and population group should be collated, summarized and integrated according to predefined best practice methods. Data on requirements may be obtained from studies focusing on dose-response relations between dietary micronutrient intake and health outcomes such as physical function or disease with measures of micronutrient status as an intermediate between intake and health (dose-response approach). Requirements may also be derived from data on micronutrient losses and maintenance and data on absorption/bioavailability (factorial approach). The two approaches for estimating micronutrient requirements are schematically shown in Figure 2.

Based on the available evidence, the expert committee has to decide whether it is possible to estimate the requirements distribution and derive a recommended intake either as an ANR+2SD or an AI. Subsequently, if appropriate, policy instruments can be selected to change the nutrition situation in order to maximize the likelihood of achieving a desired health outcome for the relevant population.

With this approach, rather than merely focusing on the prevention of severe deficiencies and maintaining stores, chronic disease and long-term outcomes related to micronutrient intake may also be considered for establishing micronutrient recommendations. Although the selection of appropriate long term health outcomes can be challenging because there is generally a lack



* Additional needs may include needs for growth, pregnancy or lactation

Figure 2 Two approaches for estimating micronutrient requirements

of established data on the precise association between dietary intake levels, nutrient status, and long term health outcomes. In addition, etiologies of such health outcomes are generally not nutrient-specific but multifactorial, reflecting a combination of genetics, environmental exposure, and lifestyle patterns. A systematic review approach for collating and summarizing data on micronutrient requirements can highlight these challenges by making transparent what evidence is available addressing populations and health outcomes of interest and what evidence is missing (17-19).

In the context of the EURRECA network, several micronutrients were prioritized for which the available evidence on requirements was systematically reviewed. These micronutrients were prioritized according to a) the amount of new scientific evidence available on the micronutrient for different life-stage groups since 2003, particularly that from randomized controlled trials, b) the public health relevance of the micronutrients, and c) variations in current micronutrient recommendations (20). One of these priority micronutrients was vitamin B12.

VITAMIN B12

Vitamin B12 is a water soluble vitamin characterized by a large and complex chemical structure containing a cobalt ion. Compounds with vitamin B12 activity are therefore also known as cobalamins. In humans, cobalamins function as a cofactor for two enzymes, methionine synthase and methylmalonyl coenzyme A (CoA) mutase (21, 22).

Methionine synthase catalyzes the remethylation proces of homocysteine to the amino acid methionine in which folate serves as donor of a methyl group. Methionine is required for the synthesis of S-adenosylmethionine, which functions as a methyl group donor in many biological methylation reactions, including the methylation of DNA and RNA (21).
Methylmalonyl CoA mutase catalyzes the conversion of Methylmalonyl-CoA (MMA) into succinyl CoA which is an important step in the production of energy from proteins and fats. Succinyl CoA is required for the synthesis of hemoglobin (22).

Intake

Humans are unable to synthesize vitamin B12 and therefore fully depend on dietary intake (23). The vitamin must be supplied by animal-source products in the diet, including meat, fish, dairy, eggs, and liver. Mean vitamin B12 intakes in Europe range from 3.8 to 9.3 µg per day in men and from 3.5 to 8.8 µg per day in women based on nine surveys including 28015 adults and elderly people (24). A recent food consumption survey in the Netherlands showed median intakes of 4.8-5.2 µg per day among 1055 men and 3.6-4.2 µg per day among 1051 women (25).

Absorption

Absorption of vitamin B12 is a complex process which is illustrated in Figure 3. After ingestion of foods containing vitamin B12, first, vitamin B12 needs to be released from the proteins in the food by pepsin, which happens in the acidic environment in the stomach. The free form of the vitamin immediately binds to R-proteins (haptocorrins) secreted by the salivary glands and the gastric mucosa. In the duodenum, pancreatic proteases degrade the R-proteins, releasing the vitamin which then binds to Intrinsic Factor (IF), a glycoprotein that the parietal cells in the stomach secrete after being stimulated by food. This vitamin B12-IF complex then proceeds to the terminal end of the ileum where it is absorbed by specific ileal receptors. The capacity of IF mediated absorption is limited, because ileal receptors become saturated at intakes of ca 1.5-3.0 µg from a single meal (26, 27).Once absorbed, vitamin B12 binds to transcobalamin II, also referred to as holoTC II. Then it enters the portal circulation and is delivered to cells in the body. Only about 20% of the circulating vitamin B12 is bound to transcobalamin II and available for cellular uptake, the other 80% is bound to the R-proteins Transcobalamin I and III (28, 29). Active absorption of

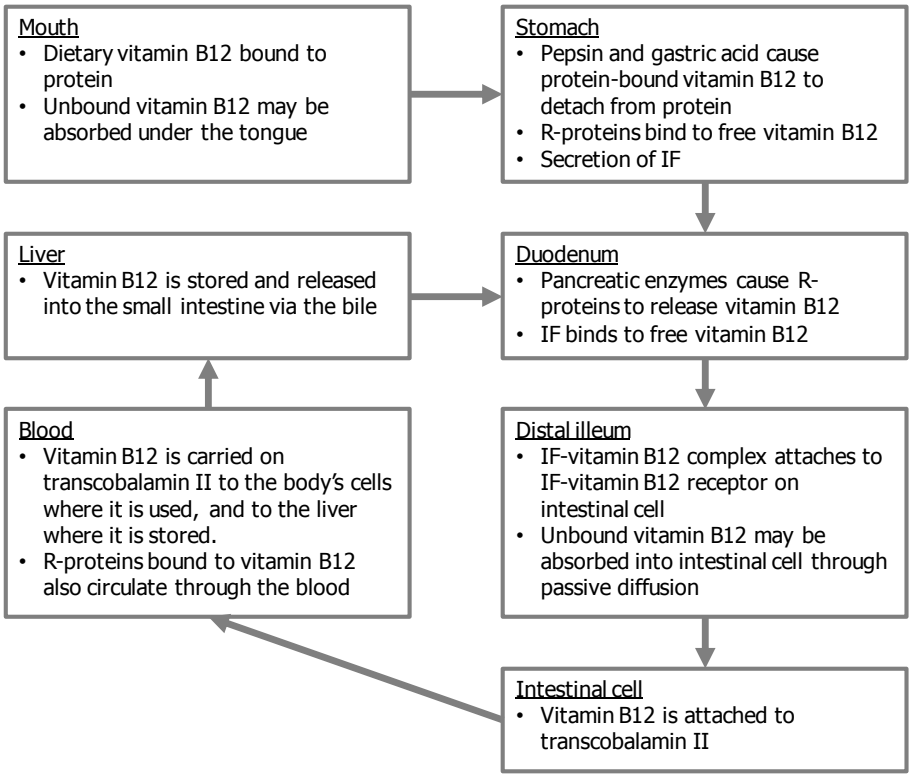


Figure 3 Schematic presentation of the absorption process of vitamin B12

vitamin B12 thus depends on the amount consumed and normal functioning of the gastrointestinal tract (30). Small amounts of vitamin B12 (1-3% of the ingested amount) can also take place by passive diffusion, which is independent of intrinsic factor secretion and normal functioning of the gastro-intestinal tract (31, 32).
Malabsorption of vitamin B12 can occur due to a lack of IF or due to intestinal abnormalities, but it may also be the result of a reduced ability to release the vitamin B12 from food as in gastric atrophy, a clinical condition common among elderly people (28, 33).

Status

Concentrations of vitamin B12 in serum or plasma are most commonly used as a marker of vitamin B12 status. Although the major fraction of circulating vitamin B12 is bound to R-proteins that are not involved in delivery of vitamin B12 to body cells, it may reflect the general underlying state of

vitamin B12 stores (34). Serum/plasma vitamin B12 is therefore better for assessing long-term than short-term intake. Functional markers of vitamin B12 status including serum or plasma concentrations of holoTC II and MMA, have been suggested as more sensitive and specific marker of vitamin B12 status. HoloTC II represents the fraction of vitamin B12 that is delivered to body cells and MMA is the substrate for the vitamin B12 dependent enzyme methylmalonyl CoA mutase, so in case of vitamin B12 deficiency MMA levels will increase. Each of these markers of vitamin B12 status have been reviewed for their effectiveness in responding to oral vitamin B12 supplementation (32, 35). The available evidence from randomized controlled trials (RCT's) suggested that plasma and serum concentrations of total vitamin B12 and MMA were effective biomarkers of a change in vitamin B12 intake; although data were limited (n=8 and n=3 respectively). The effectiveness of holoTC II as a biomarker of vitamin B12 status could not be evaluated as it was measured in only one study. Plasma homocysteine concentrations were also evaluated as a potential marker for vitamin B12 status. Although concentrations of serum and plasma total homocysteine were significantly lowered by intervention with vitamin B12, this is not very specific for measuring vitamin B12 status because folate and vitamin B12 both affect this measure (32).

Based on observations from population-based studies, the prevalence of low vitamin B12 status among adults and elderly people is 5-60% depending on the cut-off levels used to indicate deficiency (36-38). About 2% of these cases result from inadequate intake due to a strict vegetarian or vegan diet or limited access to food of animal origin (39, 40), but most cases of low vitamin B12 status in adults and elderly are caused by malabsorption of protein-bound vitamin B12.

Health outcomes

Severe vitamin B12 deficiency is associated with megaloblastic anemia, gastrointestinal effects, bone metabolism and neurological manifestations such as peripheral neuropathy, myelopathy, and most severely, subacute combined systems disease characterized by demyelination of dorsal columns and corticospinal tract (28, 37, 39, 41, 42). Furthermore low vitamin B12 status is associated with cognitive decline and it has been suggested that cognitive function is associated with vitamin B12 status across the normal range (43). Cognitive function includes processes of being aware, knowing, thinking, learning and judging (44-46). Several specific domains of cognitive

function can be distinguished including memory, executive function, processing speed and language (47). Global measures of cognitive function, such as the Mini-Mental State Examination, evaluate functioning in different domains at the same time (48). A serious loss of cognitive function is referred to as dementia and can be characterized by difficulties with many areas of cognitive function including memory, language, emotional behavior, perception, thinking and judgment (44, 45). Dementia is a very substantial cause of morbidity in any aging population, with profound social and economic effects. Alzheimer's Disease (AD) is a common form of dementia, circa 60% of the cases, that gets worse over time (49, 50).
There are many possible mechanisms through which low normal vitamin B12 status could influence cognitive function. The commonest hypothesis is that low vitamin B12 status leads to a deficiency of S-adenosylmethionine and thereby to deficient methylation reactions in the central nervous system (43). As folate is also involved in the synthesis of S-adenosylmethionine, the association of low vitamin B12 status with cognitive impairment may depend on the folate status of the population. Whether an optimal vitamin B12 status has a role in preventing cognitive impairment or dementia should be confirmed. If so, this relation could be considered when setting recommendations on vitamin B12 intake.

OUTLINE OF THIS THESIS

The general aim of this PhD-thesis is to contribute to standardization of the process for setting evidence-based, transparent and harmonized micronutrient recommendations across Europe by addressing two objectives:
1-To identify the variation in European micronutrient recommendations and to explore factors that could explain the observed variation.
2-To systematically review data on vitamin B12 requirements for adults and elderly people.

The first objective is addressed by a descriptive study including comparisons of micronutrient recommendations across European countries and a critical review of the methods used for establishing these recommendations (chapter 2 and 3).
The second objective is addressed by:
a-a systematic review on vitamin B12 requirements based on daily losses of vitamin B12 and its bioavailability (factorial approach) (chapter 4).

b-a systematic review and meta-analysis to evaluate whether the associations between vitamin B12 and cognitive function should be considered for estimating vitamin B12 requirements (dose-response approach) (chapter 5)
c-cross-sectional and prospective data analyses in cohort data from the Hordaland Homocysteine Study to evaluate whether there is an interaction between vitamin B12 and folate in relation to cognitive performance (chapter 6).
As low vitamin B12 status in Europe mainly occurs in older adults, the focus in this thesis is on adults and elderly people.

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chapter 2

Current micronutrient recommendations in Europe: towards understanding their differences and similarities

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ABSTRACT

Background: This paper gives an overview of the available micronutrient recommendations in Europe, and provides information on their origin, concepts and definitions. Furthermore this paper illustrates the diversity in European recommendations on vitamin A and vitamin D, and explores differences and commonalities in approaches that could possibly explain variations observed.

Methods: Questionnaires were sent to key informants in the field of micronutrient recommendations across Europe to collect information on the process of establishing micronutrient recommendations. Furthermore the latest reports on nutrient recommendations in Europe were collected. Recommendations for vitamin A and vitamin D were compared per sex at the ages 3m, 9m, 5y, 10y, 15y, 25y, 50y and 70y.

Information extracted from the questionnaires and reports was compared focusing on: 1) concepts of recommendations, 2) publication years of the reports (proxy for available evidence), 3) population groups defined, 4) other methodological issues such as selected criteria of adequacy, the type of evidence used, and assumptions made.

Results: In total 23 countries, WHO/FAO and the EC published their own reports on nutrient recommendations. France, the Netherlands, Latvia, the United Kingdom, WHO/FAO and the EC defined their own recommendations and the DACH-countries (Germany, Austria and Switzerland) as well as the Nordic countries (Norway, Sweden, Finland, Denmark and Iceland) cooperated in setting recommendations. Fifteen countries based their micronutrient recommendations on those from other countries or organizations.

Rather than by concepts, definitions, and defined population groups, variability in recommendations on intake of vitamins A en D seemed to result from differences in criteria for adequacy, assumptions made and type of evidence used to establish micronutrient recommendations.

Discussion: The large variation in current micronutrient recommendations for population groups as illustrated for vitamin A and vitamin D strengthens the need for guidance on setting evidence based, up-to-date European recommendations. Differences in endpoints, type of evidence used to set recommendations, experts' opinions and assumptions are all likely to contribute to the identified variation.

INTRODUCTION

Most likely, the first true dietary recommendations were proposed by Dr. Edward Smith in 1862 in response to a request from the British Privy Council. The council wanted to determine the least cost for which enough food could be purchased to prevent starvation and associated diseases among the population that was unemployed as the result of the economic depression of the time. Since then, many other nutrient recommendations have been proposed that were used for the planning of food supplies and ration scales during times of war and food shortages, focussing merely on the prevention of deficiencies (1). Due to the continuously increasing knowledge on the physiological role of nutrients, and the health consequences of micronutrient deficient diets, the concept of dietary and nutrient recommendations still receives much attention (2). Nowadays most countries in Europe have established their own nutrient recommendations to assess the adequacy of dietary intakes and to plan desirable dietary intakes both at the individual and population level (3-35). These recommendations serve as a basis for national or regional nutrition policies, nutritional education programs, food regulations and action programs. In 1993, population reference intakes (PRIs) were defined for the European Community (EC) to be used for food labelling in Europe (14).

The approach to establish nutrient recommendations has changed over the course of time. The classical paradigm focused on an adequate intake of nutrients via food to prevent deficiencies based on clinical trials. In 1994, the Food and Nutrition Board of the Institute of Medicine (IOM) introduced dietary reference intakes (DRIs) for the United States of America and Canada including many aspects of the conceptual framework from the report published in the United Kingdom (35). These DRIs represented a paradigm shift in the way nutrient recommendations were established and used by practitioners, educators, and researchers. Besides the prevention of deficiencies, DRIs were intended to help individuals optimize their health, prevent disease, and avoid consuming too much of a nutrient. Furthermore the IOM paradigm placed greater emphasis on the distribution of nutrient requirements within a population, rather than on a single value and they quantified the relationship between a nutrient and the risk of disease based on scientific evidence (36).
In Table 1 the classical paradigm and the new paradigm as presented by the IOM are shown. The DRIs included four types of nutrient-based

reference values: the average nutrient requirement (ANR), the recommended intake, the adequate intake (AI) and the upper nutrient level. The ANR is defined as the average daily nutrient intake level that is estimated to meet the requirements of half of the healthy individuals in a particular life-stage and gender group. The recommended intake represents the average daily dietary nutrient intake level that is sufficient to meet the nutrient requirements of nearly all (97-98%) healthy individuals in a particular life-stage and gender group and is derived as ANR+2SD. When an ANR+2SD cannot be determined, an AI is estimated as the lowest level of intake estimated to be sufficient for nearly all healthy people within the population. The upper nutrient level is outside the scope of this paper (36).

Pavlovic et al. (2), King et al. (38) and Prentice et al. (39) compared a selection of European nutrient recommendations to provide an overview of existing differences in terminologies and reference values. The countries/organizations included were: the Nordic countries (Norway, Sweden, Denmark, Finland and Iceland), the DACH-countries (Germany, Austria and Switzerland), the United Kingdom, the Netherlands, Italy, WHO/FAO, and EC. From these comparisons, it appeared that often nutrient recommendations have been established involving small and select committees of experts. As yet there is no standard approach for deriving nutrient recommendations they vary from country to country. This occurs even for well-defined population groups that are assumed to have the same physiological requirements. Given the lack of standardized methodologies, some nations/organizations provide, for example, one single recommendation for all adults grouped together, while others provide recommendations separately for men and women (38).
Besides such differences in methodologies, national recommendations are reviewed at different times so they may not always be based on the same most up-to-date scientific information. Furthermore, cultural and regional factors may affect the weighing of evidence and the decision process. This results in different national recommendations causing confusion for policy-makers, health professionals, industry, and consumers within Europe.

Table 1 Paradigm of nutrition science: classical and extensions of the 21st century

	Classical	21st century
Scientific domain	• Essential nutrients (± 50) • Biological effects • Adequate intake via food	• Essential nutrients and bio-active food components • Biological effects • Adequate intake via food, supplements and "functional foods" • Avoid toxic levels
Basis for nutrient recommendation (criteria for adequacy)	• Prevention of deficiency diseases	• Prevention of deficiency diseases • Prevention of chronic diseases (optimal health)
Variables taken into account for recommendation (assumptions)	• Age, sex, physical activity, body weight • Made up for groups	• Age, sex, physical activity, body weight • Ethnicity • Heredity • Genetic predisposition for disease • Made up for groups and individuals • Food patterns • Lifestyle and environment
Scientific Base (type of evidence)	• Clinical "depletion-repletion" model	• Epidemiology: Meta-analyses and RCT provide best funded evidence

Based on IOM (36) and van Staveren (37)

Harmonization will improve the objectivity and transparency of values that are derived by various national, regional and international groups. Where harmonization is not possible, transparency is needed on the approaches to establish recommendations. This will improve understanding and explanation of potential differences between recommendations and simplifies their application in policy making.

EURRECA Network of Excellence (www.eurreca.org)

EURRECA (EURopean micronutrient RECommendations Aligned) is a network of excellence funded by the EC and established to identify and address the problem of differences between countries in micronutrient recommendations. It is originally made up of 34 partners based in 17 countries, drawn not only from nutrition science but also from industry, consumer groups, national nutrition societies and health professions. Based on previous experiences of the University of the United Nations (UNU) (40) and the International Life Sciences Institute (ILSI) in the WHO South-East Asia Region (41), EURRECA works towards a general framework including harmonized approaches, methods and key terms to be used for the development of micronutrient recommendations. This general framework will supply a basis for the use of micronutrient recommendations across countries/regions for establishing public and clinical health objectives, food and nutrition policies, and for addressing trade and regulatory issues (38). Further details on the network can be found in the article by Ashwell et al. (42).

Aims and objectives

One of the research activities within EURRECA aims to collate, compare and critically evaluate existing micronutrient recommendations for all population groups set by European countries/organizations. The objective of this paper is to give an overview of the availability of micronutrient recommendations in Europe and to provide information on the origin, concepts and definitions used, and population groups defined. Furthermore this paper shows the diversity in European recommended intakes of vitamin A and vitamin D, and aims to explore differences and commonalities in approaches that could possibly explain variations observed. These two nutrients were selected because vitamin D already had some attention in earlier stages of the EURRECA network and vitamin A was selected because much work on this nutrient was already done by the authors. In the near future other nutrients will be studied and more in depth comparisons will be made. The results of

these explorations will be used to identify gaps and opportunities on which subsequent activities within EURRECA can build.

METHODS

Data collation

To obtain a comprehensive overview of currently used concepts and methods in European countries, a questionnaire was developed by the Division of Human Nutrition of Wageningen University and Research Centre (WUR) in the Netherlands in cooperation with the Food, Consumer Behaviour and Health Research Centre of the University of Surrey in the United Kingdom. Questions addressed three stages of the process of micronutrient policy development. The first stage concerned the approach for setting micronutrient recommendations, while the other two investigated the process from micronutrient recommendations to nutrition policies and options and applications for public health policy. Only the first stage will be reported in this overview.

The questionnaire included open-ended questions on the process of setting up recommendations and close-ended questions on the people involved in the process and the type of evidence used. Each of the 11 questions addressed the nutrients considered to be most relevant to public health: vitamins A, D, E, C, B1 (thiamin), B2 (riboflavin), B3 (niacin), B6 (pyridoxine), B11 (folate) and B12 (cobalamin), and the minerals sodium, potassium, calcium, magnesium, iron, zinc, copper, phosphorus, selenium, and iodine.

Questionnaires were distributed among seven EURRECA partners, (University of Oslo (Norway), National Institute of Public Health (Czech Republic), Institute for Medical Research (Serbia), Warsaw Agricultural University (Poland), University College Cork (Ireland), WHO Regional Office for Europe, and WUR, in August 2007. Subsequently, these partners sent the questionnaires to key informants in the field of micronutrient recommendations to cover all European countries/regions. Key-informants were asked to fill out the questionnaire, if necessary with help of others, and return it in September 2007 also providing the latest report on nutrient recommendations. After the deadline had expired, the key-informants of the missing countries were followed up to increase response rate.

Data extraction

Both the completed questionnaires and the recommendation reports were used to extract micronutrient recommendations, information on their origin,

the approach used for deriving them, definitions and concepts, scientific evidence used, and population groups considered. Any unclear information given in the returned documents was re-checked with the key-informants to be sure that correct information was extracted.

Comparison of recommended intakes of vitamin A and vitamin D

As micronutrient recommendations usually consist of values, ranges, multiple values applying to one population group, (for example values for different activity levels), or additional amounts for sub groups (for example pregnant women), standardization procedures were defined to enable comparison of the recommendations. In case of multiple recommendations for one population group, the mean of all given values was used. In case of a range, the mid value was used. In cases where recommendations were not given in the most common unit, values were converted into that unit. Standardized recommended intakes of vitamin A and vitamin D were compared per sex at the ages 3m, 9m, 5y, 10y, 15y, 25y, 50y and 70y. These ages were selected because they indicate points of time in the different population groups as defined by countries. More population groups were defined for children and adolescents and therefore more ages between 0 and 18 years were selected. Also comparisons of recommendations for pregnant and lactating women were made. To depict the diversity between recommended intakes of vitamin A and vitamin D, boxplots were constructed in SPSS version 12.0.

In exploring commonalities and differences between micronutrient recommendations, background information extracted from the questionnaires and recommendation reports was compared focusing on the following items that could help to explain the commonalities and differences found:

1)-The concept of recommendation (ANR+2SD or AI),

2)-The year of publication of the recommendations (proxy for available evidence),

3)-Population groups for which recommendations were defined,

4)-Other methodological issues from the paradigm used to establish recommendations as selected criteria of adequacy or health endpoints (e.g. preventing deficiencies, plasma concentration), the type of evidence used (e.g. review of randomized controlled trials (RCT), experts' opinion), and assumptions made (e.g. physical activity, body weight, sunlight).

Information on the first three items was extracted from the recommendation reports and information on the last item came mainly from the questionnaires.

RESULTS

Data collation

Of the total 35 questionnaires sent out, 32 have been completed. No reaction after follow up was received from Montenegro and the Russian Federation. No reaction from Iceland was received due to a delay in sending the questionnaire.

From 31 European countries, WHO/FAO, and the EC the latest versions of reports on nutrient recommendations were collected, including varying amounts of background documentation. Reports from Republika Srpska (entity of Bosnia and Herzegovina) and Croatia could not be collected in time and reports from Federation of Bosnia and Herzegovina (entity of Bosnia and Herzegovina) and Montenegro were not received after follow up. No national report on nutrient recommendations has been published for the Czech Republic.

Available nutrient recommendations in Europe

Current publications on nutrient recommendations in Europe are listed in Table 2. Most of the reports were published since the year 2000. The oldest report dates from 1990 (Romania) and the most recent one is published in 2007 (Spain).

Table 2 also shows that 23 countries and WHO/FAO and the EC have their own reports on nutrient recommendations (own, own+adopted and own+shared). France, Latvia, the Netherlands, the United Kingdom, WHO/FAO and the EC defined their own recommendations (own) and 15 countries based their recommendations on those from other countries or organizations (own+adopted). The DACH-countries as well as the Nordic countries cooperated in setting recommendations, indicated by 'shared' in Table 2. Greece, Portugal, Estonia and Slovenia adopted recommendations from the EC, WHO/FAO, the Nordic countries and the DACH-countries respectively. The publication from which Albania and Federation of Bosnia and Herzegovina adopted their recommendations was not clear. The origin of recommendations was unknown for 2 countries (Romania and the Russian Federation).

Table 2 List of European and key non-European countries with published micronutrient recommendations and their recommendation report's origin

Country/ Organisation	Year published	Origin			Remark
		Own	Shared	Adopted	
Albania (3)	2005	x		x	Adopted from literature, especially from the Linus Pauling institute
Austria (4)	2000		x		Shared document with Germany and Switzerland (the DACH-countries)
Belgium (5)	2006	x		x	Based on WHO (2003) (44), EC (1990) [no ref], European countries that are geographically and culturally related to Belgium, e.g. The United Kingdom (1991) (35), the Netherlands (2001) (45) and France (2001) (13)
Federation of Bosnia and Herzegovina-[1] (6)	2004			x	Adopted from Food and Nutrition Board (1989) [no ref]
Republika of Srpska-[1] (7)	2005	x		x	Based on WHO/FAO-reports (46-49), and IOM (2003) (50)
Bulgaria (8)	2005	x		x	Based on IOM-reports (50) and WHO (2003) (44)
Croatia (9)	2004	x		x	Aligned with EU legislation
Czech Republic	Not published			x	Adopted from Nutrition Society [no ref] and EC (1993) (14)
Denmark (10)	2004		x		Shared document with Finland, Iceland, Norway, Sweden (Nordic countries)
Estonia (11)	2006			x	Adopted from the Nordic countries (2004) (10). Translated in local language
Finland (10, 12)	2004 2005	x	x		Shared document with Denmark, Iceland, Norway, Sweden (the Nordic countries) and translated into own country specific document
France (13)	2001	x			
Germany (4)	2000		x		Shared document with Austria and Switzerland (the DACH-countries)
Greece	1993			x	Adopted from EC (1993) (14)
Hungary (15)	2005	x		x	Based on EC (1993) (21) and IOM-reports (50)
Iceland (10,16)	2004 2006	x	x		Shared document with Denmark, Finland, Norway, Sweden (the Nordic countries) and translated into own country specific document; Own recommendations for vitamin D and calcium
Ireland (17)	1999	x		x	Adopted from EC (1993) (14) and the United Kingdom (1991) (35); Own recommendations for folate, iron, calcium, vitamin C
Italy (18)	1996	x		x	Based on Food and Nutrition Board (1989) (51) and EC (1993) (14)

Table 2 (continued)

Country/ Organisation	Year published	Origin			Remark
		Own	Shared	Adopted	
Latvia (19)	2001	x			
Lithuania (20)	2000	x		x	Adopted from the Nordic countries (1989) [no ref]; EC (1992) [no ref]; the United Kingdom (1991) (35), Food and Nutrition Board (1989) (51)
Montenegro	2002			x	Adopted from IOM (2001) (36)
Netherlands (21-23)	1992 2000 2003	x			
Norway (10, 24)	2004 2005	x	x		Shared document with Denmark, Finland, Sweden, Iceland) (the Nordic countries) and translated into own country specific document
Poland (25)	1996	x		x	Based on Food and Nutrition Board (1989) (51), the United Kingdom (1991) (35) and EC (1993) (14)
Portugal	2004			x	Adopted from WHO/FAO (2004) (26)
Romania (27)	1990	x		x	Partly based on WHO/FAO (2004) (26)
Russian Federation (28)	1992				No data on origin
Serbia (29)	1994	x		x	Adopted from unknown source(s)
Slovakia (30)	1997	x		x	Adopted from unknown source(s)
Slovenia (31)	2000			x	Adopted from the DACH-countries (4). Translated in local language
Spain (32)	2007	x		x	Adopted from unknown sources. Published as a book chapter
Sweden (10, 33)	2004 2005	x	x		Shared document with Denmark, Finland, Iceland, Norway (the Nordic countries) and translated into own country specific document
Switzerland (4)	2000		x		Shared document with Austria and Germany (the DACH-countries); Own recommendations for Iodine
FYR Macedonia (34)	2001	x		x	Based on recommendations of Former Republics of Yugoslavia (no ref) and the United Kingdom (35)
United Kingdom (35)	1991	x			
EC (14)	1993	x			
WHO/FAO (26)	2004	x			

1 Entities of Bosnia and Herzegovina
FYR Macedonia, Former Yugolslav Republic of Macedonia; EC, European Community; WHO/FAO, World Health Organization/Food and Agricultural Organization of the United Nations; No ref, No reference available
own = country specific recommendations developed
shared = recommendations set by one collaborative committee representing different countries
adopted = recommendations adopted from at least one other country/organization

Concepts and definitions to define micronutrient recommendations

Different terms have been used for the total set of nutrient recommendations (DRIs, DRVs RDAs etc). Within these sets, various terms have been used to express the different types of reference values. Though terminology differed substantially between countries (e.g. recommended nutrient intake, recommended daily amount of absorption, population reference intake), all these concepts could be considered as equivalent to the three basic concepts: ANR+2SD, AI and an acceptable range which is defined as a range of intakes high enough to avoid deficiency and low enough to avoid undesirable toxic effects (14). Only the Hungarian and Polish publication included a deviating term, which was defined as the intake level that is sufficient for 100% of the healthy population (Table 3). For Albania, Latvia, Romania, Slovakia, Spain, and the former Yugoslav Republic of Macedonia, the concept of the recommendation (ANR+2SD or AI) was not clear from the reports and questionnaires. In general, reports provided an ANR+2SD for most nutrients, but for sodium, potassium, selenium, copper, vitamin D, vitamin E, and magnesium, an AI was often provided instead.

Population groups

Age span

Table 4 lists the population groups encountered in the collated reports on nutrient recommendations. Deviations from generally defined population groups per country are given in foot notes. Table 4 shows that except for a few countries, most recommendations cover all ages. Exceptions include the Nordic countries, Italy, and the EC, which do not give recommendations for infants under 6 months, and Serbia provides values only for children ages 1 to 14 years. Lithuania covers people up to the age of 65 years.

Children and adolescents

The first year of life is split up in two to four age categories. For the age span of 1 to 18 years, the grouping of ages differs substantially: the number of age categories varies between four and six and different age cut-off points are used. All publications, except for the Netherlands, start to separate recommendations for men and women between the age of 10 to 15 years. The report from the Netherlands provides gender-specific separate recommendations from the age of 1 year, although actual recommendations for men and women do not differ for all age groups and nutrients.

Table 3 Description of currently available recommendations across Europe

Country	Type of recommendation used	Micronutrients
Albania (3)	Insufficient information	All
Belgium (5)	• ANR+2SD • AI/AR	AI/AR for vitamin D, sodium, potassium, iodine, copper, other ANR+2SD
Bulgaria (8)	• ANR+2SD • AI	AI for calcium, sodium, potassium, other ANR+2SD
DACH-countries (4)	• ANR+2SD • AI/AR	AI/AR for vitamin E, sodium, potassium, copper, selenium, other ANR+2SD
France (13)	• ANR+2SD • AI	AI for vitamin D, vitamin E, folate, riboflavin, thiamin, selenium, iodine, other ANR+2SD
Hungary (15)	• Safe intake: Intake level that fulfils the need of 100% of the population • Maximum intake :insufficient information	Maximum intake for sodium , other safe intake
Ireland (17)	• ANR+2SD	All
Italy (18)	• ANR+2SD • AR	AR for vitamin E, sodium, magnesium, other ANR+2SD
Latvia (19)	• Insufficient info provided	All
Lithuania (20)	• ANR+2SD	All
Netherlands (21-23)	• ANR+2SD • AR • AI	ANR+2SD for vitamin B6, vitamin B12, folate, thiamin, riboflavin, niacin; AR for magnesium, zinc, copper, phosphorus, selenium; other AI
Nordic countries (10)	• ANR+2SD	All
Poland (25)	• ANR+2SD • Recommended intake: Intake level that fulfils the need of 100% of the population. • AI/AR	AI/AR for copper, sodium, potassium, other ANR+2SD and recommended intake
Romania (27)	• Insufficient information	All
Russian Federation (28)	• Insufficient information • AI	AI for copper
Serbia (29)	• ANR+2SD	All
Slovakia (30)	• Insufficient information	All
Spain (32)	• Insufficient information	Insufficient information provided
FYR Macedonia (34)	• ANR+2SD	All (ranges for copper, selenium, sodium, potassium)
United Kingdom (35)	• ANR+2SD • AI/AR	AI/AR for copper, iodine, potassium, selenium, sodium, , other ANR+2SD
EC (14)	• ANR+2SD • AR	AI/AR for vitamin D, sodium, magnesium, other ANR+2SD
WHO/FAO (26)	• ANR+2SD • Safe intake: intake level that prevents clinical signs of deficiency and allows normal growth, but is does not protect vitamin A status during prolonged periods of infection or other deceases • AI	Safe intake for vitamin A; AI for vitamin E, other ANR+2SD

DACH-countries, Germany, Austria and Switzerland; Nordic countries, Norway, Sweden, Finland, Denmark and Iceland; FYR Macedonia, former Yugoslav republic of Macedonia; EC, European Community; WHO/FAO, World Health Organization/Food and Agricultural Organization; ANR+2 SD, Average Nutrient Requirement plus two standard deviations (=Recommended intake); AR, Acceptable Range; AI, Adequate Intake

Table 4 Characteristics of population groups as observed in Recommendation reports of different European countries

country	age span	age groups 0-12 m	age groups 1-18 y	age groups adults (y)	upper age group (y)	Other Characteristics
Albania-a (3)	all ages	0-6, 7-12	1-3, 4-8, 9-13, 14-18 (m/f)	≥19 (m/f)	≥19	P and L: age ≤18y, ≥19y
Belgium-b (5)	all ages	0-12	1-3, 4-6, 7-10, 11-14, 15-18 (m/f)	19-59 (m/f), ≥60 (m/f)	≥60	P, L
Bulgaria-c (8)	all ages	0-5, 6-12	1-2, 3-6, 7-9, 10-13 (m/f), 14-18 (m/f)	19-29 (m/f), 30-59 (m/f), 60-75 (m/f), ≥76 (m/f)	≥76	P and L: age ≤18y, ≥19y
DACH-d (4)	all ages	0-3, 4-12	1-3, 4-6, 7-9, 10-12, 13-14 (m/f), 15-18 (m/f)	19-24 (m/f), 25-50 (m/f), 51-64 (m/f), ≥65 (m/f)	≥65	P: ≥4months, L
Estonia (11)	≥ 6 m	6-12	1, 2-5, 6-9, 10-13 (m/f), 14-17 (m/f)	18-30 (m/f), 31-60 (m/f), 61-74 (m/f), ≥75 (m/f)	≥75	P, L
France-e (13)	all ages	0-12	1-3, 4-6, 7-9, 10-12, 13-15 (m/f), 16-19 (m/f)	20-74 (m/f), ≥75	≥75	P, L
Hungary (15)	all ages	0-6, 7-24	2-3, 4-6, 7-10, 11-14 (m/f), 15-18 (m/f)	19-30 (m/f), 31-60 (m/f), ≥61	≥61	P, L
Ireland (17)	all ages	0-3, 4-6, 7-9, 10-12	1-3, 4-6, 7-10, 11-14 (m/f), 15-17 (m/f)	18-64 (m/f), ≥65 (m/f)	≥65	P: second half, L: first 6 months
Italy-f (18)	≥ 6 m	6-12	1-3, 4-6, 7-10, 11-14, 15-17 (m/f)	18-29 (m/f), 30-59 (m), 30-49 (f), ≥60 (m), ≥50 (f)	≥60 (m), ≥50 (f)	P, L
Latvia-g (19)	all ages	0-6, 7-12	1-3, 4-6, 7-10, 11-14, 15-18	≥19	≥19	P, L
Lithuania (20)	≤ 64 y	0-3, 4-6, 7-9, 10-12	1-3, 4-6, 7-10, 11-14 (m/f), 15-18 (m/f)	19-34 (m/f), 35-49 (m/f), 50-64 (m/f)	50-64	PAL: 4 levels (m/f) and 2 body weights (m/f) P and L: 4 PAL levels and 2 body weights.
Netherlands-h (21-23)	all ages	0-5, 6-12	1-3 (m/f), 4-6 (m/f), 7-9 (m/f), 10-12 (m/f), 13-15 (m/f), 16-18 (m/f)	19-21 (m/f), 22-49 (m/f), 50-64 (m/f), ≥65 (m/f)	≥65	P, L
Nordic countries-i (10)	all ages	0-5, 6-12	1, 2-5, 6-9, 10-13 (m/f), 14-17 (m/f)	18-30 (m/f), 31-60 (m/f), 61-74 (m/f), ≥75 (m/f)	≥75	P, L
Poland (25)	all ages	0-5, 6-12	1-3, 4-6, 7-9, 10-12 (m/f), 13-15 (m/f), 16-18 (m/f)	19-25 (m/f), 26-59 (m/f), ≥60 (m/f)	≥60	PAL: 3 levels (ages 19-59y)P, L,
Romania (27	all ages	0-12	1-3, 4-6, 7-9, 10-12, 13-15 (m/f)	16-19 (m/f), 20-45 (m/f), 46-62 (m), 46-60 (f), ≥63 (m), ≥61 (f)	≥63 (m), ≥61 (f)	PAL: 3 levels (ages 20-60y)
Russian Federation-j (28)	all ages	0-3, 4-6, 7-12	1-3, 4-6, 7-10, 11-13 (m/f), 14-17 (m/f)	18-29 (m/f), 30-39 (m/f), 40-59 (m/f), 60-74 (m/f), ≥75 (m/f)	≥75	P, L: age <7m, ≥7m, PA: 5 levels (m), 4 levels (f) (ages 18-59y)
Serbia-k (29)	1-14 y	not set	1, 2, 3-4, 5-6, 7-9, 10-11 (m/f), 12-14 (m/f)	not set	12-14	daily needs group, preschool-and-school institution group
Slovakia (30)	all ages	0-6, 7-12	1-3, 4-6, 7-10, 11-14 (m/f), 15-18 (m/f)	19-34 (m/f), 35-59 (m), 60-74 (m), 35-54 (f), 55-74 (f), ≥75 (m/f)	≥75	P, L, PAL: 4 levels (ages 19-59y (m) and 19-54y (f)), 2 levels (ages 15-18y)
Spain-l (32)	all ages	0-5, 6-12	1-3, 4-5, 6-9, 10-12 (m/f), 13-15 (m/f)	16-19 (m/f), 20-39 (m/f), 40-49 (m/f), 50-59 (m/f), ≥60 (m/f)	≥60	P: second half, L

Table 4 continued

country	age span	age groups 0-12 m	age groups 1-18 y	age groups adults (y)	upper age group (y)	Other Characteristics
The FYR Macedonia (34)	all ages	0-3, 4-6, 7-9, 10-12	1-3, 4-6, 7-10, 11-14 (m/f), 11-14 (m/f), 15-18 (m/f)	19-24 (m/f), 25-50 (m/f), 51-64 (m/f), ≥65 (m/f)	≥65	preschool-in-kindergarten and school institution group P, L
United Kingdom-m (35)	all ages	0-3, 4-6, 7-9, 10-12	1-3, 4-6, 7-10, 11-14 (m/f), 15-18 (m/f)	19-50 (m/f), ≥51 (m/f)	≥51	P, L: >4months, <4months
EC-n (14)	≥ 6 m	6-12	1-3, 4-6, 7-10, 11-14 (m/f), 15-17 (m/f)	≥18 (m/f)	≥18	P, L
WHO/FAO-o (26)	all ages	0-6, 7-12	1-3, 4-6, 7-9, 10-18 (m/f)	19-50 (m/f), 51-65 (m/f), ≥66	≥66	P, L

Croatia, Federation of Bosnia and Herzegovina and Republika of Srpska (entities of Bosnia and Herzegovina) and Montenegro are excluded from the table because no recommendation report was available for the author and information could not be extracted from the questionnaire. The Czech Republic was excluded due to lack of published report.
Nordic countries = Denmark, Finland, Iceland, Norway, Sweden; The FYR Macedonia = The former Yugoslav Republic of Macedonia; EC = European Commission; WHO/FAO = World Health Organization/Food and Agricultural Organization, m = men; f = women, m/f = recommendations defined for men and for women separately; these recommendations do not have to differ between men and women, P = pregnancy, L =lactation, PAL = physical activity level, n.a. = not applicable
a-Vit D, E, B12, folate, sodium, potassium, calcium, magnesium, iron, copper, phosphorus, selenium, iodine: no separate recommendation for men and women. Vit D, E, B6, B12, folate, riboflavin, thiamin, niacin, iron (P), sodium, potassium, selenium, copper, iodine: no separate age groups for P and L. Magnesium: ≤18y, 19-30y, ≥ 31 for P and L. Vit D: 19-50y, ≥71y. Vit B6, B12, calcium: 19-50y, ≥51y (vit B6 (19-50y), vit B12, calcium: no separate recommendations for men and women). Sodium: 19-50y, 51-70y, ≥71y and no separate recommendations for men and women. Magnesium: 19-30y, ≥31y and for 14-18y no separate recommendations for men and women.
b-Minerals: 0-5m, 6-11m. Trace elements: 0-3m, 4-5m, 6-11m. Iron (11-14y, 15-18y and 19-59y): separate recommendation for menstruating/non-menstruating women. Sodium (0-5m, 6-11m): based on weight.
c Vit C: separate recommendation for smokers.
d Vit D, C, B12, folic acid, phosphorus, copper, selenium and Vit B6 (10-12y,13-14y): no separate recommendation for men/women. Vit E, Iodine (1-3y, 4-6y, 7-9y): separate recommendation for men/women. Iron: separate recommendation for menstruating/non-menstruating women. Vit C: separate recommendation for non-smokers/smokers. Calcium, phosphorus, magnesium: separate recommendation for P and L for <19y and ≥ 19y.
e Minerals: 1-3y, 4-6y, 7-9y, 10-12y, 13-19y (m/f), 20-65y (m/f), 66-74y (m), 56-74 (f), ≥75y. Vit C: separate recommendation for smokers above 10 cigarettes/day and for pregnant women in the 3rd trimester.
f Iron: separate recommendation for menstruating/non-menstruating women. Calcium (≥50y): separate recommendation for post-menopausal women with/without oestrogen therapy.
g Minerals (≥19): separate recommendation for men and women.
h Calcium, vit D, thiamin, riboflavin, niacin: 4-8y, 9-13y, 14-18y, 19-50y, 51-70y, >70y. Vit B6: 4-8y, 9-13y, 14-18y, 19-50y, ≥51y. Vit B12, folate: age groups: 4-8y, 9-13y, 14-18y, ≥19y. Iron, zinc: for pregnancy separate recommendation for 1st/2nd/3rd trimester. Copper: pregnancy 3rd trimester. Riboflavin, thiamin: separate recommendation for men and women. Calcium, phosphorus, zinc, Vit B6 (0-5m): separate recommendation for breast-feeding/bottle feeding. Vit D: separate recommendation for no exposure to sunlight/light coloured skin/remain outdoor for at least 15 min a day with at least hands and face uncovered. Vit D: separate recommendation for 51-60y and 61-70y. Vit A: recommendation per gram PUFA. Zinc (>3m): separate recommendation.
i Vit D: separate recommendation for infants >4weeks and elderly people with little or no sun exposure. Folate (31-60y): separate recommendation for women in reproductive age/not in reproductive age. Iron: recommendation according to meal composition, and separate recommendation for post-menopausal women. Zinc: separate recommendation for vegetarians. Calcium, phosphorus (18-20y): separate recommendation.
j Copper: 0-5m, 7-10y, 11-17y, ≥18y.
k Minerals: no separate recommendation for men and women.
l Folic acid: recommendation for 1st and 2nd half of pregnancy.
m Vit D (≥51y): separate recommendation for ≤65y / >65y. Thiamin: recommendations for last trimester of pregnancy only.
n Thiamin, niacin, vit B6: recommendation according to body weight, energy or protein intake. Thiamin: recommendation for >10 weeks of pregnancy. Iron (≥18y): separate recommendations to cover of 96% and 90% of the population and postmenopausal women.
o Minerals: recommendation for 1st, 2nd, 3rd trimester of pregnancy, and 0-3m, 3-6m, 7-12m of lactation. Iodine: <5y, 6-12y, 13-18y (m/f). Zinc: separate recommendation for 3 levels of bioavailability. Zinc (0-12m): separate recommendation for breast feeding/bottle feeding. Iron: separate recommendation for 4 levels of bioavailability. Iron: separate recommendation for pre-menarche/post menarche. Calcium: separate recommendation for breast feeding/cow milk-feeding. Magnesium: separate recommendation for breastfeeding/formula feeding. Vit B6 (51-65y,m): separate recommendation for 19-50y / >50y. Vit D (51-65y, m): separate recommendation for 19-50y / 51-65y.

Adults

The number of age categories defined for adults varies from one to five but most reports include recommendations for four age categories. Discrepancies also emerge for the higher age levels. Most often the highest age group is ≥ 60, ≥ 65, or ≥ 75 years of age. The United Kingdom, however, have ≥ 51 years as an upper age group, Italy defined ≥ 51 years for women and ≥ 60 years for men, both Albania and Latvia defined the upper age group as ≥ 19 years and the EC as ≥ 18 years. All countries, except Latvia, give recommendations separately for men and women, although Latvia does separate recommendations on minerals for the two sexes.

Other criteria considered in setting micronutrient recommendations

All countries except Romania provide separate micronutrient recommendations for pregnant and lactating women. The Albanian and the Bulgarian recommendations for both pregnant and lactating women have been further split up in ≤ 18 years and ≥ 19 years. The United Kingdom and the Russian Federation split up the group of lactating women according to the period of lactation (≤ 4 months and > 4 months for the United Kingdom and < 7 months and ≥ 7 months for the Russian Federation).

Some countries distinguish physical activity levels (PAL) per age group. Lithuania provides micronutrient recommendations for four physical activity levels and two different body weights per age category and sex. The Russian Federation splits up the recommendations for adults in five physical activity levels for men (1.4, 1.6, 1.9, 2.2, and 2.5), and four levels for women (1.4, 1.6, 1.9, and 2.2). Poland, Romania and Slovakia distinguish three physical activity levels: low, moderate and high. In addition Slovakia also gives recommendations for students with and without physical workload. For specific nutrients other subgroups are sometimes distinguished, for example for vitamin C, Bulgaria and the DACH-countries provide a separate recommendation for smokers. Other characteristics influencing requirements that are mentioned in recommendations for specific nutrients are: sunlight exposure, skin colour, menstrual blood loss, energy intake (especially for thiamin, riboflavin, and niacin), protein intake (vitamin B6) or fat intake (vitamin E) and bioavailability for the nutrients iron and zinc.

Diversity in recommended intakes of vitamin A and vitamin D

Vitamin A

Figure 1 shows box plots that illustrate the variability in recommended vitamin A intakes in Europe separately for men and women. Supplemental Tables S1 and S2 present the current available standardized recommended vitamin A intakes (retinol equivalents, RE) by country and sex.

The median recommendation on intake of vitamin A for men and women are the same up to the age of 10 years, 400 µg for ages 3 and 9 months, 450 µg for age 5 and 650 µg for age 10. For the other ages the median recommendation for women is 800 µg (15 years) and 700 µg (>15 years), which is lower than for men (i.e. 900 µg for ages 15, 25 and 50 years and 850 µg for age 70 years).

Recommended vitamin A intakes from the EC, Ireland, Italy, and the United Kingdom are lower than the median for all ages for both men and women and recommendations from Belgium only for men. Recommendations higher than the median for all ages are found in reports of the DACH-countries, and Romania for both men and women.

For men the largest absolute difference in recommended vitamin A intakes between countries amounts to 500 µg RE/day (range at 10 years: 500-1000 µg; range at 15 years: 600-1100 µg). The smallest range is observed at age 3 months, i.e. 150 µg (range: 350-500 µg). For women, the largest variation is found at the ages 25 and 50y, with a range of 500-1000ug RE/day. The smallest difference is similar to that found for men. For pregnant women the median for recommended vitamin A intakes is 800 µg and for lactating women 1125 µg. The individual European recommendations range from 700 to 1100 µg and from 850 to 1500 µg for pregnant and lactating women respectively.

When comparing methodological approaches for recommended vitamin A intakes in Europe, all reports provide an ANR+2SD, except the Netherlands, which provide an AI (Table 2). The defined population groups in the different recommendations vary largely (Table 4). However, in each report recommended vitamin A intakes are mostly the same from the age of 15 up to 70 years. Comparing publication dates, Spain and the DACH-countries that were published more recently provided high values, although the relatively high recommendations from the Netherlands were published in 1992 and the low recommendations from WHO/FAO in 2004.

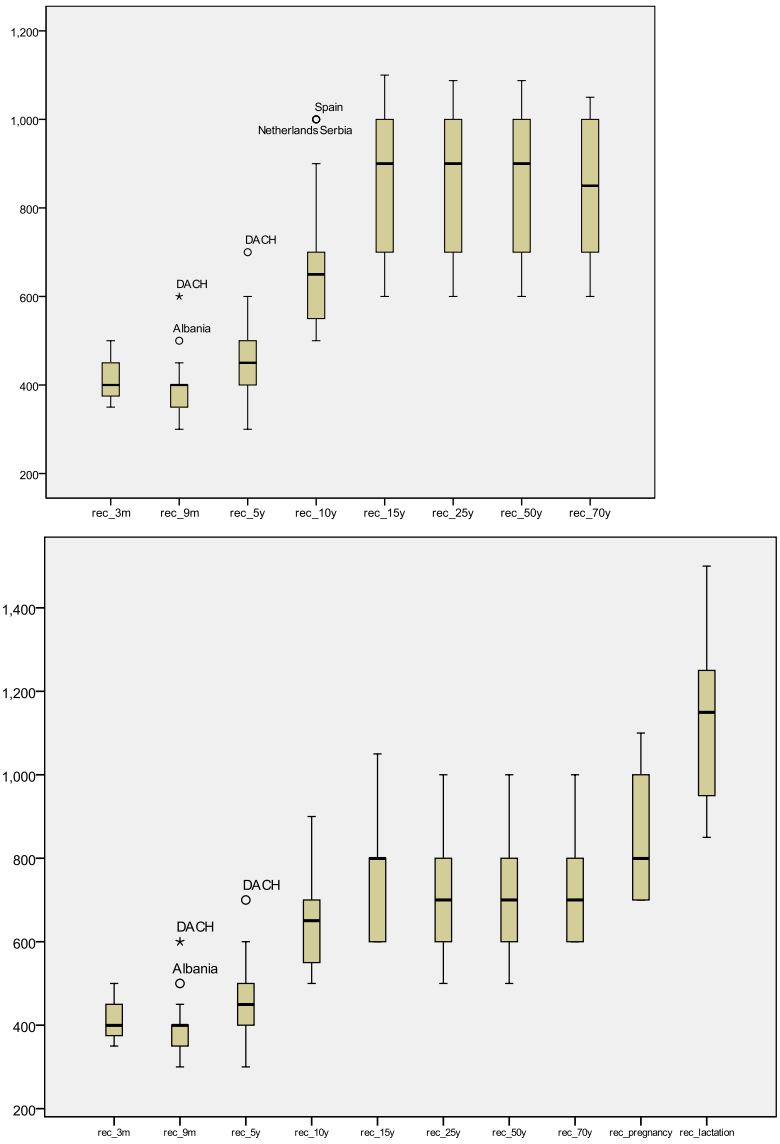

Figure 1 Diversity of recommended vitamin A intakes (µg retinol equivalents (RE)) for men (top) and women (bottom) in Europe for selected life stages (3m, 9m, 5y, 10y, 15y, 25y, 50y, 70y, (pregnancy, lactation))

The boxes indicate the interquartile range (IQR=x_{75}-x_{25}) in which the median (x_{50}) of all recommended vitamin A intakes is indicated by a horizontal line. Vertical lines connected to the upper and lower side of the box indicate values less than 1.5*IQR below the first quartile or above the third quartile. Values not included in this range are considered as an outlier and are indicated by open dots (>1.5*IQR above x_{75} or below x_{25}) and stars (>3*IQR above x_{75} or below x_{25}). DACH: Austria, Germany and Switzerland.

The criteria for adequacy or the health endpoints used for defining recommended vitamin A intakes were not always reported in the questionnaire responses or background documents (data not shown). General health and preventing deficiencies were most frequently mentioned. In the Netherlands (high values) Belgium (values just below the median) and the DACH-countries (high values) an adequate reserve in the liver was the main functional criteria of nutritional adequacy. Most recommended vitamin A intakes are based on data from observational cohort studies, or expert committees. Information on the type of evidence on which recommendations were based was, however, not always clear from the questionnaire responses and the type of evidence was unknown for some countries (Lithuania, Serbia, The former Yugoslav Republic of Macedonia) (data not shown). If, and what, assumptions were made when defining recommended vitamin A intakes was not clear from the information provided in the questionnaires response or from the reports.

Vitamin D

Figure 2 and supplemental Tables S3 and S4 present an overview of the available standardized recommended vitamin D intakes. The median of recommended intakes for both men and women is 10 µg for ages 3 and 9 months, 5 µg for ages 5, 10, 25 and 50 years, 6.9 µg for age 15 years and 7.5 µg for age 70 years. No publication includes values that are below or higher than the median for all ages. The differences between countries are largest for infants in the age of 3 and 9 months, with values ranging from 5 µg (Albania, Bulgaria, the Netherlands and WHO/FAO) to 22.5 µg (France) and smallest for the ages of 5, 10, 15, 25 and 50 years with values ranging from 2.5 µg (the Russian Federation, the Netherlands) to 10 µg a day.
Similar recommended vitamin D intakes are given for pregnant and lactating women, varying from 5 to 11.3 µg, with a median of 10 µg. The former Yugoslav Republic of Macedonia gives the highest recommendation and Albania, Bulgaria, the DACH countries, Romania, and the WHO/FAO (5 µg) the lowest.

Most countries provide an ANR+2SD for vitamin D, except for Belgium (acceptable range and AI), the EC (acceptable range), France (AI), and the Netherlands (AI). These AIs and acceptable ranges are not higher over all ages than the other recommendations. The lowest recommended vitamin D

intakes were published by the Russian Federation and the United Kingdom in 1991 but also by the Netherlands in 2000.
In general, criteria for adequacy on which recommendations are based are 'health' and 'prevention of deficiency as measured by the serum level of 25-hydroxy vitamin D3'. 'Appropriate bone formation' is mentioned as a criterion for adequacy in the questionnaire from Belgium, Italy, and the Netherlands (data not shown). Recommended vitamin D intakes appear to be based most often on expert's opinion or on values that are borrowed from another country. Several countries reported that some assumptions were made when setting up recommended vitamin D intakes: the Italian recommendations are based on the assumption that in the Italian environment sun exposure guarantees adequate physiological vitamin D production. They provide a range starting from 0 µg for people with an adequate sun exposure. The upper level of the range applies to people without sunlight exposure (10 or 25 µg depending on the age). Also recommendations from the United Kingdom for adults are 0 µg a day, based on the assumption that sun exposure will provide the amount sufficient for an adequate vitamin D status during summer and allow for stores to be laid down to support vitamin D status in winter. The Netherlands define normal exposure to sunlight as daily 15 minutes with at least hands and face uncovered, whereas the Nordic countries assume that exposure of the face, arms, hands, and legs to sunshine for 6-8 minutes, 2 to 3 times a week is more than adequate to satisfy the vitamin D requirements. However, they indicate that dietary vitamin D is essential to ensure satisfactory vitamin D at northern latitudes. In setting vitamin D recommendations for the Polish population sunlight exposure was considered to be too diverse between adults and therefore no recommended intake for adults was set (data not shown).

DISCUSSION

The results from this comparison of micronutrient recommendations show similarity in concepts and definitions used to establish recommendations on micronutrient intake in European countries, but also considerable diversity in defined population groups and levels of current recommendations was observed as illustrated by two vitamins. In exploring elements of the paradigm, differences between publications in criteria of adequacy, type of evidence used, and assumptions made, were recognized that could explain disparities in recommendations between countries

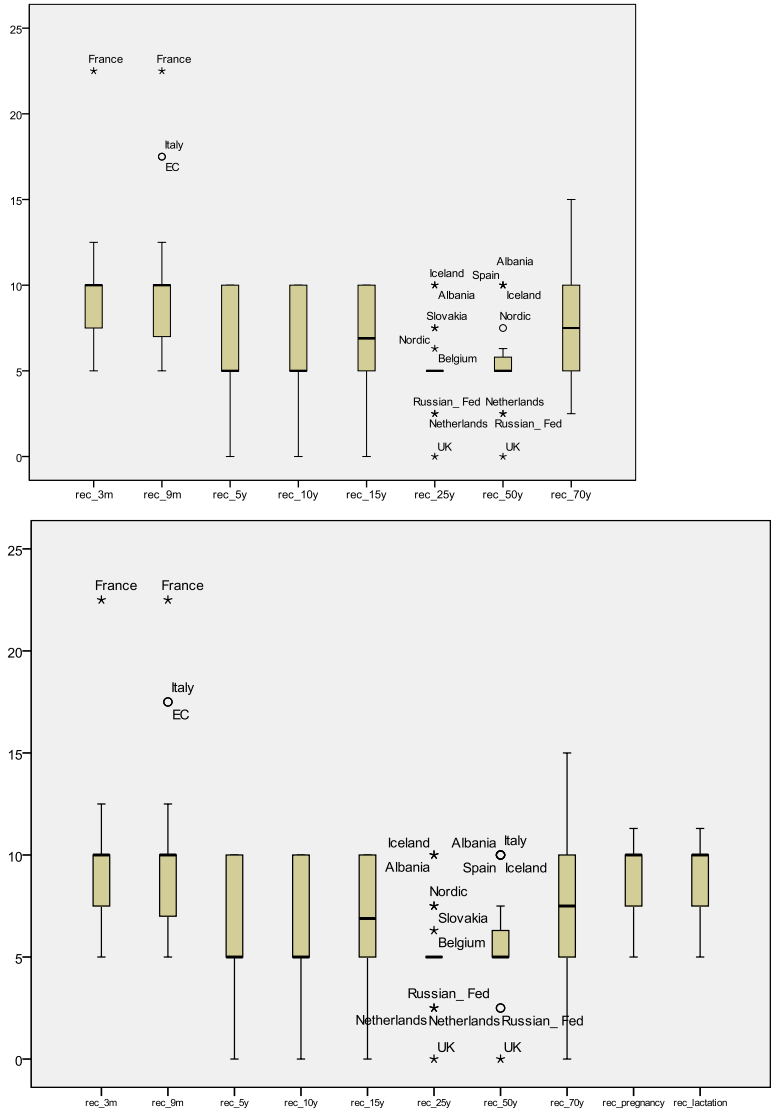

Figure 2 Diversity in recommended vitamin D intakes (µg) for men (top) and women (bottom) in Europe for selected life stages (3m, 9m, 5y, 10y, 15y, 25y 50y 70y (pregnancy, lactation))

The boxes indicate the interquartile range (IQR=x_{75}-x_{25}) in which the median (x_{50}) of all vitamin D recommendations is indicated by a horizontal line. Vertical lines connected to the upper and lower side of the box indicate values less than 1.5*IQR below the first quartile or above the third quartile. Values not included in this range are considered as an outlier and are indicated by open dots (>1.5*IQR above x_{75} or below x_{25}) and stars (>3*IQR above x_{75} or below x_{25}).
Russian_fed: Russian Federation; Nordic: Norway, Sweden, Finland, Denmark and Iceland; EC: European Community; UK: United Kingdom.

Availability of current micronutrient recommendations

Many countries (22 out of 33) adopted recommendations from other publications, most frequently from the EC, WHO/FAO and IOM (45, 43, 53). The EC report (14) was meant to provide practical advice and recommendations for a number of purposes including nutrition labelling and Community programmes on research and nutrition. The Scientific Committee for Food of the EC tried to harmonize existing national reports and also to include the most recent data. The WHO/FAO and the IOM publications both contain dietary reference values based on extensive scientific evidence evaluated by a large number of experts from all over the world or the United States/Canada respectively. They constituted an important source of information for all who work in the areas of nutrition, agriculture, food production and distribution, and health promotion (26, 50).

Due to the continuous changes in scientific knowledge, revisions of recommendations should be planned for every 5 to 10 years in order to keep them updated in the light of the most up-to-date scientific evidence (38, 52). In view of this, the present overview of recommendations is time bound, as some countries have already planned to revise their recommendations soon. Belgium, the Republika Srpska (entity in Bosnia and Herzegovina), the Czech Republic, Italy, Lithuania, Poland, Slovakia, the Netherlands and the United Kingdom will have some or all of their recommendations revised or set before 2010. The EC publication dates from 1993 and one of the tasks of the European Food Safety Authority (EFSA) is to update this advice from the Scientific Committee on Food on PRI's. The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) intends to start revising the micronutrients PRIs in 2008. EURRECA on the other hand works towards a general framework including harmonized approaches, methods and key terms to be used for the development of micronutrient recommendations. Therefore EURRECA will work in close collaboration with EFSA, to improve the process and scientific basis on which micronutrient recommendations for European populations can be developed.

Concepts and definitions

One of the factors that could explain differences between countries are the concepts and definitions that were used. However, all countries or regions included in this paper provided recommendations that were based on an ANR with the recommended intake usually defined as 2SD above the

average. In the comparison of recommended vitamin A intakes it appears that all publications provide an ANR+2SD, except for the Netherlands that provide an AI (Table 2). By definition an AI may be higher than the ANR+2SD and therefore could explain the relatively higher values from the Netherlands. Most countries provide an ANR+2SD for vitamin D, but the recommendations of countries that provide an AI or an acceptable range for vitamin D were not higher over all ages than the others.

Population groups in current micronutrient recommendations

To estimate the nutrient requirement of a specific population, first one needs data on requirements of a population with similar characteristics (38). The definitions of age groups, each considered as relatively homogenous with regard to nutrient requirement, differ between countries, especially during childhood, puberty and at older age. This may be due to differences in reasoning in defining population groups; however in most publications these arguments are not clearly described. An example of an argument is given in the EC report that provides only one age group for adults (≥ 18 years) (14). The report states that although elderly are prone to suffer from deficiencies due to a reduced food intake, inability to care for themselves or illness causing malnutrition, there is no evidence that micronutrient requirements of the elderly differ from those of middle-aged adults. Except for vitamin D the EC provides no different values for elderly. On the contrary, the Netherlands (22, 23), defined four age groups in adulthood, 19-21, 22-49, 50-64, and ≥ 65 years, using reference weights and heights from a representative sample of the population, and in doing so they follow the IOM (50). These examples illustrate the differences in underpinning the definition of population groups.

In general, when data specific to physiological state are not available to estimate nutrient requirements, extrapolation from other growth states or a factorial approach, which estimates nutrient requirement based on the expected nutrient losses via e.g. urine, feces, and skin and accounting for differences in assumed bioavailability, are used instead (52, 53). For some population groups, especially infants, children, adolescents, elderly, post-menopausal women, pregnant and lactating women, nutrient requirements are often extrapolated from the recommendation applying for adults. For the recommendations included in this overview it is not clear whether values are originally based on average requirements of the population group, or if they

are based on requirements estimated by the factorial approach or extrapolation. Only for infants (0 to 1 years) it is often indicated that nutrient values are extrapolated from the composition of breast milk. Prentice et al. (39) showed that the wide differences in perceived nutrient requirements between countries might be partly attributed to real physiological and environmental differences, but were mostly due to the differences in judgements about the best methodological approach to use and in the way theoretical approaches were applied. Unless sufficient data on nutrient requirements will be available for all life-stage groups some time, extrapolation from one group to another is necessary. The scientific basis for the method chosen should be completely transparent and thoroughly described for each nutrient and life stage group (53). Atkinson and Koletzko (53) recommend that for the harmonization of dietary reference values, standardization of age groups should be biologically based (growth and pubertal stages) with consideration of relevant developmental milestones throughout childhood. This requires agreement and transparency on which data to use concerning growth standards, body sizes and composition, fetal and maternal accretion in pregnancy and milk composition, and on inclusion of appropriate adjustments (metabolic efficiency, weight change or physical activity).

In the comparison of recommended intakes of vitamin A and vitamin D in this paper, values were mostly the same for the age of 15 up to 70 years within publications. Therefore it seems unlikely that differences in population groups are a key issue for disparities between publications. The definition of population groups might be an issue for other nutrients, but this needs to be studied further.

A few countries, Lithuania, Latvia, Poland, Romania and Slovakia, provide recommendations for different physical activity levels. An explanation could be that these countries are all from Eastern Europe where lifestyles might be less sedentary than in Western Europe (54).

Criteria for adequacy, assumptions and type of evidence

As they may explain differences between recommendations, questions on the criteria/endpoint(s) used to determine adequate intake per nutrient were included in the questionnaire. This information was, unfortunately, often not included in the received reports. However, the answers were often

formulated in a very general manner, for example 'health' or 'prevention of deficiencies'. This limited the comparison of criteria for adequacy. In all probability the question as included in the questionnaire was not clearly enough formulated, resulting in answers that were too less informative.

It is obvious that estimated requirements may vary with the endpoint or criteria for adequacy chosen. Nutrients have multiple sites of action in human metabolism and therefore it is possible to demonstrate abnormal function in one parameter measured or observed as a result of inadequate intake of a nutrient, while other parameters requiring the same nutrient intake appear adequate. For example a nutrient requirement based on the amount that prevents the clinical symptoms of a nutrient deficiency will be lower than one based on the amount that sustains nutrient stores or reserves (38). Nutrient adequacy is a matter of definition, and may be a policy decision. Thus it is possible to have multiple requirements, each corresponding to a different indicator or criterion of adequacy. It is then up to nutrition and public health policy planners to determine which level of adequacy is desirable or possibly attainable in the population group of interest (52).

Besides criteria for adequacy, the type of evidence used when establishing recommendations could explain differences between recommendations. In the questionnaire informants were asked to indicate the type of evidence that was used for each nutrient against a list with different possibilities (including RCT's, observational studies, mechanistic studies, experts' opinion, and borrowed from another country). Experts' opinion was the most frequently given answer, which unfortunately does not reveal on which types of evidence the experts have based their opinion, and how. Although some reports list all the experts that were included in the working groups, for the countries without clear background reports, this information was often missing.

The year of publication was used as a proxy for the age of the available scientific evidence. Even though publication years varied between 1990 and 2006, we cannot conclude that the year of publication was related to the level of the recommendation: For both vitamin A and vitamin D high intakes were recommended in both relatively old and recent publications, and the same was true for low values.

Opportunities: What can EURRECA do with this information on the status quo?

From the results of this comparison of micronutrient recommendations across Europe, it became clear that the concepts and definition used for setting them is quite similar throughout Europe. But even though many countries adopt and adapt recommendations from other publications, disparities remain. In our search for possible explanations for these disparities, we aimed at comparing different aspects of underlying approaches for setting up micronutrient values, but unfortunately there was a lack of transparency and completeness of the available information. An important opportunity for EURRECA is to develop tools to show how micronutrient recommendations can be devised for different population groups in a transparent manner. These tools should provide guidance on how to interpret data, how to take into account different criteria for adequacy and how to weigh different types of evidence in defining requirements. Expert committees throughout Europe can then use these tools to make decisions in a harmonized way and provide transparently based micronutrient recommendations.

In the EC call which led to the commissioning of EURRECA, vulnerable groups were identified as population groups that are prone to extremes of intake and those identified were: infants, children, adolescents, adults, pregnant and lactating women, post-menopausal women, elderly, immigrants and low income groups. Our overview shows that immigrants, low-income, post-menopausal women were never a specified target groups in any recommendation report. The only exception is iron recommendations for post menopausal women. Within EURRECA variation in micronutrient needs based on micronutrient status, functional status and especially health and physiological status will be identified and parameters (biomarkers) for vulnerability will be explored. Based on this information population groups to be considered vulnerable will be identified. Guidance can then be given on whether and in what way micronutrient recommendations should be extended to these groups.

CONCLUSIONS

This paper provides an overview of the availability of micronutrient recommendations in Europe and provides information on the origin, concepts and definitions used, and population groups defined used for

setting current micronutrient recommendations in Europe. A comparison of recommended intakes of vitamin A and vitamin D is included in order to explore possible explanations for disparities between publications. The large variation in current recommendations for population groups as illustrated for vitamin A and vitamin D strengthens the need for guidance on setting evidence based, up-to-date European recommendations. Differences in health endpoints, studies used to set recommendations, experts' opinions and assumptions are all likely to contribute to the identified variation, but the background information we collated does not allow us to disentangle the relative contribution of these different aspects due to lack of transparency. EURRECA has an excellent opportunity to develop tools to improve transparency on the approaches used, including the selection of criteria for adequacy, weighing of evidence, and interpretation of data to support those who develop quality assured, evidence-based harmonized micronutrient recommendations across Europe and elsewhere.

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Table S1 Overview of recommended vitamin A intakes (µg RE) for selected population groups in Europe: men

Country	3m	9m	5y	10y	15y	25y	50y	70y
Albania [3]	400	500	400	600	900	900	900	900
Belgium [5]	350	350	400	500	700	700	700	700
Bulgaria [8]	375	400	450	600	800	800	800	800
DACH [4]	500	600	700	900	1100	1000	1000	1000
France [13]	350	350	450	550	700	800	800	800
Hungary [15]	420	400	500	700	1000	1000	1000	1000
Ireland [17]	350	350	400	500	700	700	700	700
Italy [18]		350	400	500	700	700	700	700
Latvia [19]	375	375	500	700	1000	1000	1000	1000
Lithuania [20]	420	400	500	700	1000	800	800	800
Netherlands [21]	450	400	500	1000	1000	1000	1000	1000
Nordic countries [10]		300	350	600	900	900	900	900
Poland [25]	450	450	500	600	700	700	700	700
Romania [27]	450	450	600	900	1050	1087.5	1087.5	1050
Russian Federation [28]	400	400	500	700	1000	1000	1000	1000
Serbia [29]			500	1000				
Slovakia [30]	400	400	500	700	1000	950	950	850
Spain [32]	450	450	300	1000	1000	1000	1000	1000
The FYR Macedonia [34]	375	375	400	700	1000	1000	1000	1000
United Kingdom [35]	350	350	400	500	700	700	700	700
EC [14]		350	400	500	700	700	700	700
WHO/FAO [26]	375	400	450	600	600	600	600	600

* DACH countries = Austria, Germany, Switzerland; Nordic countries = Denmark, Finland, Iceland, Norway, Sweden; The FYR Macedonia = The former Yugoslav Republic of Macedonia; EC = European Commission; WHO/FAO = World Health Organization/Food and Agricultural Organization.

Table S2 Overview of recommended vitamin A intakes (µg RE) for selected population groups in Europe: women

Country	3m	9m	5y	10y	15y	25y	50y	70y	pregnancy	lactation
Albania [3]	400	500	400	600	700	700	700	700	760	1250
Belgium [5]	350	350	400	500	800	600	600	600	700	950
Bulgaria [8]	375	400	450	600	700	700	700	700	775	1150
DACH [4]	500	600	700	900	900	800	800	800	1100	1500
France [13]	350	350	450	550	600	600	600	600	700	950
Hungary [15]	420	400	500	700	800	800	800	800	1000	1200
Ireland [17]	350	350	400	500	600	600	600	600	700	950
Italy [18]		350	400	500	600	600	600	600	700	950
Latvia [19]	375	375	500	700	1000	1000	1000	1000	1100	1300
Lithuania [20]	420	400	500	700	800	800	800	800	1000	1200
Netherlands [21]	450	400	500	800	800	800	800	800	1000	1250
Nordic countries [10]		300	350	600	700	700	700	700	800	1100
Poland [25]	450	450	500	600	600	600	600	600	950	950
Romania [27]	450	450	600	900	1050	950	950	900	900	900
Russian Federation [28]	400	400	500	700	800	900	900	800	1100	1300
Serbia [29]			500	800						
Slovakia [30]	400	400	500	700	900	850	850	800	1100	1200
Spain [32]	450	450	300	800	800	800	800	800	800	1300
The FYR Macedonia [34]	375	375	400	700	800	800	800	800	1000	1200
United Kingdom [35]	350	350	400	500	600	600	600	600	700	950
EC [14]		350	400	500	600	600	600	600	700	950
WHO/FAO [26]	375	400	450	600	600	500	500	600	800	850

DACH countries = Austria, Germany, Switzerland; Nordic countries = Denmark, Finland, Iceland, Norway, Sweden; The former YR Macedonia = The former Yugoslav Republic of Macedonia; EC = European Commission; WHO/FAO = World Health Organization/Food and Agricultural Organization.

Table S3 Overview of recommended vitamin D intakes (µg) for selected population groups in Europe: men

Country	3m	9m	5y	10y	15y	25y	50y	70y
Albania [3]	5	5	5	5	5	10	10	
Belgium [5]	12.5	12.5	7.5	6.3	6.3	6.3	6.3	10
Bulgaria [8]	5	5	5	5	5	5	5	10
DACH [4]	10	10	5	5	5	5	5	5
France [13]	22.5	22.5	5	5	5	5	5	5
Hungary [15]	10	10	10	10	10	5	5	5
Iceland [16]		10	10	10	10	10	10	15
Ireland [17]	8.5	7	5	5	7.5	5	5	10
Italy [18]		17.5	5	5	7.5	5	5	10
Latvia [19]	10	10	10	10	10	5	5	5
Lithuania [20]	10	10	5	5	5	5	5	5
Netherlands [21]	5	5	2.5	2.5	2.5	2.5	2.5	7.5
Nordic countries [10]		10	7.5	7.5	7.5	7.5	7.5	10
Poland [25]	10	10	10	10	10			5
Romania [27]	10	10	10	10	7.5	5	5	5
Russian Federation [28]	10	10	2.5	2.5	2.5	2.5	2.5	2.5
Serbia [29]			10	10				
Slovakia [30]	7.5	10	7.5	7.5	10	7.5	5.8	5
Spain [32]	10	10	10	5	5	5	10	15
The FYR Macedonia [34]	7.5	10	10	10	10	5	5	5
United Kingdom [35]	8.5	7	0	0	0	0	0	10
EC [14]		17.5	5	5	7.5	5	5	10
WHO/FAO [26]	5	5	5	5	5	5	5	15

DACH countries = Austria, Germany, Switzerland; Nordic countries = Denmark, Finland, Norway, Sweden; The FYR Macedonia = The former Yugoslav Republic of Macedonia; EC = European Commission; WHO/FAO = World Health Organization/Food and Agricultural Organization.
Croatia, Federation of Bosnia and Herzegovina and Republika of Srpska (entities of Bosnia and Herzegovina) and Montenegro are excluded from the table because no recommendation report was available for the author. The Czech Republic was excluded due to lack of published source

Table S4 Overview of recommended vitamin D intakes (µg) for selected population groups in Europe: women

Country	3m	9m	5y	10y	15y	25y	50y	70y	pregnancy	lactation
Albania [3]	5	5	5	5	5	10	10		5	5
Belgium [5]	12.5	12.5	7.5	6.3	6.3	6.3	6.3	10	10	10
Bulgaria [8]	5	5	5	5	5	5	5	10	5	5
DACH [4]	10	10	5	5	5	5	5	10	5	5
France [13]	22.5	22.5	5	5	5	5	5	5	10	10
Hungary [15]	10	10	10	10	10	5	6	6	10	10
Iceland [16]	10	10	10	10	10	10	10	15	10	10
Ireland [17]	8.5	7	5	5	7.5	5	5	10	10	10
Italy [18]		17.5	5	5	7.5	5	10	10	10	10
Latvia [19]	10	10	10	10	10	5	5	5	10	10
Lithuania [20]	10	10	5	5	5	5	5	5	10	10
Netherlands [21]	5	5	2.5	2.5	2.5	2.5	2.5	7.5	7.5	7.5
Nordic countries [10]		10	7.5	7.5	7.5	7.5	7.5	10	10	10
Poland [25]	10	10	10	10	10	0	0	5	0	0
Romania [27]	10	10	10	10	7.5	5	5	5	5	5
Russian Federation [28]	10	10	2.5	2.5	2.5	2.5	2.5	2.5	10	10
Serbia [29]			10	10						
Slovakia [30]	7.5	10	7.5	7.5	10	7.5	5.8	5	10	10
Spain [32]	10	10	10	5	5	5	10	15	10	10
The FYR Macedonia [34]	7.5	10	10	10	10	5	5	5	11.3	11.3
United Kingdom [35]	8.5	7	0	0	0	0	0	10	10	10
EC [14]		17.5	5	5	7.5	5	5	10	10	10
WHO/FAO [26]	5	5	5	5	5	5	5	15	5	5

DACH countries = Austria, Germany, Switzerland; Nordic countries = Denmark, Finland, Norway, Sweden; The FYR Macedonia = The former Yugoslav Republic of Macedonia; EC = European Commission; WHO/FAO = World Health Organization/Food and Agricultural Organization.
Croatia, Federation of Bosnia and Herzegovina and Republika of Srpska (entities of Bosnia and Herzegovina) and Montenegro are excluded from the table because no recommendation report was available for the author. The Czech Republic was excluded due to lack of published source

chapter 3

Explaining the variability in recommended intakes of folate, vitamin B12, iron and zinc for adults and elderly people

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ABSTRACT

Objective: To signal key issues for harmonizing approaches for establishing micronutrient recommendations by explaining observed variation in recommended intakes of folate, vitamin B12, iron and zinc for adults and elderly people.

Design: We explored differences in recommended intakes of folate, vitamin B12, iron and zinc for adults between nine reports on micronutrient recommendations. Approaches used for setting recommendations were compared as well as eminence-based decisions regarding the selection of health indicators indicating adequacy of intakes and the consulted evidence-base.

Results: In nearly all reports, recommendations were based on the average nutrient requirement. Variation in recommended folate intakes (200-400 µg/d) was related to differences in the consulted evidence-base, whereas variation in vitamin B12 recommendations (1.4-3.0 µg/d) was due to the selection of different CVs (10-20%) and health indicators (maintenance of haematological status or basal losses).

Variation in recommended iron intakes (men 8-10 mg/d, premenopausal women 14.8-19.6 mg/d, postmenopausal women 7.5-10 mg/d) was explained by different assumed reference weights and bioavailability factors (10-18%). Variation in zinc recommendations (men 7-14 mg/d, women 4.9-9 mg/d) was also explained by different bioavailability factors (24-48%) as well as differences in the consulted evidence-base.

Conclusions: For the harmonization of approaches for setting recommended intakes of folate, vitamin B12, iron and zinc across European countries, standardized methods are needed to a)-select health indicators and define adequate biomarker concentrations, b)-make assumptions about inter-individual variation in requirements, c)-derive bioavailability factors, and d)-collate, select, interpret and integrate evidence on requirements.

INTRODUCTION

Adequate nutrient intakes are critical to health maintenance and contribute to the prevention of chronic diseases and functional decline (1-13). Many countries and organizations across Europe provide micronutrient recommendations that serve as a basis for good health(14). Although different terms are used to express such recommendations, e.g. Population Reference Intakes, Recommended Intake, Recommended Daily Allowance, they all refer to the daily intake level that is sufficient to fulfil the requirements of nearly all healthy individuals in a defined population (14, 15).

There are two main approaches for establishing micronutrient recommendations: the requirement-based approach, and the intake-based approach. The first approach evaluates evidence across intervention and observational studies on the relation between intake and selected health indicators. These health indicators indicate the adequacy of intake to fulfil physiological needs and may include physiological, biochemical and functional measures; equilibrium maintenance and disease incidence (16). Based on the distribution of nutrient requirements both the average nutrient requirement (ANR), also referred to as average requirement or estimated average requirement, and the recommended intake can be derived (ANR+2SD).

The intake-based approach is used when evidence needed to estimate the distribution of nutrient requirements is lacking or incomplete. Consequently, recommendations are based on the lowest level of intake estimated to be sufficient for nearly all healthy people within the population, also referred to as Adequate Intake (AI).

Micronutrient recommendations serve as an important basis for public health nutrition policy. If the distribution of requirements can be estimated, the ANR can be used to assess the prevalence of adequate intakes within a population. Furthermore, if the prevalence of adequate intakes is not satisfactory, recommended intakes can be used for planning interventions to improve the situation (17-19)

Currently, there is considerable variation in micronutrient recommendations between countries and this can cause confusion among policy makers, consumers, food industry and health professionals (14, 20-22). This variation may be due to differences between populations, e.g. in bioavailability from national diets, but it may also be due to differences in approaches used for establishing recommendations (ANR+2SD vs. AI) (23),

and eminence-based decisions regarding the selection of relevant health indicators and data underlying recommendations (16, 19, 23, 24). Harmonization of the process for establishing micronutrient recommendations will increase transparency, objectivity and reliability of recommendations and contribute to aligned nutrition policy across Europe(19, 23)

This paper aims to contribute to the understanding of the variation in recommendations and to signal key issues for harmonizing approaches for establishing recommended intakes for adults and elderly people of four micronutrients (folate, vitamin B12, iron and zinc) that were prioritized by the Network of Excellence EURRECA(25-27). First we explored differences in current recommendations between reports from key European and non-European countries/organizations. Secondly, we examined whether the approaches used and the eminence-based decisions made for establishing recommendations contribute to the observed variation.

METHODS

Collection and comparison of recommended micronutrient intakes

By the end of 2007, and early 2008, the latest reports on micronutrient recommendations were collected from 31 European countries and organizations in collaboration with local key-informants involved in setting these recommendations as described elsewhere (14).

Eight European countries, clusters of countries or organizations provided reports on micronutrient recommendations based on a review of the available literature on nutrient requirements (14): United Kingdom (GB) (28), Netherlands (NL) (29, 30), France (FR) (31), Latvia (32), Germany-Austria-Switzerland (DACH) (33), Norway-Sweden-Finland-Denmark-Iceland (NNR) (34), European Community (EC) (35) and WHO/FAO (36). The latter two defined recommendations to be used in an international perspective. Seven of these reports were included in this study: the Latvian report was excluded, because no information was available on the approaches and eminence-based decisions underlying recommendations.

In other European countries, micronutrient recommendations were adopted from at least one of the reports mentioned above or from guidance provided in USA/Canada (14, 37, 38). Therefore, we also included the report of USA/Canada (US/CA) in this study. In addition the report of Australia/New-Zealand (AU/NZ) (39) was included, because it includes an extensive

evaluation of the recommendations previously published in USA/Canada and considers the literature up to 2003.
From the nine reports, we extracted recommended intakes of folate, vitamin B12, iron and zinc for adults and elderly people (age ≥18 y). To quantify the extent of heterogeneity between reports the ratio of the highest to the lowest recommendation observed per gender was calculated. Within-report comparisons were used to identify differences between men and women and between younger and older adults.

Collection and comparison of approaches

From each report we retrieved information on the approach used for establishing the recommendations of interest (ANR+2SD , or AI). If an ANR+2SD was reported, we obtained the ANR and inter-individual variation coefficient (CV=SD/ANR). In case the ANR or CV was not explicitly reported, the missing value was calculated based on the following equation: ANR+2SD=ANR*(1+2*CV/100). We evaluated whether variation in ANR+2SD was more strongly related to between-report differences in ANRs or CVs. For each combination of two reports with a different ANR+2SD, we checked in what way applying the same CV would change the difference between the recommendations.

Collection and comparison of eminence-based decisions

In general there are two ways to establish an ANR: 1)-based on intake-health associations or 2)-based on physiological needs estimated by basal losses (i.e. factorial approach). If intake-health associations are used, the ANR is estimated by the mean intake needed to reach a specified cut-off level of the selected health indicator. The factorial approach includes the summation of basal losses via skin, faeces, urine and additional needs for accretion. By correcting total needs for bioavailability from the usual diet, the ANR is estimated (ANR=mean physiological needs*100/bioavailability factor). To explain potential variation in ANRs between reports we collected and compared health indicators and cut-off levels or basal losses and bioavailability factors depending on the method used to establish the ANR.
Finally, to assess the data underlying recommendations the references that were given greatest weight by the authors of the reports were compared between reports that used similar methods for deriving an ANR or AI.

RESULTS

Recommended intakes of folate, vitamin B12, iron and zinc for adults (>18 y) from European countries, US/CA and AU/NZ are presented in Tables 1 and 2. Since recommendations were similar across adult ages, for each report we only present one recommendation per gender. Due to the diminished iron needs after onset of menopause, all reports provided recommended iron intakes for pre- and postmenopausal women separately.

WHO/FAO and FR provided recommended intakes of iron and zinc for different bioavailability factors. In our comparison, we included the recommendations estimated with the bioavailability factor most frequently used in the other reports (iron 15%, zinc 30%).

Variation in recommendations

Between-report ratios of highest to lowest recommendations were about 2.0 for folate (200-400 µg), vitamin B12 (1.3-3 µg) and zinc (men 7-14 mg, women 4.9-9 mg), whereas for iron the variation was less with a ratio around 1.3 (men 8-10 mg, premenopausal women 14.8-19.6 mg, postmenopausal women 7.5-9 mg).

Within reports, we observed no gender differences for folate and vitamin B12; only FR recommended a higher folate intake for men (+30 µg). For iron, higher recommendations were established for premenopausal as compared to postmenopausal women (+3 mg to +12 mg), whereas recommendations for men and postmenopausal women were similar or slightly different (EC, NL, WHO/FAO; maximum difference 1.6 mg). For zinc all reports provided higher recommendations for men than for women (+1 to +6 mg).

In general few differences existed between recommendations for younger and older adults, due to limited knowledge of the effects of the aging process on utilization and absorption of folate, vitamin B12, iron and zinc. However, all reports, except the one from GB, indicated that consumption of vitamin B12 via fortified foods or supplements is recommended for elderly people suffering from food-bound malabsorption. Due to the high prevalence of atrophic gastritis among elderly people (10-30%), US/CA applied this recommendation for all those aged 51y and older.

Both for folate and vitamin B12 we observed a clear difference in recommended intakes between reports of GB and EC published until 1993 (folate 200 µg, vitamin B12 1.4-1.5 µg) and the other reports published since 1998 (folate 300-400 µg, vitamin B12 2.0-3.0 µg).

Variation in approaches

In general, reports suggested that recommendations were established using the requirements-based approach (ANR+2SD); only FR indicated providing an AI for folate, iron and zinc (Tables 1 and 2).

To derive recommended iron intakes for premenopausal women, five reports (EC, NNR, WHO/FAO, US/CA, GB) used an alternative approach since the distribution of menstrual iron losses was found to be skewed. EC, NNR, WHO/FAO and US/CA estimated the distribution of iron requirements for this population group as the convolution of the distributions of menstrual and basal iron losses and subsequently derived recommended iron intakes as the 90^{th} (EC, NNR), 95^{th} (EC, WHO/FAO) or 97.5^{th} (US/CA) percentile of iron requirements. EC and NNR argued that considering a usual diet, an iron intake fulfilling requirements of 95% would be unrealistically high for the majority of premenopausal women. GB derived an ANR for iron as the sum of mean basal losses and the 75^{th} percentile of menstrual iron losses corrected for the bioavailability factor. The ANR+2SD was estimated by applying a CV of 15%.

Between reports CVs varied from 10-20% (vitamin B12 and iron) or 10-25% (folate and zinc). Part of the observed between-report differences in ANR+2SD can be explained by variation in CVs. In general for folate, zinc and iron, between-report differences in ANRs contributed more to the variation in recommendations than differences in CVs, as between-report differences in ANR+2SD stayed similar, only slightly decreased or even increased after applying the same CV (data not shown). The variation in CVs only fully explained the differences in ANR+2SD for vitamin B12 established by France, NL and US/CA, and differences in ANR+2SD for zinc established by NL and US/CA. In these cases, ANRs were the same in all specified reports and only CVs varied. For zinc, some variation in ANR+2SD was more strongly related to between-report differences in CVs than in ANRs both for men (GB-NL, EC-NL) and women (NL-AU/NZ).

Variation in eminence-based decisions

For folate and vitamin B12, the maintenance of blood or tissue levels at a concentration not accompanied by deficiency symptoms was generally considered as health indicator for establishing an ANR (Table 1). For folate all countries/organizations used at least one biomarker of folate status as primary health indicator for establishing an ANR. Cut-off levels indicating

Table 1 Recommended intakes of folate and vitamin B12 for adults (age ≥18 y) and underlying approaches

	Year	Recommended intake (µg)*	Approach	ANR (µg)	CV (%)	Method for estimating ANR/AI	
						Health indicator (cut-off level)	Daily losses
Folate							
GB (28)	1991	200	ANR+2SD	150	*15*	**Folate levels in liver (>3 µg/g)** **Erythrocyte folate (>327nmol/l)**	
EC (35)	1993	200	ANR+2SD	140	20	**Serum folate (n.a.)** **Erythrocyte folate (>327 nmol/l)**	
DACH (33)	2000	400	ANR+2SD	n.a.	10-15	**Plasma homocysteine (n.s)** Correct deficiency symptoms (100-200 µg)	
FR (31)	2001	m:330, f:300	AI	x	x	**Plasma homocysteine (<10 µmol/ l)**	
NL (30)	2003	300	ANR+2SD	200	25	**Serum folate (>10 nmol/l)** **Erythrocyte folate (>300 nmol/l)** Plasma homocysteine (<15 µmol/ l)	Compensate losses (n.a.)
NNR (34)	2004	300	ANR+2SD	200	*25*	**Serum folate (>6.8 nmol/l)** **Erythrocyte folate (>317nmol/l)**	
US/CA (37)†	1998	400	ANR+2SD	320	10	**Serum folate (>7 nmol/l)** **Erythrocyte folate (>305 nmol/l)** Plasma homocysteine (<16 mmol/l)	
Vitamin B12							
GB (28)	1991	1.5	ANR+2SD	1.25	*10*	**Haematological status ‡ (1.0 µg)** **Total vitamin B12 stores (n.s.)**	
EC (35)	1993	1.4	ANR+2SD	1.0	20	**Haematological status ‡ (1.0 µg)** Serum vitamin B12 (>113 pmol/l)	
DACH (33)	2000	3.0	ANR+2SD	n.a.	10-15	**Haematological status ‡** **Plasma vitamin B12 (n.s.)**	0.1% of total stores (2-5 mg)
FR (31)	2001	2.4	ANR+2SD	2.0	10		**0.8 µg**
NL (30)	2003	2.8	ANR+2SD	2.0	20		**0.2% of liver stores (500 µg)**
NNR (34)	2004	2.0	ANR+2SD	1.4	15	**Haematological status ‡ (0.7 µg)** Serum vitamin B12 (n.a.) Total vitamin B12 stores (1000 µg)	
US/CA (37)†	1998	2.4	ANR+2SD	2.0	10	**Haematological status ‡ (1.0 µg)** Serum vitamin B12 (≥150 pmol/l)	

GB, United Kingdom; EC, European Community, DACH, Germany-Austria-Switzerland; FR, France; NL, Netherlands; NNR, Norway-Sweden-Finland-Denmark-Iceland; US/CA, USA/Canada; n.a., not available in report; x, not available because the intake-based approach was used

Values in italics could not be retrieved from the reports, but were calculated: CV=0.5*((recommendation /ANR)-1)

Bold text indicates the primary indicator used to estimate the ANR, whereas the text not in bold indicates a secondary health indicator

* Recommended folate intakes were expressed as dietary folate equivalents (DFE): 1 DFE=1 ug food folate=0.6 µg folic acid from fortified foods=0.5 µg folic acid from supplements

† World Health Organization/Food and Agricultural Organization and Australia/New-Zealand adopted recommendations from US/CA, therefore the reports of WHO/FAO and AU/NZ were not considered separately.

Table 2 Recommended intakes of iron and zinc for adults (age ≥18 y) and underlying approaches

	Year	Recommended intake (mg/d)			Approach	ANR (mg)/50th pct			CV (%)	Method for estimating ANR/AI: Daily losses							Bioav (%)
										Component losses (mg/d)				Total losses (mg/d)			
Iron		**m**	**f pre**	**f post**		**m**	**f pre**	**f post**	**m+f**	**feces**	**urine**	**sweat**	**menstr**	**m**	**f pre**	**f post**	
GB (28)	1991	8.7	14.8	8.7	ANR+2SD	6.7	11.4	6.7	15	0.76	0.1	n	0.7	0.86	1.56	0.86	15
EC (35)	1993	9.1	19.6*	7.5	ANR+2SD†	7	9	5.8	15†			n		1.05	1.46	0.87	15
DACH (33)	2000	10	15	10	ANR+2SD	n.a.	n.a.	n.a.	10-15					1.0	1.5	1.0	10-15
FR (31)	2001	9	16	9	AI	x	x	x	x	0.6	0.2-0.3	0.1	0.4-0.5	0.9-1.0	1.3-1.5	0.9-1.0	10
NL (29)	1992	9‡	15‡	8	ANR+2SD	8	13	7	20				0.8	0.9	1.6	0.8	12
NNR (34)	2004	9	15§	9	ANR+2SD†	7	9	5.8	15†			n		1.05	1.46	0.87	15
WHO/FAO (36)	2004	9.1❙	19.6❙	7.5❙	ANR+2SD†	7	9	5.8	15†			n		1.05	1.46	0.87	15❙
US/CA (38)¶	2001	8	18	8	ANR+2SD	6.0	8.1	5.0	n.a.**				0.51	1.08	1.40	0.90	18
Zinc		**m**	**f**	**Δ m-f**		**m**	**f**			**feces**	**urine**	**sweat**	**semen**	**m**	**f**		
GB (28)	1991	9.5	7	2.5	ANR+2SD	7.3	5.5		*15*					2.2	1.6		30
EC (35)	1993	9.5	7	2.6	ANR+2SD	7.5	5.5		15					2.2	1.6		30
DACH (33)	2000	10	7	3	ANR+2SD	n.a.	n.a.		10-15					2.2	1.6		30
FR (31)	2001	9❙,††	7❙,††	2	AI	x	x		X					n.a.	n.a.		30❙
NL (29)	1992	10	9	1.2	ANR+2SD	7.6	6.8		20	0.3-0.5	0.3-0.7	0.5	0.27	1.3-1.9	1.1-1.7		25
NNR (34)	2004	9	7	0.9	ANR+2SD	6.4	5.7		15	1.4	Urine+sweat+semen: m: 1.27, f: 1.0			1.4	1.0		30
WHO/FAO (36)	2004	7❙	4.9❙	2.2	ANR+2SD	4.7	3.2		25					2.67	2.4		40❙
US/CA (38)	2001	11	8	3.1	ANR+2SD	9.4	6.8		10	m:2.57, f::2.3	0.63	0.54	0.10	3.84	3.3		m:41, f:48
AU/NZ (39)	2005	14	8	6.6	ANR+2SD	12	6.5		10	m:1.54, f:1.06	0.63	0.54	0.10	2.81	1.96		m:24, f:31

GB, United Kingdom; EC, European Community; DACH, Germany-Austria-Switzerland; FR, France; NL, Netherlands; NNR, Norway-Sweden-Finland-Denmark-Iceland; WHO/FAO, World Health Organization/Food and Agricultural Organization; US/CA, USA/Canada; AU/NZ, Australia/New-Zealand; pre, premenopausal women; post, post-menopausal women; menstr, menstrual losses; n.a., not available in report; x, not available because the intake-based approach was used; n, negligible

Values in italics could not be retrieved from the reports, but were calculated: CV=0.5* ((recommended intake/ANR)-1)

* EC also presents the 90th percentile of intakes to fulfil physiological requirements, 15.8 mg

† Not applicable for premenopausal women: the distribution of requirements was skewed and recommended iron intake could therefore not be derived as ANR+2SD.

‡ A separate recommended iron intake was provided for men and women aged 19-21y, m: 11.0 mg, f: 16.0 mg

§ The recommended iron intake was set at the 90th percentile of iron requirements

❙ Recommended intakes of iron and zinc were also available for other bioavailability coefficients (WHO/FAO: for iron 5, 10 and 12%, zinc 15 and 50%, France: zinc 20%)

¶ AU/NZ adopted recommendations from US/CA, therefore the reports of AU/NZ was not considered separately.

** Distribution of iron requirements for both men and women on the median and variability in body weights recorded in NHANES III

†† For men and women aged ≥75 y, the recommended zinc intake was 8.0 mg

adequate concentrations of biomarkers showed some variation between reports: erythrocyte folate >300-340 nmol/l, serum folate >6.8-10 nmol/l, serum homocysteine <10-16 µmol/l. EC, NL, NNR and US/CA all used adequate serum and erythrocyte folate as health indicators, but ANRs varied from 140 to 320 µg. All four reports referred to the same depletion-repletion study by Sauberlich et al. (40). In addition, EC based their ANR on two older depletion-repletion studies (41, 42), and NL, NNR and US/CA on a more recent depletion-repletion study (43) and a balance study (44). NL and US/CA also considered the RCT by O'Keefe et al.(45). However, NL did not use this study for estimating the ANR because requirements based on the results of this study were much higher than those in other studies. In contrast, in the report of US/CA this study was given greatest weight, as subjects in the other depletion-repletion studies would have received more folate than reported due to underestimation of the folate content in food.

GB based recommended folate intakes on observational data on the relation between long-term folate intake and adequate concentrations of folate in liver and erythrocytes (43). An optimal concentration of homocysteine was selected as health indicator for folate in reports of FR (46) and DACH (47, 48), which was explained as the level at which the risk for cardiovascular diseases is minimized.

On the contrary, NL, NNR and US/CA reported that available evidence on minimizing cardiovascular disease risk by lowering homocysteine concentrations is not strong enough to take this biomarker into account for estimating folate requirements.

For vitamin B12, GB, EC, NNR and US/CA selected the maintenance of adequate haematological status as primary health indicator for estimating the ANR (1.0-2.0 µg). EC, NNR and US/CA mainly based the ANR of vitamin B12 on Darby et al. (49), studying the effects of various intramuscular doses of vitamin B12 on subjects with pernicious anemia. NNR and US/CA corrected these levels for reabsorption of biliary vitamin B12 as occurs in healthy people (50-52). EC did not perform these corrections, but assumed that adjustments for reabsorption will cancel out adjustments needed for incomplete absorption from the diet. Studies in vegetarians were used as supportive data (53). In GB (ANR: 1.25 µg) studies given largest weight were different (53-57).

FR and NL estimated the ANR for vitamin B12 following the factorial approach using estimates of basal losses (0.8-1.0 µg) and a bioavailability of 60 and 50% respectively. Estimations of basal losses were based on different studies (FR (51, 58), NL (50, 52), but ANRs were similar (2.0 µg).
The DACH report mentioned several health indicators, but from the information provided we could not deduct which indicator and data were used to estimate an ANR.

For iron and zinc all reports used the factorial approach to estimate the ANR (Table 2). For iron, the maximum difference between reports in basal losses was 0.3 mg (premenopausal women DACH vs. NL). All reports, except GB and NL, stated that basal iron losses were based on physiological needs per kg body weight, namely 14 µg/kg per day. Reference body weights selected for estimating an ANR varied between reports (men 74-77.4 kg, women 59-64 kg). Using the highest or lowest observed reference weight would result in a difference in ANR of ≤1.2 mg.
In most countries/organizations, basal iron losses were based on a single experimental study by Green et al. (59) using radio-labelled iron to measure losses from different body compartments. US/CA reported that this was the only study with reliable quantitative data for basal iron losses in humans. A previous report on recommendations published by FAO (60) was also indicated three times as an important reference.
In contrast, FR, DACH and NL did not refer to the experimental study by Green et al. (59): FR referred to the FAO report (60), NL used data from other balance, turnover and depletion-repletion studies (61-63) and DACH did not provide information on their evidence-base.
For estimates of menstrual iron losses, all reports, except FR and NL, referred to the same study from year 1966 (64). Although the evidence-base used in FR and NL was different, their estimated basal losses were similar to estimates provided in the other reports.
Bioavailability factors for iron varied between reports from 5-18%. They were mainly based on studies investigating bioavailability from diets with heme:non-heme ratios representative of national (NL, FR, NNR, US/CA) or Western/mixed diets (GB, DACH, WHO/FAO, EC) in iron replete subjects with minimal or normal iron stores. FR applied a bioavailability factor of 10%, but indicated that in subjects without iron stores bioavailability could reach 15-20%, whereas US/CA estimated an ANR for those with normal iron status but minimal stores and applied a factor of 18%.

For zinc, total losses varied substantially (men:1.3-3.8 mg; women:1.0-3.3 mg); on intestinal losses in particular there was no agreement (men:0.3-2.57 mg; women:0.3-2.3 mg). All countries/organizations used different combinations of studies to estimate the ANR, but all, except FR and NNR refer to the balance study by Milne et al (65). Other important sources of evidence reported in at least three reports were a depletion-repletion study (66) (NL, NNR, WHO/FAO), the study by Hess et al. (67) measuring zinc excretion in young women on low zinc intakes (NNR, WHO/FAO, US/CA, AU/NZ), a book chapter (68) (GB, EC, DACH) and a cohort study (69) (WHO/FAO, US/CA, AU/NZ).

Bioavailability factors for zinc varied between reports from 15-50%. US/CA and AU/NZ applied higher bioavailability factors for women than for men, which contributed to observed gender differences in ANR (respectively 2.6 mg and 5.5 mg). More recent reports (NNR, US/CA, AU/NZ) based bioavailability factors on the relationship between the amount of zinc absorbed and that excreted via the intestine based on mixed diets. AU/NZ estimated their factor with results from IZiNCG (70), which was lower than factors provided by NNR and US/CA (40-48%). The other reports based bioavailability factors on studies investigating absorption efficiency from different types of diets. Although the evidence base varied between reports bioavailability factors in these reports were similar (25-30%).

DISCUSSION

Based on our comparisons of approaches and eminence-based decisions underlying the establishment of recommended intakes of folate, vitamin B12, iron and zinc, we identified explanations for the variation in current recommendations. For folate selected health indicators were similar between reports and the variation was mainly related to differences in the underlying data. In contrast, for vitamin B12 variation in CVs and selected health indicators (maintenance of haematological status or basal losses) seemed to contribute most to the observed variation. For iron, differences in reference weights and bioavailability factors were the main explanatory factors for the variation in recommendations as the underlying data and consequently the estimates of basal losses were similar in most reports. For zinc the variation in recommended intakes was related to differences in CVs, bioavailability factors and the large variation in the evidence-base.

Although the reports that we considered were published over 13-14 years, access to new data and changes in concepts of nutrition did not seem to have altered the estimates made by the newer panels, except for folate. Recommended folate intakes published until year 1993 (GB and EC) were based on older studies as compared to the other reports published since 1998.
This paper describes the situation at the beginning of 2008. Since then several countries have published updates for one or more micronutrients. However, for the countries/organizations, micronutrients and age groups included in this comparison, no updates were identified. All most recent recommendations and ANRs provided by European countries and organizations are collated in EURRECA's web-based tool Nutri-RecQuest (71).

In general, the requirements-based approach was used to establish recommendations. Only FR indicated providing an AI for folate, iron and zinc, although the type of health indicators and data selected as the basis for recommendations were similar to other reports providing an ANR+2SD. Apparently there is no consensus on the use of this term.
In 2007, a working group of international experts reviewed the harmonization of approaches for developing recommendations on nutrient intakes. They proposed new terminology and developed a new statistical approach for establishing recommendations. With this new approach, recommendations do not necessarily reflect ANR+2SD covering the needs of 97.5 % of the population, but lower levels in the distribution of requirements may also be chosen when this is more likely to be achieved with current nutrition policies (23). Clearly, estimates of inter-individual variation depend on the health indicator selected to be most reflective of meeting nutritional needs. However, for folate, vitamin B12 and zinc, CVs varied largely between reports using the same health indicators, as data on inter-individual variation in requirements is unavailable, inadequate or inconsistent and therefore assumptions are made based on variation in metabolic rates, variation in bioavailability and/or the level of uncertainty in the ANR estimate. More research is needed to define CVs, but as long as appropriate data are lacking, international consensus on how to make assumptions regarding inter-individual variation in requirements of folate, vitamin B12 and zinc will help to harmonize approaches for setting recommendations.

Health indicators selected for estimating requirements for folate, vitamin B12, iron and zinc varied between reports and included biomarker concentrations rather than associations between intake and health outcomes such as organ function or chronic disease risk. Cut-off levels indicating adequate concentrations of biomarkers were based on the prevention of deficiency symptoms, whereas their predictive value for health outcomes was not often considered. Moreover, along with the observation that similarities in the consulted evidence-base often lead to different ANRs of folate and vitamin B12, the example on optimal concentrations of homocysteine for the prevention of CVD illustrates that methods used for the selection, evaluation and integration of data were not consistent between reports. The Institute of Medicine (IOM) developed an analytical framework for setting nutrient reference values for optimal health based on associations between dietary exposure (intake) and clinical health outcomes (chronic diseases) (72). The use of standard systematic review methodology including meta-analyses for evaluating data on these associations will contribute to increased transparency of the decision-making process and could therefore be an important step in harmonizing approaches for setting micronutrient recommendations (72, 73). However, the use of chronic disease outcomes for setting recommended micronutrient intakes may be challenging due to the lack of data on associations between intake and such clinical outcomes and due to the multi-factorial nature of chronic diseases including factors such as genetics, age, diet, environment and lifestyle (73). In addition, it could be questioned whether establishing an ANR based on associations between intake and chronic disease risk would be possible as it seems very challenging to estimate the intake at which 50% of the population is at risk and 50% is not. Setting an AI seems more feasible as it represents the intake level that resulted in the greatest reduction of risk of disease (72, 74). As data on direct associations between intake and clinical outcomes are often lacking, biomarkers that correlate both with intake and with a disease or physiological state may be used as an intermediary between intake and health (72, 73). Recently, within the scope of the EURRECA Network of Excellence, best practice methods for assessing intake (75, 76) and best practice biomarkers have been evaluated for all nutrients of interest (11, 77-79) by means of standardized systematic review methodology (80). Up to now, these best practice biomarkers have not yet been used for estimating requirements, except for folate (erythrocyte folate and serum/plasma folate). Currently within EURRECA, systematic reviews including meta-analyses are

carried out to evaluate the strength and quality of dose-response relations between intake and status of the priority micronutrients and between micronutrient intake or status and various health outcomes. Following standardized protocols, these reviews will make transparent what evidence is available addressing populations and outcomes of interest and they may enhance the selection of health indicators (73).

Dose-response relations for iron and zinc might be especially difficult to estimate due to a lack of sensitive and specific biomarkers reflecting status and due to the strong homeostatic regulation of body iron and zinc (81). The factorial approach may therefore be the most appropriate way of estimating ANRs on both minerals assuming a certain level of bioavailability. Bioavailability of iron and zinc is influenced by the composition of the diet as well as individual characteristics including micronutrient status, age, genotype and clinical conditions influencing absorption and utilization (81-83). Absorption of iron depends on the form of iron (heme or non-heme), and the presence of factors enhancing or inhibiting absorption like vitamin C and phytate (81, 84). In addition, homeostatic adaptations occur, increasing or decreasing fractional absorption when intake or status is low or high (81). For zinc, bioavailability is also influenced by factors enhancing or inhibiting absorption, by individual zinc intake and status, and it may also be influenced by ageing (85). Variation in bioavailability factors between reports was related both to differences in diet composition between countries, and differences in methodologies for deriving bioavailability factors. To align methods of deriving bioavailability factors for iron and zinc, agreement is needed on how to estimate the effects of meal composition and individual characteristics such as micronutrient status, genetic variation (single nucleotide polymorphisms) and age on bioavailability (83, 86, 87).

In conclusion, for the harmonization of approaches for setting recommended intakes of folate, vitamin B12, iron and zinc across European countries, standardized methods are needed to a)-select health indicators and define adequate biomarker concentrations, b)-make assumptions about inter-individual variation in requirements, c)-derive bioavailability factors, and d)-select and interpret evidence on requirements.

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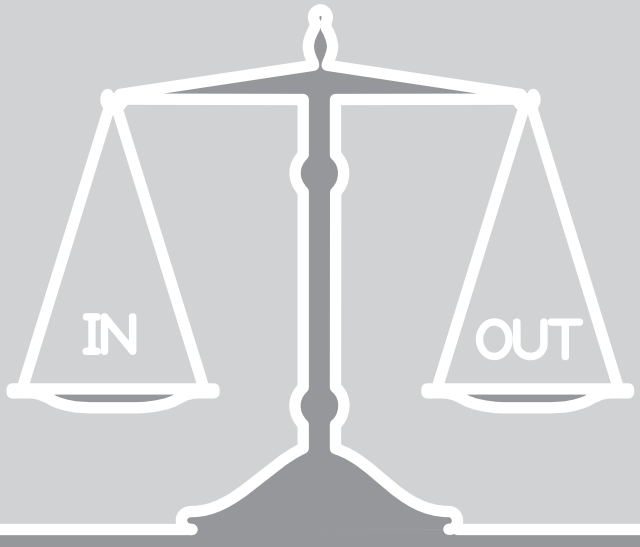
chapter 4



Systematic review on daily vitamin B12 losses and bioavailability for deriving recommendations on vitamin B12 intake with the factorial approach

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ABSTRACT

Aims: To systematically review literature on daily losses and bioavailability of vitamin B12. These estimates could be used for deriving recommendations on vitamin B12 intake for adults and elderly.

Methods: We identified publications on daily vitamin B12 losses (July 2011) and publications on bioavailability of vitamin B12 from foods or diets (June 2010) in MEDLINE, EMBASE and Cochrane Library.

Results: A pooled analysis of five studies (52 subjects) showed that 0.13±0.03% of total body stores is lost per day. Absorption of vitamin B12 ranged from 4.5% (dose of 38 µg from consumption of liver) to 83% (dose of 3.0 µg from consumption of mutton meat). Data from eight studies including 83 subjects suggested that the amount of vitamin B12 absorbed from food (A_i) increased with increasing doses of vitamin B12 (D_i) as described by the equation: $\ln(A_i)=0.7694*\ln(D_i)-0.9614$.

Conclusion: Daily vitamin B12 losses in apparently healthy adults and elderly probably range between 2.6-3.9 µg. Based on usual intake data, bioavailability from the diet may range between 29-37% rather than the generally assumed 50%. These results suggest that a vitamin B12 intake of 7.0-13.4 µg is needed to compensate daily vitamin B12 losses in apparently healthy adults and elderly people.

INTRODUCTION

Current recommendations on vitamin B12 intake for adults and elderly vary between European countries from 1.4 to 3 µg per day. The variation in these recommendations results from different approaches used for estimating vitamin B12 requirements and from different assumptions regarding the inter-individual variation in requirements (1).

One of the approaches used for estimating vitamin B12 requirements for adults and elderly in Europe (The Netherlands and France) is the factorial approach, which includes a summation of daily vitamin B12 losses that need to be compensated for by dietary intake of vitamin B12. Daily vitamin B12 losses can be measured with the whole body counting method (WBC). Subjects are given labelled vitamin B12 and the rate of loss of vitamin B12 (µg per day) is calculated based on the decrease in radio-activity during follow-up.

As an approximation to this approach, daily vitamin B12 losses can be estimated by determining vitamin B12 excretion in bile and accounting for the partial reabsorption of this excreted vitamin B12 in the small intestine. This approximation assumes that excretion of vitamin B12 via other routes, e.g. skin and urine can be neglected (2).

To estimate vitamin B12 requirements, estimates of daily vitamin B12 losses need to be corrected for bioavailability of the vitamin from the usual diet. Bioavailability is defined as 'the efficiency with which a dietary component is used systematically through normal body functions' and expressed as a percentage of intake (3). Currently, bioavailability of vitamin B12 is generally assumed to be 40 or 50% for healthy adults with normal gastrointestinal functioning (2, 4-7). This assumption is based on the absorption of labelled vitamin B12 from a few food products including mutton meat, chicken meat, rainbow trout, eggs or fortified foods (8-12).

Recommended vitamin B12 intakes indicate the daily intake that is sufficient to fulfil requirements of nearly all individuals within a defined population. When the distribution of vitamin B12 requirements is known, the recommended vitamin B12 intake is derived as the average nutrient requirement (ANR) plus twice the SD of the ANR, with the ANR defined as the intake level that is sufficient to fulfil requirements in 50% of individuals in a defined population. As there is very limited evidence on the shape of this distribution, usually a standard assumption of a CV of 10 to 20% is made.

Box 1 Calculations used for establishing vitamin B12 recommendations with the factorial approach

General formula:

$$\text{ANR} = \frac{\text{sum of losses (faeces, urine, skins, menstrual losses) + needs for growth}}{\text{bioavailability factor}}$$

The Netherlands (6):
Assumption 1: Daily vitamin B12 losses are maximally 0.2% of vitamin B12 stores (11, 13, 14)
Assumption 2: Minimal body stores are 500 µg (14)
Assumption 3: Bioavailability is 50% (8-10, 12)
Assumption 4: CV=20%
Daily losses=0.2%*500 µg=1.0 µg
ANR=1.0/0.5=2.0 µg.
Recommended intake[a]=2.0*1.4=2.8 µg.

France (2)
Assumption 1: Daily vitamin B12 excretion in bile is 1.4 µg (15)
Assumption 2: 40% of vitamin B12 excreted in bile is reabsorbed in the small intestine (16, 17)
Assumption 3: Bioavailability is 40% (17)
Assumption 4: CV=10%
Daily losses= 1.3*60%=0.8 µg
ANR=0.8/0.4=2.0 µg
Recommended intake[a]=2.0*1.2=2.4 µg.

ANR, Average nutrient requirement; CV, coefficient of variation
a-) the recommended intake is derived as (1+2*CV/100)*ANR

As shown previously, European recommendations on vitamin B12 intake for adults and elderly established using the factorial approach (The Netherlands and France) are based on a limited number of studies that were published between 1963 and 2001 (Box 1) (1). The aim of this article was to systematically review the available literature on daily vitamin B12 losses and bioavailability of vitamin B12 from different food sources including estimates of inter-individual variation. The results of this review could serve as input for establishing vitamin B12 recommendations using the factorial approach.

METHODS

This systematic review was conducted according to standardized methodology as developed within the scope of the EURRECA (EUROpean micronutrient RECommendations Aligned) Network of Excellence (18) , which is shortly described below.

Search

We conducted two systematic literature searches to identify publications on 1) basal vitamin B12 losses (MEDLINE, 12 July 2010) and 2) bioavailability of vitamin B12 from diets (MEDLINE, EMBASE and Cochrane Library, 15 June 2010). The first search included terms on vitamin B12 or labelled vitamin B12 in combination with terms on excretion routes, kinetics or maintenance and the second search included terms on vitamin B12 in combination with terms on absorption. Both searches were restricted to studies in humans. To be able to use the same search to identify publications for younger population groups, no terms regarding the age of the study population were added. The second search also included terms regarding folate to identify papers on folate bioavailability as will be described elsewhere. The search strategies are included in Supplemental file 1. Additional studies were identified by hand searching, i.e. screening of reference lists from all selected articles, review articles and reports including recommendations on micronutrient intakes.

Study selection and data extraction

In general, studies were eligible for inclusion when they had an experimental design and the study population included apparently healthy adults (age 18 years and older) or adult patients with diseases not associated with disturbance of vitamin B12 metabolism or absorption. Studies on losses were included when 1) the exposure was oral or parenteral administration of vitamin B12 and 2) the outcome was an estimate of daily vitamin B12 losses measured with WBC or estimated based on vitamin B12 excretion in bile. Studies on bioavailability were included when 1) the exposure concerned diets, meals or individual foods in- or extrinsically labelled with vitamin B12 and 2) the outcome was a measure of fractional vitamin B12 absorption. Four reviewers screened all titles and abstracts of papers identified through the literature searches according to the inclusion criteria and full-text of potentially relevant papers were evaluated against the same criteria (PitV, AS, MD, ED). For the purpose of

alignment and quality control, each reviewer screened and evaluated 10% of the total number of references in duplicate with another reviewer. Each included study was extracted by a single reviewer (PitV or AS) and a second reviewer independently verified the extracted data (ED). Any disagreements during the selection and extraction process were resolved by discussion.

From all studies we extracted general characteristics of the study design (duration, dose), and study population (number of participants, age, gender, ethnicity). In addition, from each study reporting on total vitamin B12 losses measured with WBC, we extracted the rate of loss indicating the mean decay in radioactivity per day as a percentage of the radioactivity measured after redistribution of the labelled vitamin B12 through different body compartments (i.e. at the time that a constant excretion rate was established). The underlying assumption is that labelled vitamin B12 is distributed throughout the body in a manner similar to non-labelled vitamin B12. We did not merely extract estimates based on measurements in healthy subjects (11, 13, 19-21), but also on subjects with low serum concentrations of vitamin B12 (20) and patients with pernicious anemia (PA) (11, 13), because it appeared that the rate of loss does not significantly differ between these subpopulations (11, 13, 20). One study reported the absolute mean loss of vitamin B12 per day and the total body content of vitamin B12 (21). To allow comparison with the other studies, the rate of loss was calculated as (mean loss per day/ total body content) * 100%.

From each study reporting on vitamin B12 excretion in bile, we extracted the rate of excretion, the vitamin B12 concentration of bile, or the absolute vitamin B12 excreted in bile per day. The rate of excretion reflected the radioactivity measured in excreted bile as a percentage of the ingested dose minus total losses during the first four days (i.e. period of redistribution of vitamin B12 throughout the body) after administration of labelled vitamin B12 (22, 23).

From studies on bioavailability of vitamin B12 we extracted the percentage of the ingested dose that was absorbed. Furthermore we extracted the absolute amount of vitamin B12 that was absorbed. In case this latter estimate was not reported, it was calculated as % absorbed*ingested dose (9, 24-26).

To describe the relation between the amount absorbed (A_i) and the dose administered (D_i) via the foods provided in the intervention studies we performed linear regression with both variables transformed to their natural logarithm in order to achieve linearity of the regression.

As an upper limit of inter-individual variation we calculated the coefficient of variation (CV) for each outcome measure reported per study (standard deviation (SD)/mean)*100%., ignoring the measurement errors and within-subject variation. When no SD was provided we estimated it based on individual subject data ($SD=\sqrt{[\sum(x_i-x_{mean})^2/(N-1)]}$) (8, 10, 11, 13, 19, 20, 27), on the standard error (SE) ($SD=SE*\sqrt{n}$) (22, 23, 28), or we approximated it by the total range ($SD\approx0.25*$total range) (11, 22).
Where applicable, amounts of vitamin B12 were converted from nmol to µg using the molar weight of vitamin B12 (1355.38 g/mol) (15, 26).

If three or more studies were comparable with regard to exposure and outcome measures, we calculated summary estimates using random effects meta-analysis. Applying the methods of DerSimonian and Laird, the between-study variance is estimated which is then used to modify the weights for calculating the summary estimate (29). SE's were calculated as $SD/\sqrt{n}$. If no SD was reported and no individual data were available, we imputed an SE based on the pooled SD from the other studies included in the pooled analysis: $SD_{pooled}=\sqrt{[\sum((n_i-1)*SD_i^2)/\sum(n_i-1)]}$ (21). Heterogeneity between studies was evaluated using the I^2 statistic, which expresses the percentage of variation attributable to between-study heterogeneity rather than chance (30). Meta-analyses were performed using STATA version 11.0 (College Station, TX), with statistical significance defined as a p-value <0.05.

RESULTS

Study characteristics

In total, we identified 523 potentially relevant papers on daily vitamin B12 losses and 5556 on bioavailability of vitamin B12 and folate (combined search). After evaluation of titles, abstracts and full texts, ten papers were included for vitamin B12 losses (five papers for daily vitamin B12 losses measured with WBC, and five papers for daily vitamin B12 excretion in bile) and eight papers were included for bioavailability of vitamin B12 (Figure 1). All studies were performed in Western countries (USA, United Kingdom, Sweden, Norway or Finland). Unfortunately, information on age, sex and ethnicity of the study populations was not provided for most of the studies. Serum vitamin B12 concentrations were reported to be normal in all but one study including subjects with low serum concentrations (20), but only five studies (8-10, 24, 25, 31) actually reported concentrations (137-546 pmol/l).

Figure 1 Selection of studies for systematic review on daily vitamin B12 losses and bioavailability of vitamin B12

*515 from MEDLINE, 8 identified from reference lists of selected papers and review papers
**5551 from databases MEDLINE, EMBASE, COCHRANE, 5 identified from reference lists of selected papers, review papers or recommendations reports

Vitamin B12 losses measured with WBC

Table 1 presents estimates of daily vitamin B12 losses measured with WBC expressed as the percentage of total body stores lost per day. Estimates were based on measurements in healthy subjects (11, 13, 19-21), subjects with low serum concentrations of vitamin B12 (20) and PA patients (11, 13). Absolute amounts of vitamin B12 lost per day varied in these subpopulations, as the absolute amount depends on the size of total body stores: the larger the body stores, the larger the losses (13, 21). Across studies, the rate of loss varied from 0.04% (SD not available) to 0.17% per day (SD=0.017). Reizenstein et al. (1966) (21) observed the lowest rate (0.04%), which was based on observations in only two individuals. This result deviated from the other four studies that reported a rate of loss in the range 0.13-0.17% per day (SD_{pooled}=0.03%). A meta-analysis of all five studies showed a summary estimate for the rate of loss of 0.13% per day with high heterogeneity between studies (I^2=91.5%, p<0.0001) (Figure 2). When the estimate of the rate of loss from the study by Reizenstein et al. (1966) (21) was omitted from the meta-analysis, the summary estimate only slightly changed to 0.14 % per day (95% confidence interval: 0.12, 0.16), and the observed heterogeneity between studies remained large (I^2=87.6%, p<0.0001).

Vitamin B12 excretion in bile

Studies reporting on vitamin B12 excretion in bile included healthy subjects or patients with normal vitamin B12 metabolism subjected to duodenal lavage (22, 23), patients with post-operative drain in common bile duct (22, 23, 27, 32), patients undergoing surgery for lithiasis of the bile duct (15), patients undergoing cholesystectomy (27) or autopsy (27). Results of the included studies are shown in Table 2. Two studies (22, 23) reported on the rate of excretion, indicating similar amounts of vitamin B12 excreted in bile per day as a percentage of total body stores (rate of loss 1.1% and 1.5%, respectively). One study measured absolute vitamin B12 excretion in bile and concentrations of the vitamin in bile (15), and two studies only measured concentrations of vitamin B12 in bile. Halsted et al. (32) observed no excretion of vitamin B12 in bile in one subject, whereas the two other studies showed mean vitamin B12 concentrations in bile of 3248 pg/ml (27) and 2955 pg/ml (15).

Table 1 Daily vitamin B12 losses measured with whole body counting

Reference	Population characteristics: N (% men)	Population characteristics: Health status, age, n	Exposure: Dose vitamin B12 labelled with 57/58/60 Co (µg)[a]	Exposure: Administration route	Outcome: Rate of loss (% per day)[b] Mean	SD	CV
Boddy and Adams 1968 [20]	14 (50)	Healthy, 42 y, n=1 Low vitamin B12, 34-74 y, n=13	5000	IV or IM	*0.13*	*0.028*	21
Bozian 1963 [13]	16 (69)	Healthy, 25-41 y, n=3 Patients without PA, 40-65 y n=2 PA, 57-85 y, n=11	0.5-1.0	oral or IV	*0.14*	*0.039*	27
Adams and Boddy 1968 [19]	6 (100)	Healthy, n.a., n=6	0.1	IV	*0.17*	*0.017*	10
Heyssel 1966 [11]	14 (n.a.)	Healthy, n.a., n=3 PA, n.a., n=11	?	IM	0.13	*0.02*	15
Reizenstein 1966 [21]	2 (n.a.)	Healthy, n.a., n=2	0.5	IV	*0.04*	0.03[c]	n.a.

IV=Intra-venous; IM=Intramuscular; PA=pernicious anaemia; n.a.=not available

Values in italic are based on reported estimates or individual subject data

a-) in the studies of Bozian (1963) and Adams and Boddy (1968), the radioactivity of a dose labelled vitamin B12 was 0.5 µCi

b-) expressed as a percentage of the radioactivity measured after redistribution of the labelled vitamin B12 through different body compartments

c-) pooled SD of other studies included in the pooled analysis

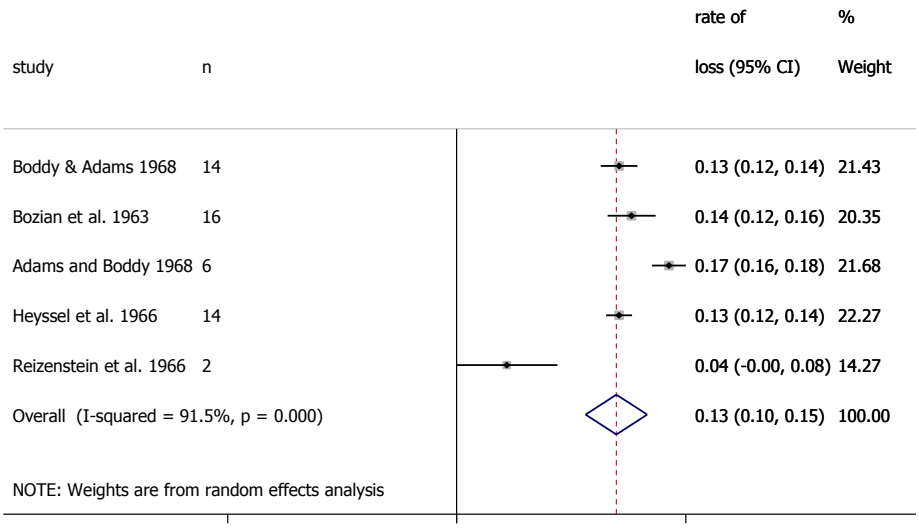

Figure 2 Pooled analysis of the daily rate of loss of vitamin B12 as a percentage of total body stores - CI, Confidence interval

Bioavailability of vitamin B12

We did not identify any study that assessed bioavailability of vitamin B12 from specific diets. All studies rather addressed absorption of vitamin B12 from a specific food product alone or in combination with a meal free of vitamin B12. Study populations included healthy subjects and subjects with a disease not affecting vitamin B12 absorption. Table 3 shows quantitative estimates of absorption obtained by the faecal excretion method (33), or WBC based on observations in 2-13 subjects per study (11). Overall, absorption ranged from 4.5% (dose of 38 µg from consumption of liver) to 83% (dose of 3.03 µg from consumption of mutton meat) (11). CV's for absorption estimates within studies ranged from 11% to 84%, whereas SD's were more similar ranging from 1.7% (11) to 22% (26), with most estimates in the range 5.2-14%. Per specific food groups and doses absorption ranged from 24% to 36% for egg products (dose 0.3-0.94 µg), from 52% to 83% for lean meat (dose 0.54-5.11 µg), from 30% to 42% for fish (dose 2.1-13.1 µg) and from 4.5% to 49% for liver products (dose 0.5-38 µg).

Four studies measured absorption from a food product with varying vitamin B12 contents. In two out of four studies, the percentage absorption decreased with increasing vitamin B12 content (10, 26), whereas the other two did not show such clear trend (8, 11). Overall, the amount of vitamin B12 absorbed

Table 2 Daily vitamin B12 losses in bile

	Population characteristics		Exposure		Outcome			
Reference	N	Health status, age, n	Dose vitamin B12 labelled with 57/58/60 Co (µg)[a]	Administration route	Outcome measure	Result		
						Mean	SD	CV
Reizenstein 1959 [23]	14	Healthy, 20-34 y, n.a. Patients with irrelevant diseases[b], 16-69 y, n.a. Patients with post-operative drain in common bile duct, n.a., n.a.	0.3-0.6	IM	Rate of loss (%/day)[c]	1.5	*1.2*	77
Grasbeck 1958 [22]	7	Healthy, n.a., n=2 Patients with post-operative t-tube drain common bile duct, n.a., n=5	0.5	IM	Rate of loss (%/day)[c]	1.1	*0.8*	72
El Kolthy 1991 [15]	8	Patients undergoing surgery for lithiasis of the bile duct, 62-70 y, n=8	?	Oral	Absolute loss (µg/day) Concentration in bile (pg/ml)	1.4 2955	0.60 1003	42 34
Ardeman 1965 [27]	6	Drain in bile duct, n.a., n=4 Cholesystectomy, n.a., n=1 Autopsy, n.a., n=1	none	-	Concentration in bile (pg/ml)	*3248*	*4314*	133
Halsted 1956 [32]	1	T-tube in common bile duct, n.a., n=1	2.0	Oral	Concentration in bile (pg/ml)	None	-	-

IM=Intramuscular

Values in italic are based on reported estimates or individual subject data

a-the radioactivity of a dose labelled vitamin B12 ranged from 0.1 to 1.2 µCi

b-diagnosis were asthenia and achlorhydria, allergic bronchial asthma, diabetes mellitus and acute or subacute neprhitis. All patients showed normal serum vitamin B12 concentrations and normal vitamin B12 absorption tests

c-expressed as % of total body stores

Table 3 Bioavailability of vitamin B12 from different food products

Reference	N	Population characteristics: Health status, age	Exposure: Food product	Exposure: Dose vitamin B12 labelled with 57/58/60 Co (µg)[a]	Outcome: % absorbed mean	% absorbed SD	% absorbed CV	Outcome: Mean amount absorbed (µg)
Fecal excretion method								
Doscherholmen & Swaim 1973 [25]	11	Healthy, 23-55 y	Scrambled whole egg	0.56	28	-	-	*0.15*
Doscherholmen 1975 [9]	5	Healthy	Scrambled egg yolk	0.5	36	-	-	*0.18*
	6	Healthy	Scrambled whole egg	0.56	28			*0.15*
	4	Healthy	Boiled egg	0.51-0.94	24			*0.12-0.23*
	4	Healthy	Fried egg	0.51-0.94	24			*0.12-0.23*
Doscherholmen 1976 [24]	13	Healthy	Scrambled egg white	0.30-0.62	36	-	-	*0.11-0.22*
Doscherholmen 1978[b] [10]	3	Healthy	Cooked chicken meat	0.42-0.64	*65*	*8.4*	13	*0.35*
	3		(100, 200 or 300 g)	0.84-1.28	*63*	*13.9*	22	*0.68*
	3			1.26-1.92	*61*	*13.1*	22	*1.0*
Doscherholmen 1981[b] [8]	2	Healthy	Cooked rainbow trout	1.95-2.18	*42*	*5.9*	14	*0.87*
	3		(50, 100, 200 or 300 g)	3.90	*38*	*8.1*	22	*1.5*
	3			7.80-10.90	*41*	*7.5*	18	*3.9*
	3			11.7-15.6	*30*	*5.2*	17	*4.0*
Reizenstein & Nyberg 1959 [28]	11	Healthy or irrelevant diseases[c], 17-38 y	Raw pig/calf liver	22	30	*14*	47	6.0
Kittang 1985[b] [26]	6	Healthy, mean 31 y	Boiled/fried rabbit liver	0.50	49	22	45	*0.24*
	6			1.0	34	10	29	*0.34*
	6			1.5	26	8.4	32	*0.39*
	6			3.0	10	8.4	84	*0.31*
Whole body counting								
Heyssel 1966 [11]	3	Healthy young	Mutton lean meat	0.95	65	*11*	17	0.62
	2	Healthy young	Mutton lean meat	3.03	83	*9.2*	11	2.5
	2	Healthy young	Mutton lean meat	5.11	52	*16*	32	2.7
	6	Healthy young	Mutton liver pate	38	11	*6.0*	55	4.1
	4	Healthy old	Mutton liver pate	38	4.5	*1.7*	38	1.7

Values in italic are based on reported estimates or individual subject data

a-) in the studies of Doscherholmen (1973, 1975, 1981), the radioactivity of a dose labelled vitamin B12 ranged from 0.05 to 1.02 µCi

b-) repeated measures in same subjects

c-) irrelevant diseases included hypoplastic anaemia and mediastinal tuberculosis

(A_i) increased with increasing doses of vitamin B12 (D_i) and the relation was estimated as $\ln(A_i) = 0.7694*\ln(D_i)-0.9614$ as illustrated in Figure 3. Since we applied a natural logarithmic transformation on both the vitamin B12 dose and the amount of vitamin B12 absorbed, the regression coefficient of 0.7694 means that for every doubling in vitamin B12 dose, the difference in absorbed amount is $2^{0.7694}$ (=1.70), which is 70%.

DISCUSSION

Main findings

Currently in Europe, ANRs for vitamin B12 established with the factorial approach are based on a selection of the studies as summarized in this review. This systematic review indicated that the total amount of vitamin B12 lost per day is 0.13% (CV=23%) of total body stores. Observations of the rate of vitamin B12 excretion in bile were similar (1-1.5% of total body stores per day, CV=72-77%), whereas the absolute amount of vitamin B12 excreted in bile per day and vitamin B12 concentrations in bile varied largely between and within studies (CV within studies= 34-133%). Estimates of vitamin B12 absorption varied between 4.5% and 83% (CV 11-84%) based on studies with varying vitamin B12 contents and different types of food products. The relation between absorbed amount (A_i) and ingested dose of vitamin B12 (D_i) was estimated as $\ln(A_i)= 0.7694*\ln(D_i)-0.9614$.

Implications of our findings for estimates of vitamin B12 requirements

Inter-individual variation in requirements

Most studies reporting on daily vitamin B12 losses or absorption are old, include a small number of subjects and details on the study subjects are very limited. Moreover, we observed a large variation between and within studies, especially among studies reporting on vitamin B12 excretion in bile. Estimating vitamin B12 requirements with the factorial approach, as in the Netherlands, requires assumptions regarding the rate of loss, total body stores and bioavailability (Box 1). Each assumption is a component of uncertainty and following the simple error-propagation formula for independent measurements, for any given value of body stores the CV of the ANR for vitamin B12 can be estimated as: $\sqrt{(CV^2_{\text{rate of loss}}+CV^2_{\text{bioavailability}})}$. Based on the observations of within–study variation for the rate of loss (CV=23%, based on the pooled SD) and bioavailability (CV=8-81%), this would imply a CV for the ANR of at least 24%. These calculations suggest

that the CV is larger than the 20% which is currently assumed for estimating recommended intakes of vitamin B12 in the Netherlands. However it must be noted that the CVs used in these calculations are based on studies with few subjects, which may result in an overestimation of the inter-individual variation. Moreover, there is some evidence that lower body stores of vitamin B12 go together with higher absorption (23), as for many other nutrients. If bioavailability of vitamin B12 depends on total body stores, the CV of vitamin B12 requirements may be less than predicted with the error-propagation formula.
The French recommended intake of vitamin B12 is derived as the ANR multiplied by 1.2 (2 times a CV of 10%). However, this review showed that measures of vitamin B12 excretion bile vary largely between and within studies (CV: 34-133%). So, considering the additional assumptions required for estimating vitamin B12 requirements based on daily losses in bile (% reabsorption from the intestine, % bioavailability) it seems likely that a CV of 10% underestimates the inter-individual variation and uncertainty in requirements.

Total vitamin B12 losses measured with WBC

Current recommendations based on the factorial approach include the assumption that compensation of daily losses will be sufficient to maintain vitamin B12 body stores, and thereby prevent deficiency disorders. Estimates of body stores (500 µg) were based on observations in patients suffering from malabsorption that remained both hematologically normal (no megaloblastic changes of the bone marrow, no anemia, no macrocytosis) and serum vitamin B12 concentrations at 96-148 pmol/l. As recommendations on micronutrient intake are designed for apparently healthy populations, it may be more appropriate to use estimates of body stores based on apparently healthy subjects for estimating daily losses. The Food and Nutrition Board/Institute of Medicine summarized mean total body stores in adults with most estimates between 2 and 3 mg (5). Based on these stores and a rate of loss of 0.13% of stores per day, total losses would range between 2.6 and 3.9 µg per day under the assumption that size of the total body stores of vitamin B12 is the major determinant of the amount of vitamin B12 lost per day (Table 4).

Vitamin B12 excretion in bile

The absolute amount excreted in bile per day was 1.4 µg as reported in one study. Based on the reported mean rate of loss of 1.1 or 1.5% of total body stores and assuming body stores to range between 2 and 3 mg as also referred to above (5), daily vitamin B12 excretion in bile may range between 22 and 45 µg. Daily biliary excretion of vitamin B12 derived from the reported concentration of the vitamin in bile was 1.6 µg per day, assuming a daily output of bile of 493 ml (15). So, using the French assumption that 40% of vitamin B12 excreted in bile is reabsorbed in the small intestine (Box 1), total losses of vitamin B12 range between 0.8 µg and 27 µg per day as shown in Table 4. It must be noted however, that in two studies included in this review, excretion of vitamin B12 was measured both in bile and in faeces and results showed that losses in faeces were about one third (28-33%) of the amount of vitamin B12 excreted in bile (22, 23). This would imply that more than two thirds of the vitamin B12 excreted in bile is reabsorbed in the small intestine and that daily losses range between 0.5 µg and 14.9 µg per day (Table 4).

Bioavailability of vitamin B12

Absorption of vitamin B12 from food is influenced by the type of food source and its vitamin B12 content as shown in this review and in other publications (4, 7). Studies included in our review measured absorbed amounts up to 6 µg from a single meal. This is not consistent with previous data showing that the average maximum amount of vitamin B12 that can be absorbed from amounts usually consumed with a meal is about 1.5 µg due to saturation of the vitamin B12-Intrinsic factor receptors, which are key-factors in the absorption of vitamin B12 from the ileum (34). Although, passive diffusion of 1% of the ingested dose also takes place, this only becomes relevant when high doses are ingested, e.g. via supplements.

In general, absorption of 50% from the diet was assumed for estimating vitamin B12 requirements. Although two studies observed vitamin B12 absorption of more than 50% (chicken or mutton mean), absorption from other food products was lower than 50%. So, it seems that the generally assumed 50% overestimates real absorption, leading to an underestimation of the requirements. Moreover, as it is suggested that there is a maximum amount of vitamin B12 that can be absorbed per meal, it does not seem right to report on the percentage absorption of a specific food without referring to the actual dose ingested (4). Usual vitamin B12 intakes among

Table 4 Summary of vitamin B12 requirements estimated with the factorial approach

Approach	Number of studies	Measure	Assumption	Total losses (µg)	Bioavailability[a]	Requirements (µg)
Daily losses (based on WBC)	5	Rate of loss 0.13%	Body stores: 2-3 mg	2.6-3.9	29 37	9.0-13.4 7.0-10.5
Daily losses (based on excretion in bile)	2	Rate of loss 1.1-1.5%	Body stores: 2-3 mg 67% reabsorption	7.3-14.9	29 37	25-51 20-40
	1	Bile concentrations 3248 pg/ml	Daily output of bile:493 ml 67% reabsorption	0.5	29 37	3.3 2.6
	1	Absolute amounts in bile 1.4 µg	67% reabsorption	0.5	29 37	1.6 1.2

a- bioavailability of 29% based on a meal content of 3.1 µg vitamin B12 and bioavailability of 37% based on a meal content of 1.2 µg

adults and elderly people across Europe range from 3.5 µg to 9.3 µg per day (35). Assuming that each day three meals contribute to vitamin B12 intake, and that these three meals contain equal amounts of vitamin B12 (1.2 µg to 3.1 µg per meal), the regression equation derived in this review ($\ln(A_i)= 0.7694*\ln(D_i)-0.9614$), could be used to predict the bioavailability as follows:
$\ln(\text{Bioavailability}) = \ln(A/D) = \ln(A) - \ln(D)= (0.7694 -1) * \ln(D)-0.9614$
and bioavailability is = $(1.2 \text{ to } 3.1 \text{ µg})^{(0.7694-1)} * (e^{-0.9614})$ which is 29-37%.

Vitamin B12 requirements

Combing the estimates of daily vitamin B12 losses based on WBC measurements or excretion in bile and a bioavailability from diet of 29-37%, the dietary vitamin B12 intake needed to compensate daily losses would range from 7.0 to13.4 µg per day or from 1.2 to 51 µg per day, respectively as shown in Table 4. These intake ranges indicate the daily intake necessary to maintain body stores in apparently healthy adults and elderly people rather than the intake level at which requirements of 50% of individuals in a population are fulfilled. Therefore the intake ranges reflect the recommended vitamin B12 intake rather than the ANR.

As vitamin B12 requirements based on excretion in bile vary with a factor 30, these estimates seem less adequate for deriving recommended vitamin B12 intakes. Although vitamin B12 requirements based on total losses measured with WBC vary with a factor two, these estimates are in line with findings from five studies that showed that plasma concentrations of markers for vitamin B12 status (total vitamin B12, methylmalonic acid and holotranscobalamin II) leveled off at daily intakes between 4 and 10 µg per day (36-40). Daily intakes between 4 and 10 µg per day will therefore likely be adequate for the prevention of subclinical deficiencies of vitamin B12 that may be associated with potential health risks including neurologic and cognitive risks (41). However, more evidence is needed on the relation between plasma concentrations of markers for vitamin B12 status, vitamin B12 body stores and long-term health outcomes to define whether it is beneficial to maintain maximal vitamin B12 status and stores of 2-3 mg.

It must be noted that all studies included in our review were based on subjects with normal absorption. From the age of 60y on, the prevalence of food-bound malabsorption increases, which is a primary cause for vitamin B12 deficiency affecting about 20% of elderly populations in industrialized countries (42). Food-bound malabsorption is characterized by the inability to

release vitamin B12 from food or from intestinal transport proteins. Therefore this vitamin B12 cannot or only partly bind to intrinsic factor and so absorption of vitamin B12 does not take place or only to a lesser extent. Absorption of crystalline vitamin B12 is usually not affected in subjects with food-bound malabsorption and therefore most European countries recommend elderly people to consume a high proportion of their vitamin B12 intake as fortified foods and supplements (1). In two studies investigating absorption from bread, squash or milk fortified with 0.25-0.5 µg vitamin B12, an absorption between 55 and 74% was observed (12, 31).

CONCLUSIONS

This review showed that daily vitamin B12 losses in apparently healthy adults and elderly probably range between 2.6-3.9 µg. Based on usual intake data and the relation between vitamin B12 intake and the amount absorbed, bioavailability from the diet seems to range between 29 and 37% rather than the generally assumed 50%. These results suggest that a vitamin B12 intake between 7.0 and 13.4 µg is needed to compensate daily vitamin B12 losses in apparently healthy adults and elderly people, which is 2.5 to 5 times higher than the amount needed to prevent deficiency. Current recommendations on vitamin B12 intake of 1.4-3.0 µg may be inadequate to maintain body stores of 2-3 mg and optimal plasma concentrations of markers of vitamin B12 status. However, more evidence is needed on the relation between plasma concentrations of markers for vitamin B12 status, vitamin B12 body stores and long-term health outcomes to evaluate whether current recommended vitamin B12 intake need to be changed.

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Supplemental file 1:

Search strategy to identify papers on vitamin B12 losses in MEDLINE (12-07-2010)

	Searches
1	("vitamin b12" or vitamin-b12 or "vitamin b 12" or "vitamin-b 12" or cobalamin* or cyanocobalamin* or hydroxocobalamin* or methylcobalamin* or adenosylcobalamin*).mp. [mp=title, original title, abstract, name of substance word, subject heading word, unique identifier]
2	exp Transcobalamins/
3	exp Vitamin B 12/
4	1 or 2 or 3
5	(radio-isotop* or radioisotop* or radio-label* or radiolabel* or radio-active or radioactive or "isotopically labelled").mp. [mp=title, original title, abstract, name of substance word, subject heading word, unique identifier]
6	radioisotopes/ or exp radioactive tracers/
7	5 or 6
8	4 and 7
9	("Cobalt-57" or "Co-57" or "57Co" or "(57)Co").mp. [mp=title, original title, abstract, name of substance word, subject heading word, unique identifier]
10	4 or 8 or 9
11	exp urine/
12	exp sweat/
13	exp feces/
14	exp bile/
15	exp skin/
16	exp menstruation/
17	exp integumentary system/
18	11 or 12 or 13 or 14 or 15 or 16 or 17
19	10 and 18
20	exp kinetics/
21	exp tissue distribution/
22	exp enterohepatic circulation/
23	20 or 21 or 22
24	10 and 23
25	exp maintenance/
26	10 and 25
27	19 or 24
28	limit 27 to (english language and humans)

Search strategy to identify papers on vitamin B12 bioavailability in MEDLINE (15-06-2010)*

	Searches
1	("vitamin b12" or vitamin-b12 or "vitamin b 12" or "vitamin-b 12" or cobalamin* or cyanocobalamin* or hydroxocobalamin* or methylcobalamin* or adenosylcobalamin*).mp. [mp=title, original title, abstract, name of substance word, subject heading word, unique identifier]
2	exp Vitamin B 12/
3	exp Transcobalamins/
4	(radio-isotop* or radioisotop* or radio-label* or radiolabel*).mp. [mp=title, original title, abstract, name of substance word, subject heading word, unique identifier]
5	radioisotopes/ or exp radioactive tracers/
6	1 or 2 or 3 or 4 or 5
7	(bioavailab* or bio-availab* or bioaccess* or bio-access* or absorption).mp. [mp=title, original title, abstract, name of substance word, subject heading word, unique identifier]
8	exp Intestinal absorption/ or exp Absorption/ or exp biological availability/
9	(("vitamin b12" or "vitamin-b12" or "vitamin b 12" or "vitamin-b 12" or $cobalamin*) adj5 (status or metabol* or retention* or concentrat* or homocysteine* or hcy or tHcy or holo-tc or Holotranscobalamin* or holo-transcobalamin* or holoTC or plasma or serum)).mp. [mp=title, original title, abstract, name of substance word, subject heading word, unique identifier]
10	(diet* or meal* or food* or lactat*).mp. [mp=title, original title, abstract, name of substance word, subject heading word, unique identifier]
11	bottle feeding/ or breast feeding/ or weaning/ or infant food/ or infant formula/ or milk, human/
12	10 or 11
13	7 or 8 or 9
14	6 and 12 and 13
15	limit 14 to humans

*The search terms presented here were part of a larger search that also contained terms to identify papers on folate bioavailability. Here we only present the terms included for vitamin B12

chapter 5

Vitamin B12 intake and status and cognitive function in elderly people: a systematic review with meta-analyses

Epidemiologic reviews (accepted with modifications)

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ABSTRACT

The objective of this systematic review was to evaluate whether the relation between vitamin B12 intake and cognitive function should be considered for underpinning vitamin B12 recommendations in the future. The authors summarized dose-response evidence from randomized controlled trials (RCTs) and prospective cohort studies on the relation of vitamin B12 intake and status with cognitive function in adults and elderly people. Random-effect meta-analyses were used to pool the relative risk (RR) (binary outcomes) or regression coefficients (β) (continuous outcomes) from multiple adjusted models. Two RCTs and 4 cohort studies showed no or inconsistent associations between vitamin B12 intake and cognitive function. Serum/plasma vitamin B12 (50 pmol/l) was not associated with dementia (4 cohort studies, RR=1.01, 95% confidence interval (CI)=0.99, 1.03) and 5 cohort studies reported no association between vitamin B12 status (serum vitamin B12 n=3, holotranscobalamin II n=2) and Alzheimer's disease. Baseline serum/plasma vitamin B12 (50 pmol/l) was not associated with the additional rate of change in global cognition z-scores (4 cohort studies, β=0.00, 95% CI= -0.00, 0.01) or memory z-scores at follow-up (4 cohort studies, β=0.01; 95% CI= -0.01, 0.03). Five cohort studies reported no associations between serum/plasma vitamin B12 and executive function, language or speed. Current evidence on the relation between vitamin B12 intake or status and cognitive function does not suffice for being considered in deriving vitamin B12 recommendations.

INTRODUCTION

Dietary recommendations provide guidance on nutrient intakes that should be sufficient to fulfil requirements of nearly all apparently healthy people in a specified population. Traditionally, these recommendations were intended to prevent deficiency disorders, but today the focus is slowly changing towards optimal health also including relations between diet and prevention of chronic diseases (1, 2). Current recommendations on vitamin B12 intake are similar for adults and elderly people and vary from 1.4-3.0 µg per day in Europe. They are based on the amount needed for the maintenance of hematological status and on the amount needed to compensate obligatory losses (3-5). Relations between vitamin B12 intake and health related outcomes, e.g. cardiovascular diseases, cognitive function, and osteoporosis, are not yet taken into account when deriving vitamin B12 recommendations. To support transparent decision-making on whether these relations should be considered for setting vitamin B12 recommendations in the future, systematic reviews and meta-analyses are needed to objectively evaluate and integrate the available evidence (6). Previously, 5 systematic reviews addressed the relation between vitamin B12 intake or status and cognitive function in a qualitative manner (7-11). The aim of this review was to summarize dose-response evidence from randomized controlled trials (RCTs) and prospective cohort studies on the relation of vitamin B12 intake and status with cognitive function in adults and elderly people and to identify research gaps relevant for deriving vitamin B12 recommendations.

METHODS

This systematic review with dose-response meta-analyses was conducted according to standardized methodology as developed within the scope of the EURRECA (EUROpean micronutrient RECommendations Aligned) Network of Excellence, which is shortly described below.

Search

We conducted a systematic literature search in the databases MEDLINE, EMBASE and Cochrane library Central through February 17, 2009, using search terms on study designs in humans AND vitamin B12 AND (intake OR status). The search terms both included MeSH terms and words to be found in title or abstract. The strategy was adapted for each database to fit database specific features. To be able to use the same search to identify publications on other health related outcomes both in adults and elderly and

in younger population groups, no terms were added to limit the search to health outcome or study population. Moreover, by using a broad search we expected a more complete retrieval of relevant publications. The search was not limited by language. Supplemental file 1 shows the full MEDLINE search strategy. The initial search yielded 5,219 references after exclusion of duplicates using Endnote XII. In addition, we reviewed reference lists of 10 review articles reporting on the relation between vitamin B12 intake or status and cognitive function to identify potentially relevant references that were not yet collected on basis of the database search (n=134). We updated the searches on February 11, 2010 (n=560) and from February 2010 to July 2011 we checked database alerts.

Selection of studies

For the selection of relevant papers for our systematic review, we used predefined in- and exclusion criteria. In general, studies were eligible for inclusion if they were conducted in apparently healthy human subjects aged ≥18 y, addressed cognitive function as a health outcome and reported baseline data of the outcome measure. We defined 4 categories of cognitive function: incident dementia, incident Alzheimer's disease (AD), global cognition, and domain-specific cognition. The specific domains of cognitive function used in this review were based on the classification of cognitive tests proposed by Wald et al. 2010 (12): memory, speed, language and executive function. Global cognition comprised assessment methods addressing different domains of cognition e.g. a compound z-score combining z-scores of different cognitive performance tests or the mini-mental state examination (MMSE) combining aspects of orientation, memory and attention into one questionnaire (13). Domain-specific cognition includes cognitive performance tests assessing a single domain of cognitive function.

Observational studies were included if they 1) had a prospective cohort or nested case-control design, and 2) addressed exposure by either validated dietary assessment methods or by serum/plasma concentration of markers indicating vitamin B12 status (total vitamin B12, methylmalonic acid (MMA) or holotranscobalamin II (holoTC II)). Intervention studies were included if they 1) had a randomized controlled trial design, 2) studied the effects of supplements, fortified foods or micronutrient intake from natural food sources and included a placebo or untreated comparison group, and 3) had minimum intervention duration of 2 weeks. Studies were excluded if they only included patients with pre-existing disease because relations of vitamin

B12 intake or status with cognitive function in such study populations may not be representative for the general apparently healthy population.
First, 2 reviewers (ED, SB) screened titles and abstracts of all references identified with the searches according to the in- and exclusion criteria. Secondly, 4 reviewers (ED, JvW, AS, MP) evaluated full texts of the remaining references against the same criteria. For the purpose of alignment and quality control, each reviewer screened and evaluated 10 percent of the total number of references in duplicate with another reviewer. The rare discrepancies were resolved by group consultation among all reviewers until consensus was reached. During the selection process all reasons for exclusion were registered and details are shown in Figure 1.

Data extraction

Data extraction was performed by a single reviewer (ED, JvW, or AS) using standardized data extraction forms in an Access database. A second reviewer verified the data. Disagreements were discussed and settled, if needed in consultation with a third reviewer. Data extracted included general characteristics of the study design and study population; details on measures of intake, status and cognitive function; details on data analysis and results. In addition, we extracted information concerning the validity of studies including sequence allocation (RCT), blinding (RCT), compliance (RCT), reproducibility and between-population comparability of intake, status and cognitive measures, control for confounders (at least age, sex, education, vascular disease, ApoE-ε4) and other forms of bias. We assessed the overall risk of bias of each individual study using standardized procedures largely based on guidance from the Cochrane Collaboration (14), resulting in one of the following judgments: low, moderate or high risk of bias.

Data-synthesis

Opportunities for meta-analysis were evaluated for comparability with regard to cognitive outcome, intake or status marker and the study population. If less than 3 comparable studies were available, results were qualitatively described. If 3 or more comparable studies were available, we carried out a dose-response meta-analysis that pools the relative risk (RR) per change in unit of exposure (binary outcomes) or the regression coefficient (β)
(continuous outcomes) from multiple adjusted models. For serum/plasma vitamin B12 we chose to express association measures per 50 pmol/l.

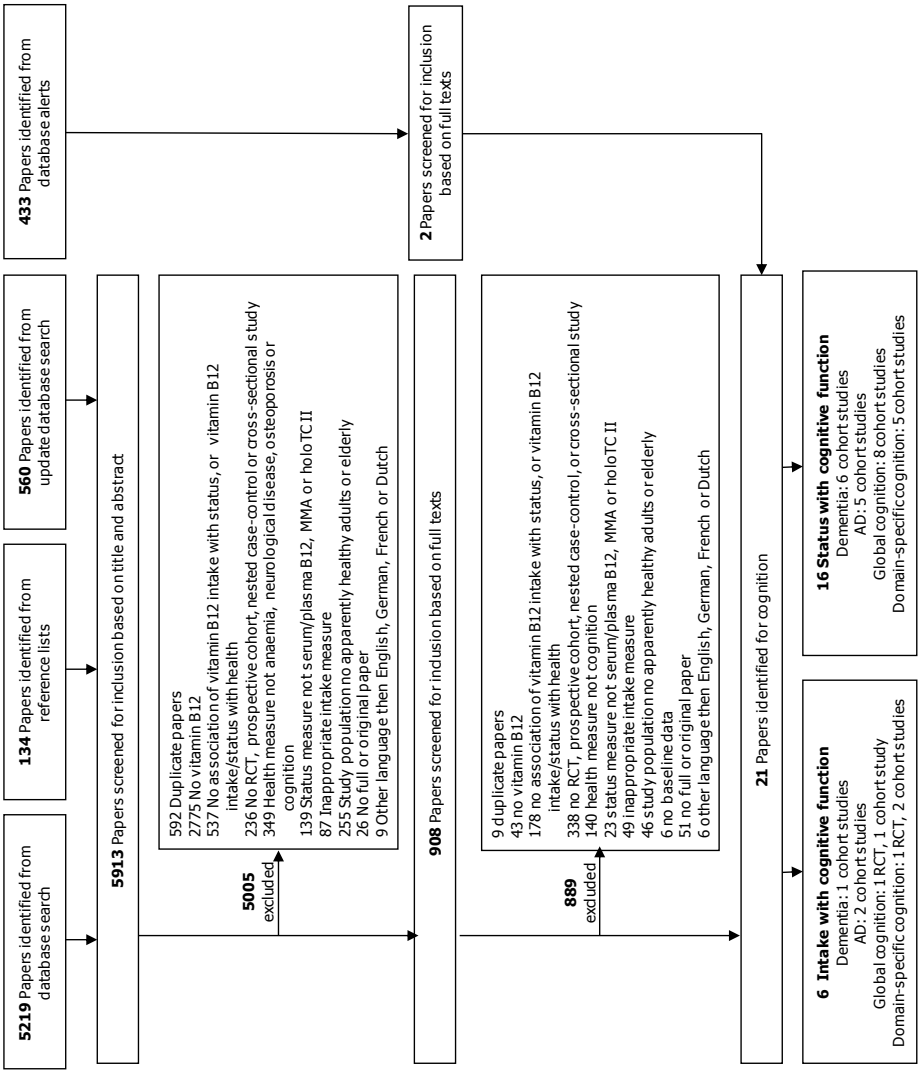

Figure 1 Study selection process for systematic review

RCT, Randomized Controlled Trial; MMA, methylmalonic acid, holoTC II, holotranscobalamin II

Hazard ratios (HR) and odds ratios (OR) were considered as relative risk because the outcome was relatively rare.

If articles reported insufficient data (missing data, inconsistencies or any other uncertainties), we requested corresponding authors for additional information.
None of the studies on incidence of dementia or AD provided data in the desired format, but rather presented the risk of dementia or AD comparing subjects with low versus normal vitamin B12 status (15-18) or the number of cases and controls among subjects with and without vitamin B12 deficiency (19). One author provided us with the log (RR) and its standard error (SE) upon request (15, 16) and for 3 other studies we were able to derive the log (RR) (SE) based on reported data (16, 19) and data provided by the authors (18) as described in detail in Supplemental file 2**.** One author did not respond to our requests, and data were insufficient to derive the log (RR) (SE) (17).

All studies on global cognition included repeated measures of global cognition scores. Associations with vitamin B12 status were most frequently assessed by the use of linear mixed models including serum/plasma concentrations of a vitamin B12 status marker, a time variable and the interaction term of vitamin B12 status and time (20-22). In such models, the regression coefficient for the interaction term represents the rate of change in global cognition attributable to an increase in vitamin B12 status additional to the deterioration in global cognition as a result of aging. Other studies presented associations between baseline serum vitamin B12 concentrations and serial (23) or follow-up (24) global cognition scores, associations between baseline serum vitamin B12 and changes in cognitive decline during follow-up (25, 26), or Spearman rank correlations between changes in serum vitamin B12 and MMSE-scores (27). In 4 studies concentrations of vitamin B12 status markers were log-transformed (20, 23-25) and 1 study presented associations per SD increase in serum vitamin B12 (21). We requested corresponding authors to provide us with the regression coefficient (SE) for the interaction term including untransformed continuous serum vitamin B12 concentrations. One study already reported data in the desired format (22) and 3 authors (21, 24, 26) provided the data upon request.

We requested authors of studies only providing data on MMSE-scores (20, 23-25, 27) to repeat the analyses with MMSE expressed as a z-score, one author provided us with the requested data (24).

Studies identified for domain-specific cognition addressed several cognitive domains, however, only for the domains executive function (21, 24, 28) and memory (21, 24, 26, 28) 3 or more comparable studies were available. Although studies were comparable, there was still variation with regard to the cognitive performance tests used, the frequency of cognitive performance testing, and statistical methods used to assess the association between executive function or memory and vitamin B12 status. Therefore we requested corresponding authors to provide us with the regression coefficient (SE) for the association between untransformed vitamin B12 status measured at baseline and cognitive performance test scores at follow-up expressed as z-scores. Four authors provided these data upon request (21, 24, 26, 29), enabling us to perform a meta-analysis for the domain memory.

Statistical analyses

We calculated summary estimates of comparable studies using random effects meta-analysis. Applying the methods of DerSimonian and Laird, the between-study variance is estimated which is then used to modify the weights for calculating the summary estimate (30). Heterogeneity between studies was evaluated using the I^2 statistic, which expresses the percentage of variation attributable to between-study heterogeneity rather than chance (31). All statistical analyses were performed using STATA version 11.0 (College Station, TX), with statistical significance defined as a P-value <0.05.

RESULTS

In total, we identified 5,913 potentially relevant papers, of which 5,005 papers were excluded based on title and abstract. Of the remaining 908 papers, 889 were excluded based on full texts, leaving 19 papers addressing the relation between vitamin B12 intake or status and cognitive function. Two additional papers were identified from the database alerts (Figure 1). From the 21 included studies, 2 RCTs and 4 prospective cohort studies addressed the relation between vitamin B12 intake and cognitive function.

Table 1 Intake of Vitamin B12 in Relation to Incidence of Dementia, Incidence of Alzheimer's Disease (AD), Global and Domain-Specific Indicators of Cognition: RCTs and Prospective Cohort Studies

Author Year	Population characteristics: N (% men) Age (year)	Study characteristics: Study design Duration/Follow-up Risk of bias	Exposure: Vitamin B12 intake (µg/d)	Cognitive outcome (number of cases or specific test):	Association/effect measure:	Results:
Nelson 2009 [32]	3634 (43) *74.7 (6.7)* (a)	Cohort 9 years Low risk	9.6 (10.2)	Incidence of dementia (352 cases) Incidence of AD (212 cases)	HR (95% CI) by quintile of intake (Q5/Q1) (e)	0.87 (0.52-1.46) 0.91 (0.52-1.60)
Morris 2006 [33]	1041 (*38*) *72.7* (b)	Cohort 3.9 years Moderate risk	*11.1* (0.5-127.2) (c)	Incidence of AD (162 cases)	OR (95% CI) by quintile of intake (Q5/Q1) (f)	0.6 (0.2-1.6)
Seal 2002 [34]	10 µg:10 (40), 50 µg:10 (50), Control:11 (45) 10 µg:82, 50 µg:84.9, Control:77.6 Low vitamin B12 status at baseline	RCT 4 weeks Moderate risk	10 or 50 vs placebo	Global cognition (MMSE)	Difference between treatment groups	P=0.494
Tucker 2005 [24]	321 (100) 67 (7)	Cohort 3 years Moderate risk	9.57 (5.73)	Global cognition (MMSE) Executive function (spatial copying) Memory (working memory) Memory (recall memory) Language (verbal fluency)	β for association between cognition at 3 y and baseline intake (g)	0.14, n.s. 0.37 (p<0.05) 0.12, n.s. -0.01, n.s. 0.38, n.s.
Eussen 2006 [35]	Intervention: 54 (23), Control: 57(22) Intervention :82 (5), Control: 82 (5) Low vitamin B12 status at baseline	RCT 24 weeks Moderate risk	1000 vs placebo	Executive function (compound z-score, n=7) Memory (compound z-score, n=6) Speed (compound z-score, n=3)	Difference between treatment groups	n.s. p<0.05 (h) n.s.
La Rue 1997 [28]	122 (49*) *71.7*	Cohort 6 years High risk	5.4 (2.9-11.7) (d)	Executive function (Rey Osterrieth copy) Executive function (Shipley-Hartford abstraction) Memory (Rey-Osterrieth recall) Memory (WMS logical memory) Memory (WMS visual reproduction)	Spearman correlations between cognition at 6 y and baseline intake (i)	n.s. 0.20 (p<0.05) 0.19 (p<0.05) n.s. n.s.

RCT, randomized controlled trial; MMSE: mini-mental state examination; HR, hazard ratio; OR, odds ratio; n.s. not significant, but no p-value provided
Values in italics are calculated based on provided values. *Values Based on total population
(a) mean (SD) all such values, unless stated otherwise; (b) mean, unless stated otherwise; (c) Mean (range); (d) Median (IQR); (e) Nelson et al. adjusted for gender, education, Body Mass Index, total energy, physical activity, ApoE4 status, alcohol, smoking, Myocardial Infarction, stroke, Diabetes and the other B-vitamins; (f) Morris et al. adjusted for age, gender, ethnicity, education, vitamin E intake, niacin intake, ApoE4 status, participation in cognitive activities; (g) Tucker et al. adjusted for age, education, Body Mass Index, alcohol, smoking, diabetes, systolic blood pressure, baseline cognitive measures, time of second measure relative to folic acid fortification, time interval between the 2 cognitive measures, total energy intake; (h) Memory function improved in all treatment groups, but the improvement in the placebo group was significantly better than the improvement in the vitamin B12 group; (i) La Rue et al. adjusted for age

Table 2 Vitamin B12 Status in Relation to Incidence of Dementia and Incidence of Alzheimer's Disease (AD): Prospective Cohort Studies

Author Year	Population characteristics: N (% men) Age (years)	Study characteristics: Study design Follow-up (years) Risk of bias	Exposure: Marker of vitamin B12 status Concentration	Outcome: Incidence of dementia/AD (cases)	Association measure:	Result:
Crystal 1994 [19]	410 (?) 75-85 (a)	Cohort 5 High risk	Serum vitamin B12 (pmol/l) 412 (74-3660)(c)	Dementia (60) AD (30)	RR (95%CI) per 50 units increase in baseline status (e) RR (95%CI) per 50 units increase in baseline status (e)	*1.00 (0.96-1.03)* *0.98 (0.92-1.05)*
Haan 2007 [15]	1332 (42*) 60-101	Cohort 4.5 Moderate risk	Plasma vitamin B12 (pmol/l) 334 (150)	Dementia or cognitive impairment no dementia (CIND) (80)	HR (95% CI) per unit increase in sqrt baseline status (f) HR (95%CI) per 50 units increase in baseline status (f)	1.05 (1.01-1.09) 1.03 (0.98-1.07)
Kim 2008 [16]	518 (43) *71.8* (*5.0)* (b)	Cohort 2.4 Moderate risk	Serum vitamin B12 (pmol/l) *380.7 (149.3)*	Dementia (45)	OR (95% CI) for low (<258 pmol/l) versus normal baseline status (g) OR (95%CI) per 50 units increase in baseline status (g)	1.53 (0.69-3.38) *1.08 (0.93-1.26)*
Wang 2001 [18]	370 (20) 75-101	Cohort 3 Moderate risk	Serum vitamin B12 (pmol/l) 324 (510)	Dementia (78) AD (60)	RR (95% CI) for low (≤250 pmol/l) versus normal baseline status (h) RR (95%CI) per 50 units increase in baseline status (h) RR (95% CI) for low (≤250 pmol/l) versus normal baseline status (h) RR[4] (95%CI) per 50 units increase in baseline status (h)	1.3 (0.8-2.1) *1.0 (1.0-1.1)* 1.8 (1.0-3.0) *1.0 (1.0-1.1)*
Ravaglia 2005 [17]	816 (47) 73.6 (6.3)	Cohort 4 Moderate risk	Serum vitamin B12 (pmol/l) tHcy≤15 µmol/l: 259 (94-708) (d) tHcy>15 µmol/l: 212 (73-612) (d)	Dementia (112) AD (70)	HR (95% CI) for low (≤251 pmol/l) versus normal baseline status (i)	0.83 (0.56-1.24) 0.66 (0.40-1.09)
Kivipelto 2009 [42]	213 (25) 81.0 (4.6)	Cohort 6.7 Moderate risk	Holo-TC (pmol/l) 105 (88)	Dementia (83) AD (61)	RR (95%CI) per unit increase in baseline status (j)	1.00 (0.99–1.00) 1.00 (0.99-1.00)

Table 2 (continued)

Author Year	Population characteristics: N (% men) Age (years)	Study characteristics: Study design Follow-up (years) Risk of bias	Exposure: Marker of vitamin B12 status Concentration	Outcome: Incidence of dementia/AD (cases)	Association measure:	Result:
Hooshmand 2010 [43]	271 (38) 70.7 (3.6)	Cohort 7.4 Low risk	HoloTC II (pmol/l) *91.3 (51.0)*	AD (17)	OR (95%CI) per unit increase in baseline status (k)	0.977 (0.958-0.997)

AD, Alzheimer's disease; HoloTC II, holotranscobalamin II; RR, relative risk; HR, hazard ratio; OR, odds ratio; CI, confidence interval
Values in italics are calculated based on provided values. Underlined values are additionaly provided by the author *Values based on total population
(a) range all such values
(b) mean (SD) all such values, unless stated otherwise
(c) mean (range)
(d) geometric means (95% confidence Interval)
(e) Crystal et al. unadjusted
(f) Haan et al. adjusted for age, sex, education, baseline stroke, plasma homocysteine, red blood cell folate,
(g) Kim et al. adjusted for age, sex, education, smoking, alcohol, physical activity, body weight, disability, depression, vascular risk score, vitamin intake, serum creatinine
(h)Wang et al. adjusted for age, sex, education
(i) Ravaglia et al. adjusted for age, sex, education, ApoE4 status, history of stroke, serum creatinine, plasma homocysteine, serum folate,
(j) Kivipelto et al. adjusted for age, sex, education
(k) Hooshmand et al. adjusted for age, sex, education, duration of follow-up, ApoE4 status, Body Mass Index, Mini Mental State Examination-score, systolic blood pressure, diastolic blood pressure, smoking, history of stroke

Seventeen prospective cohort studies addressed the relation between vitamin B12 status, measured by serum/plasma vitamin B12 (n=15), MMA (n=2) or holoTC II (n=2) and cognitive function**.**

Vitamin B12 intake and cognitive function

Details on the studies addressing the relation between vitamin B12 intake and cognitive function are presented in Table 1. Due to the large variation in cognitive outcomes, no meta-analyses could be performed on this relation. Studies on incident dementia (32), incident AD (32, 33) or global cognition assessed by MMSE (24, 34) did not show significant associations with vitamin B12 intake. One RCT (35) and 2 prospective cohort studies (24, 28) addressed the association between vitamin B12 intake and domain-specific cognition. For the domain executive function the 2 prospective cohort studies found that higher vitamin B12 intakes at baseline were associated with a better cognitive performance after 3 or 6 years of follow-up (β=0.37, $p<0.05$ (24), Spearman correlation=0.20, $p<0.05$ (28)) whereas the RCT did not show beneficial effects of daily vitamin B12 supplementation (35). For the domain memory results were largely inconsistent showing a positive (28), negative (35) or no (24, 28) association. The cognitive domains speed and language were only addressed in single studies and no associations with vitamin B12 intake were observed (24, 35).

Vitamin B12 status and incident dementia or AD

Table 2 presents details of the prospective cohort studies investigating the association between vitamin B12 status and incidence of dementia or AD. Dementia and AD were diagnosed based on similar criteria (dementia: (36-39); AD: (39-41)). Four studies involving 2,630 elderly people (age range: 60-101 year) were included in a meta-analysis pooling relative risks for incidence of dementia (263 cases) during 2.4 to 4.5y follow-up per 50 pmol/l increase in serum/plasma vitamin B12 concentrations at baseline (15, 16, 18, 19). The summary estimate showed no association between serum/plasma vitamin B12 and incidence of dementia (RR=1.01, 95% CI: 0.99, 1.03) with no heterogeneity between studies (I^2=0%, p=0.61) (Figure 2). Similar to this finding, Ravaglia et al. (2005) (17) reported that low serum vitamin B12 concentrations (≤250 pmol/l) did not significantly increase the risk of dementia (HR=0.83, 95% CI: 0.56, 1.24) and Kivipelto et al. (2009) (42) reported a relative risk of 1.00 for the risk of dementia per pmol/l increase in holoTC II concentrations (95% CI: 0.99, 1.00).

Five studies reported on the association between vitamin B12 status (serum vitamin B12 n=3, holoTC II n=2) and incidence of AD. Hooshmand et al. (2010) (43) reported a borderline significant odds ratio per pmol/l increase in holoTC II concentrations at baseline, but all other studies did not show significant associations (17-19, 42).

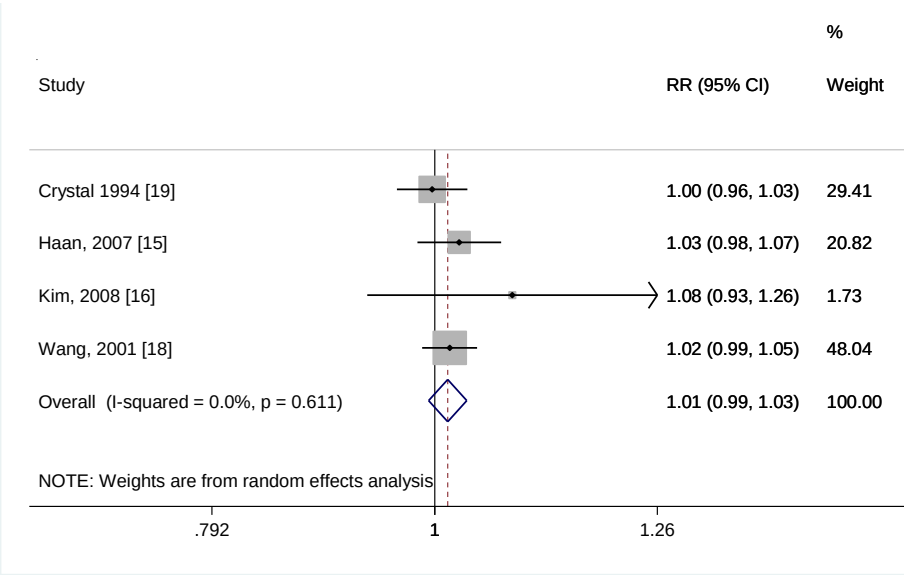

Figure 2 Forest plot of association between serum/plasma vitamin B12 (50 pmol/l) and risk of dementia: Meta-Analysis of 4 prospective cohort studies (n=2630, 363 cases)
RR, relative risk; CI, confidence interval

Vitamin B12 status and global cognition

For global cognition, we identified 3 prospective cohort studies on serum/plasma vitamin B12 and compound z-scores (21, 22, 26), and 5 prospective cohort studies on serum/plasma vitamin B12 and MMSE-scores (20, 21, 23, 24, 35) (Table 3).

From 4 cohort studies including 1,579 elderly people, we obtained the regression coefficient for associations between additional annual change in compound z-score (21, 22, 26) or MMSE z-score (24) and serum/plasma vitamin B12 concentrations (50 pmol/l). A pooled analysis of the results showed no overall association (β=0.00, 95% CI: -0.00, 0.01), with moderate heterogeneity between studies (I^2=42.6%, p=0.16) (Figure 3).

Table 3 Vitamin B12 Status in Relation to Global Cognition Scores: Prospective Cohort Studies

Author Year	Population characteristics: N (% men) Age (years)	Study characteristics: Study design Follow-up (years) Risk of bias	Exposure: Marker of vitamin B12 status Concentration	Outcome: Global cognition score	Association measure:	Result:
Clarke 2007 [20]	691 (40) 71.9 (5.2) (a)	Cohort 10 Low risk	Serum vitamin B12 (pmol/l) 280 *(106*) MMA (µmol/l) 0.35*(0.30*) HoloTC II (pmol/l) 73* (43*)	MMSE	β (95%CI) for additional change in MMSE-score during 10y associated with a doubling of baseline status (d)	Serum vitamin B12 0.22 (-0.02; 0.46) MMA: -0.65 (-0.98; -0.32)** Holo-TC: 0.59 (0.30; 0.88)
Eussen 2002 [27][	189 (49*) 75-80 (b)	Cohort 5 High risk	Plasma vitamin B12 (pmol/l) *338 (323)*	MMSE	Spearman rank correlation between changes in MMSE scores and status after 5y follow-up (e)	-0.069, n.s.
Feng 2009 [23]	539 (40) 64.9 (7.2)	Cohort 3.2 Low risk	Serum vitamin B12 (pmol/l) 396 (181)	MMSE	β (SE) for associations between repeated MMSE-scores and log-transformed baseline status (f)	1.07 (0.35), p=0.003
Tucker 2005 [24]	321 (100) 67 (7)	Cohort 3 Moderate risk	Serum vitamin B12 (pmol/l) 335 (136)	MMSE	β (SE) for association between 3y MMSE scores and log-transformed baseline status (g)	-0.16 (0.22), n.s.
					β (SE) for additional annual change in MMSE per 50 units increase in baseline status (g)	0.041 (0.033), p=0.21
				MMSE z-score*	β (SE) for the additional annual change in MMSE z-score per 50 units increase in baseline status (g)	0.024 (0.020), p=0.21
Mooijaart 2005 [21]	351 (34*) 85(c)	Cohort 4 Moderate risk	Serum vitamin B12 (pmol/l) *315 (184)*	MMSE	β (SE) for the additional annual change in MMSE per SD increase in baseline status (h)	-0.009 (0.07), p=0.89
					β (SE) for the additional annual change in MMSE per 50 units increase in baseline status (h)	0.012 (0.023), p=0.60
				Compound z-score (n=4)	β (SE) for the additional annual change in z-score per 50 units increase in baseline status (h)	-0.0023 (0.0028), p=0.42

Table 3 (continued)

Author Year	Population characteristics: N (% men) Age (years)	Study characteristics: Study design Follow-up (years) Risk of bias	Exposure: Marker of vitamin B12 status Concentration	Outcome: Global cognition score	Association measure:	Result:
Kang 2006 [26]	391 (0) 63	Cohort 4 Moderate risk	Plasma vitamin B12 (pmol/l) 337	Compound z-score (n=6)	Mean difference in rate of cognitive decline over 4 yr by quartile baseline status (Q1-Q4) (i) β (SE) for additional annual change in z-score per 50 units increase in baseline status (i)	0.00 (-0.05-0.05) *0.0010 (0.0046), p=0.8255*
Tangney 2009 [22]	516 (40) 80 (6)	Cohort 6 Moderate risk	Serum vitamin B12 (pmol/l) 337 (127) MMA (nmol/l) 279.2 (173.4)	Compound z-score (n=4)	β (SE) for the additional annual change in z-score per 50 units/per unit increase in baseline status (j)	Serum vitamin B12 0.0088 (<0.005), p=0.005 MMA -0.00016 (0.0001), p=0.004
Kado 2005 USA [25]	370 (42) 74.3 (2.7)	Cohort 7 Moderate risk	Plasma vitamin B12 (pmol/l) 325 (264)	Total score of 5 tests	RR (95%CI) of being in the worst quartile of cognitive decline after 7y comparing those in the lowest quartile of baseline status versus the rest (k)	1.42 (0.91-2.06), p=0.11

MMA, methylmalonic acid; HoloTC II, holotranscobalamin II; MMSE, Mini-Mental State Examination; CI, confidence interval; SE, standard error; n.s. not significant, but no p-value provided
Values in italics are calculated based on provided values. Underlined values are additionally provided by the author *Values based on total population ** Higher MMA values indicate a low vitamin B12 status
(a) mean (SD), all such values unless stated otherwise
(b) range
(c) mean, all such values unless stated otherwise
(d) Clarke et al. adjusted for sex, education, smoking, ApoE4 status, vascular disease, Systolic blood pressure
(e) Eussen et al. 2002 unadjusted
(f) Feng et al. adjusted for age, sex, education, smoking, alcohol, physical activity, ApoE4 status, hypertension, diabetes mellitus, Cardiovascular diseases, ApoE4 status *vitamin B12
(g) Tucker et al. adjusted for age, education, smoking, alcohol, Body Mass Index, diabetes, systolic blood pressure, time of second measure relative to folic acid fortification, time between cognitive measures, serum creatinine
(h) Mooijaart et al. adjusted for sex, education
(i) Kang et al. adjusted for age, education, smoking, alcohol, physical activity, Body Mass Index, diabetes, history of high blood pressure, history of high cholesterol, postmenopausal hormone use, age at menopause, antidepressant use, aspirin use, mental health index, energy-fatigue index, assay batch, time between blood draw and cognitive interview, vitamin E supplement intake
(j) Tangney et al. adjusted for age, sex, race, education, smoking, alcohol, frequency of cognitive activities, serum creatinine, calorie adjusted intake of saturated fat, trans unsaturated fat intake, vitamin E in food, total vitamin C, fish intake
(k) Kado et al. adjusted for age, sex, education, smoking, baseline physical function

For MMSE-scores we did not have sufficient data available in the desired format to perform a meta-analysis and therefore results are qualitatively described. In line with the finding from the pooled analysis, 3 studies showed that the additional rate of change in MMSE-score was not significantly associated with serum vitamin B12 concentrations (20, 21, 24). Eussen et al. (2002) (27) and Kado et al. (2005) (25) reported no significant associations between serum/plasma vitamin B12 concentrations and global cognition (Spearman correlation between changes in MMSE and changes in plasma vitamin B12, r=-0.069; risk of being in the worst quartile of cognitive decline comparing those in the lowest quartile of baseline status versus the rest, RR=1.42, 95% CI: 0.91, 2.06) (Table 3). In contrast, Feng et al. (2009) (23) showed that higher serum vitamin B12 concentrations at baseline (natural log transformed, pmol/l) were significantly associated with better repeated MMSE-scores during follow-up (β=1.07, SE=0.35, p=0.003).

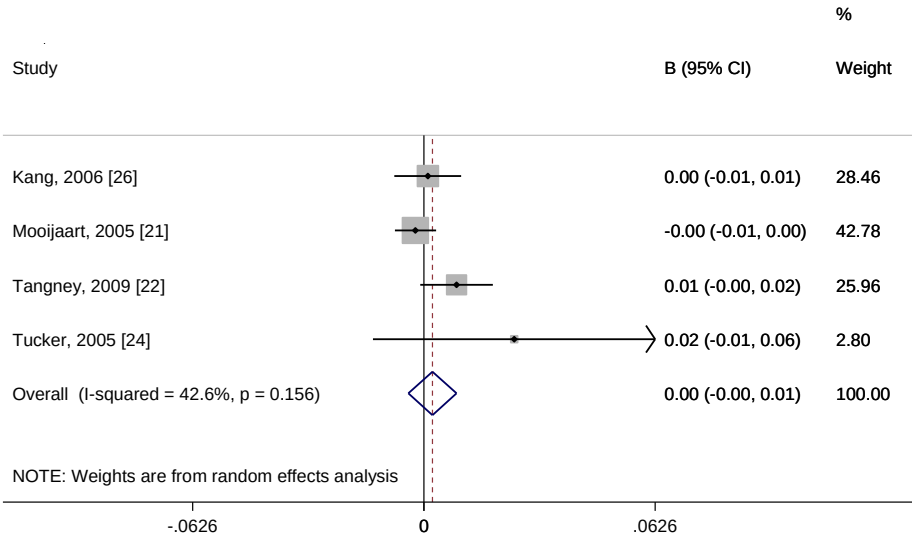

Figure 3 Forest plot of association between serum vitamin B12 (50 pmol/l) and additional rate of change in global cognition z-scores: Meta-Analysis of 4 Prospective Cohort Studies (n=1579)
B, regression coefficient; CI, confidence interval

Longitudinal data on MMA and holoTC II in relation to global cognition scores were scarce. Clarke et al. (2007) (20) reported the additional change in MMSE-score during 10 y related to a doubling in MMA (μmol/l) (β=-0.65,

95% CI=-0.98; -0.32) and holoTC II concentrations (pmol/l) at baseline (β=0.59, 95% CI: 0.30,0.88); and showed that a doubling of MMA concentrations was associated with about 60 percent faster rate of cognitive decline and a doubling of holoTC II concentrations was associated with a 40 percent slower rate of cognitive decline. In addition, Tangney et al. (2009) (22) reported that higher MMA concentrations (µmol/l) were associated with a more rapid deterioration of global cognition assessed with a compound z-score of 4 tests (β=-0.00016, SE=0.0001).

Vitamin B12 status and domain specific cognition

Five prospective cohort studies investigated associations between serum/plasma vitamin B12 and memory (n=5), executive function (n=3), speed (n=1) and language (n=1) (Table 4**)**.

For the domain memory we pooled associations between memory z-scores at 3, 4, or 6 years follow-up and serum/plasma vitamin B12 concentrations at baseline (50 pmol/l) from 4 studies (21, 24, 26, 29) including 3,460 elderly people. The studies of Mooijaart et al. (2005) (21) and Tucker et al. (2005) (24) both included 2 memory scores (word list and word list recall at 30 min (21) or word list and backward-digit span (24)). Therefore we performed 2 meta-analyses, one with the word list scores and one with the other 2 memory scores, but for both analyses the overall associations were similar (β=0.01, 95% CI:-0.01, 0.03), with no evidence for heterogeneity between studies (I^2=0.0%, p=0.99).

In line with these results, La Rue et al. (1995) (28) reported that Spearman correlations between plasma vitamin B12 at baseline and 3 different memory scores at 6 years follow-up were not significant (no data available).

Associations between serum vitamin B12 concentrations at baseline and executive function at follow-up were assessed in 3 cohort studies (21, 24, 28). La Rue et al.(28) and Mooijaart et al. (21) showed no significant associations, whereas Tucker et al. (24) observed that higher serum vitamin B12 concentrations (50 pmol/l) were associated with a better executive function score at 3 years follow-up (β =0.026, SE=0.016 , p<0.05).

The cognitive domains language (24) and speed (21) were both assessed in single studies, but no associations with serum vitamin B12 were found.

Table 4 Vitamin B12 Status in Relation to Domain-Specific Cognition Scores: Prospective Cohort Studies

Author Year	Population characteristics: N (% men) Age (years)	Study characteristics: Study design Follow-up (years) Risk of bias	Exposure: Marker of vitamin B12 status Concentration	Outcome: Cognitive domain (specific test)	Association measure:	Result:
Kang 2006 [26]	391 (0) 63(a)	Cohort 4 Moderate risk	Plasma vitamin B12 (pmol/l) 337	Memory (compound z-score, n=4)	Mean difference (95%CI) in rate of cognitive decline over 4 y by quartile baseline status (c) β (SE) for cognition z-score at 4 y per 50 units increase in baseline status (c)	0.00 (-0.06; 0.07) *0.011 (0.012)*
Nurk 2005 [29]	1678 (45*) 72	Cohort 6 Low risk	Serum vitamin B12 (pmol/l) *347*	Memory (KOLT)	OR (95%CI) for memory deficit by quintiles of baseline status (d) β (SE) for cognition z-score at 6 y per 50 units increase in baseline status (e)	Q1/Q5 1.61 (1.00-2.64), p trend 0.042 0.70 (6.7)
Mooijaart 2005 [21]	559 (34) 85	Cohort 4 Moderate risk	Serum vitamin B12 (pmol/l) *315 (184)*	Memory (word list) Memory (word list delayed recall) Speed and executive function (letter-digit coding) Speed and executive function (stroop)	β for additional rate of change in cognitive performance for each 1-SD increase in baseline status (e) β (SE) for cognition z-score at 4 y per 50 units increase in baseline status (e)	-0.008, p=0.92 0.053, p=0.19 -0.060, p=0.39 -0.21, p=0.66 0.0077 (0.05), p=0.80 0.010 (0.05), p=0.75 0.00085 (0.05) n.a.
Tucker 2005 [24]	321 (100) 67 (7) (b)	Cohort 3 Moderate risk	Serum vitamin B12 (pmol/l) 335 (136)	Memory (backward digit span) Memory (word list) Executive function (spatial copying) Language (verbal fluency)	β for association between cognition at 3 y and log-transformed baseline status (f) β (SE) for association between cognition z-score at 3 y per 50 units increase in baseline status (f)	0.18, n.s. -0.20, n.s. 0.59, p<0.05 0.06, n.s. *0.017 (0.016), n.s.* *0.0045 (0.015), n.s.* *0.026 (0.016), p<0.05* n.a.

Table 4 (continued)

Author Year	Population characteristics: N (% men) Age (years)	Study characteristics: Study design Follow-up (years) Risk of bias	Exposure: Marker of vitamin B12 status Concentration	Outcome: Cognitive domain (specific test)	Association measure:	Result:
La Rue 1997 [28]	133 (49*) 71.7	Cohort 6 High risk	Serum vitamin B12 (pmol/l) 437 (186)	Memory (Rey-Osterrieth Recall) Memory (WMS Visual reproduction) Memory (WMS logical memory) Executive function (Rey-Osterrieth copy) Executive function (Shipley-Hartford abstraction)	Spearman correlations between cognition at 6 y and baseline status (g)	n.s. n.s. n.s. n.s. 0.11, n.s.

CI, confidence interval; SE, standard error; n.s. not significant; n.a., not available
Values in italics are calculated based on provided values. Underlined values are additionally provided by the author *Values based on total population
(a) mean, all such values unless stated otherwise
(b) mean (SD), all such values unless stated otherwise
(c) Kang adjusted for age, education, smoking, alcohol, physical activity, Body Mass Index, diabetes, history of high blood pressure, history of high cholesterol, postmenopausal hormone use, age at menopause, antidepressant use, aspirin use, mental health index, energy-fatigue index, assay batch, time between blood draw and cognitive interview, vitamin E supplement intake
(d) Nurk adjusted for sex, education, ApoE4 status, history of Cardiovascular disease and hypertension, depression
(e) Mooijaart adjusted for sex, education
(f) Tucker adjusted for age, education, smoking, alcohol, Body Mass Index, diabetes, Systolic blood pressure, baseline cognitive measures, time of second measure relative to folic acid fortification, time between cognitive measures, serum creatinine
(g) La Rue adjusted for age

DISCUSSION

Main findings

The available evidence from a limited number of RCTs and prospective cohort studies in elderly people does not support the hypothesis that vitamin B12 intake or status is related with dementia, AD or global cognition. For domain-specific cognition, some prospective cohort studies observed associations between vitamin B12 intake or serum vitamin B12 and executive function or memory, however, results were inconsistent. Significant associations were shown between MMA or holoTC II and global cognition scores indicating better performance with better vitamin B12 status, but these findings originate from only 2 prospective cohort studies. Overall, these results suggest that current evidence on the relation of vitamin B12 intake or status with cognitive function does not suffice for being involved in deriving recommendations on vitamin B12 intake as the evidence-base for the presence of an association is limited and not supportive.

Methodological considerations of this meta-analysis

Previous systematic reviews on the relation of vitamin B12 intake or status with cognitive function reported a large heterogeneity between studies, mainly with regard to cognitive outcomes, cut-off levels indicating low vitamin B12 status and data analyses (7-11). This heterogeneity limited previous meta-analyses. A strength of our review is that we dealt with these sources of heterogeneity by defining 4 clusters of cognitive outcomes that were separately reviewed: dementia, AD, global cognition and domain-specific cognition. The domain-specific outcomes were further categorized as measuring memory, executive function, speed or language to cover the large variation in cognitive performance tests used between studies.

To overcome the variation in cut-off levels for low vitamin B12 status and to allow comparison and subsequent combination of individual studies, we expressed results of individual studies in a standardized format: Relative risk (binary outcomes) or regression coefficient (continuous outcomes) per change in untransformed unit of exposure. Most of the included studies reported results based on untransformed vitamin B12 concentrations whereas few studies reported results based on log-transformed (23, 24) or square root transformed (15) units of exposure because the distribution of serum vitamin B12 concentration within the study population was skewed. We chose to use untransformed data as there is no evidence to assume

thatthe association between vitamin B12 and cognitive function is not continuous across the common range of exposure (44). Meta-analyses of associations based on log-transformed data would probably not alter our conclusions as both results from studies using log-transformed data and those using untransformed data were similar.

A common concern in meta-analyses is statistical heterogeneity between studies. Among the meta-analyses reported here, we only observed moderate statistical heterogeneity for the association between serum vitamin B12 and global cognition. We were not able to explain this heterogeneity with meta-regression due to the limited number of studies included, however potential sources of heterogeneity are: age of the study populations (mean age 63-85 years), tests used to measure global cognition (MMSE, or compound z-score of 4-6 tests), duration of follow-up (3-10 years) and level of adjustment for confounders. Although most included studies adjusted for a wide range of confounders for cognitive function, residual confounding by other unmeasured or inadequately measured factors cannot be ruled out.

As the quality of included studies determines the quality of the meta-analysis, we assessed the risk of bias for each study identified in our review. Five prospective cohort studies were evaluated as having a low risk of bias, as confounders were appropriately dealt with, measures of vitamin B12 intake or status were adequate and no other serious risks of bias were identified (20, 23, 29, 32, 43). The other 16 studies (2 RCTs and 14 prospective cohort studies) were evaluated as having moderate or high risk of bias as methods used for sequence generation or allocation concealment were unclear (34, 35), important risk factors were not evaluated for their confounding potential (ApoE-ε4 (15, 16, 18, 21, 22, 24-26), vascular disease (33, 42)), or data were not reported in sufficient detail to be used in meta-analysis (17, 19, 27, 28). Due to the limited number of studies included in the different meta-analyses, we were not able to study the effect of study quality on the pooled effect measures.

Reflection on our results and considerations for future research

Observational studies

Prospective cohorts studying associations between vitamin B12 intake and cognitive function were limited. This is likely to be related to difficulties in

interpreting values on vitamin B12 intake in elderly people. Although intakes of vitamin B12 generally exceed the current recommended amounts, the prevalence of vitamin B12 deficiency among elderly people in Western countries is estimated to be 20 percent (45). The main cause for vitamin B12 deficiency in elderly people is food-bound malabsorption due to atrophic gastritis, a clinical condition accompanied by limited or absent secretion of gastric acid (46). A study including elderly with this condition showed that plasma vitamin B12 levels were significantly correlated with vitamin B12 intake from supplements and fortified foods, but not with vitamin B12 intake from unfortified foods (47). In addition intestinal absorption of vitamin B12 may be compromised by the use of acid lowering agents including proton-pump inhibitors and H2-blockers. These medicines are commonly used by elderly people, however available data on the association between vitamin B12 status and the use of these acid lowering agents is inconsistent (48, 49). To deal with the potential issue of malabsorption the use of markers for vitamin B12 status is preferred over measures of vitamin B12 intake when studying associations with cognitive function in elderly people.

Prospective cohort studies on associations between vitamin B12 status and cognitive function mainly addressed serum vitamin B12 concentrations which is considered to be a less sensitive and specific marker of vitamin B12 status than MMA or holoTC II (50). MMA is a metabolic marker of vitamin B12 status and holoTC II represents the fraction of vitamin B12 that is available for metabolic activity (50-53). Supported by findings from studies with a case-control or cross-sectional design MMA and holoTC II seem to be more sensitive to global cognition and domain-specific cognitive performance than serum vitamin B12 (20, 22, 54, 55) whereas results on incidence of AD were inconsistent (56). However, more prospective cohort studies are needed to confirm these observations.

RCTs

As shown in this review, the limited evidence from RCTs showed no effects of oral vitamin B12 supplementation on cognitive performance. RCTs on the effects of intramuscular injections with vitamin B12 were excluded from this review as they do not reflect dietary intake of vitamin B12, however these trials did not provide evidence for a beneficial effect of supplementation on cognitive performance either (57-59). Data from short-duration (<4 months) trials on the effect of vitamin B12 in combination with other B-vitamins (folic acid, vitamin B6) on cognitive performance tests did not show any effects

(35, 60-63). In contrast, 2 trials with a duration of 2 years did show a beneficial effect of B-vitamin supplementation on executive function/speed (trail making test-part B) (64, 65), although the latter study only observed a significant benefit in MCI patients with high homocysteine levels at baseline (≥11.3 µmol/l). When baseline homocysteine levels were not considered, no beneficial effect was observed. More RCTs on the effect of vitamin B12 on cognitive function are needed that are designed according to the recommendations from previous consensus meetings regarding sample sizes, selection of study populations, study duration and doses (44, 66-70).

Measures of cognitive function

Many different mechanisms have been suggested for the potential relation between vitamin B12 and cognitive performance as summarized by Smith and Refsum (2009)(44). A commonly suggested mechanism is that a low vitamin B12 status compromises methylation reactions in the central nervous system. Besides, low-normal vitamin B12 status may affect the brain via elevated levels of homocysteine, raised concentrations of MMA or changes in cytokine concentrations. Neuro-imaging studies have shown that changes in brain structures and brain volume are associated with cognitive decline (71). In addition, low-normal vitamin B12 status has been associated with loss of brain tissue (atrophy) (72-74)} and damage to the white matter (72, 75) in healthy elderly people. A recent trial including patients with mild cognitive impairment showed a 30 percent lower rate of brain atrophy in participants after 2 year supplementation with high doses of vitamin B6, folic acid and vitamin B12 as compared to placebo. In participants with elevated homocysteine levels, the rate of atrophy was even 53 percent lower after supplementation. The rate of atrophy was significantly inversely associated with change in vitamin B12 status as measured by serum vitamin B12 or holoTC II (76). These data indicate that measures of brain atrophy alone or in combination with domain-specific tests may be more sensitive for examining associations between vitamin B12 intake or status and cognitive performance.

CONCLUSIONS

Current evidence on the relation between vitamin B12 intake or status and cognitive function does not suffice for being involved in deriving recommendations on vitamin B12 intake as the evidence-base for the presence of an association is too narrow. Further studies should consider the

selection of sensitive markers of vitamin B12 status (MMA and holoTC II) as a proxy for vitamin B12 intake, and measures of brain atrophy alone or in combination with domain-specific tests as cognitive outcomes.

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Supplemental file 1 Search Strategy in Medline

	Search term
1	Randomized controlled trial.pt.
2	Controlled clinical trial.pt.
3	Randomized.ab.
4	Placebo.ab.
5	Randomly.ab.
6	Clinical trials as topic.sh.
7	Trial.ab.
8	Randomised.ab.
9	6 or 3 or 7 or 8 or 2 or 1 or 4 or 5
10	(animals not (human and animals)).sh.
11	9 not 10
12	(cohort* or "case control$" or cross-sectional$ or "cross sectional" or case-control$ or prospective or "systematic$ review$").mp. [mp=title, original title, abstract, name of substance word, subject heading word]
13	Exp meta-analysis/ or exp multicenter study/ or follow-up studies/ or prospective studies/ or intervention studies/ or epidemiologic studies/ or case-control studies/ or exp cohort studies/ or longitudinal studies/ or cross-sectional studies/
14	12 or 13
15	14 not 10
16	15 or 11
17	(("vitamin b12" or vitamin-b12 or "vitamin b 12" or "vitamin-b 12" or cobalamin$ or cyanocobalamin$ or hydroxocobalamin$ or methylcobalamin* or adenosylcobalamin*) adj5 (intake* or diet* or supplement$ or deplet$ or status or serum or plasma or "methylmalonic acid" or MMA or methylmalonate or "propanedioic acid" or "methylpropanedioic acid" or "malonic acid" or Holotranscobalamin$ or holo-transcobalamin$ or holoTC or holo-tc or concentration$ or expos$ or fortif$)).ti,ab.
18	Nutritional support/ or Dietary supplements/ or nutritional requirements/
19	Exp Nutritional Status/ or exp Deficiency Diseases/ or supplementation/ or diet supplementation/ or dietary intake/ or exp diet therapy/ or Diet/ or Food, Fortified/ or nutrition assessment/ or Nutritive Value/
20	(intake$ or diet$ or supplement$ or deplet$ or status or serum or plasma or "methylmalonic acid" or MMA or methylmalonate or "propanedioic acid" or "methylpropanedioic acid" or "malonic acid" or Holotranscobalamin$ or holo-transcobalamin$ or holoTC or holo-TC or concentration$ or expos$ or fortif$).ti,ab.
21	Methylmalonic acid/
22	18 or 19 or 20 or 21
23	Hydroxocobalamin/
24	Vitamin b 12/
25	23 or 24
26	25 and 22
27	Vitamin b 12 deficiency/
28	17 or 26 or 27
29	28 and 16

Supplemental file 2 Details on Transformations

Transformation to convert units of exposure

Serum/plasma vitamin B12 concentrations were expressed in pmol/l, if necessary using the following conversion factor: 1 pg/ml= 1 ng/l= 0.738 pmol/l.

β per pmol/l= β per pg/ml/0.738

Standard error (se) per pmol/l= se per pg/ml/0.738

Transformation to derive β(SE) for incidence dementia or Alzheimer's Disease with continuous vitamin B12 levels

Applied to studies of Crystal 1994 (19) (step 1-5) and Wang 2001 (18) (step 3-5), Kim 2008 (16) (step 3-5):

Step 1: Derive the relative risk (RR) (95% confidence interval (CI)) comparing subjects below and above the specified cut-off (111 pmol/l):

N_1 = N exposed

N_0= N unexposed

p_1= exposed cases/N_1

p_0= unexposed cases/N_0

RR= $(p_1)/(p_0)$

β= ln(RR)

$$se = \sqrt{\frac{1-p_1}{N_1 p_1} + \frac{1-p_0}{N_0 p_0}}$$

Upper limit 95% CI= $e^{(\beta+1.96*se)}$

Lower limit 95% CI= $e^{(\beta-1.96*se)}$

Step 2: Approximate the standard deviation (SD) of serum/plasma vitamin B12 concentrations in the population by assuming it to be equal to one quarter of the range of data values

Step 3: Calculation of the standard normal deviate, $z_{cut\text{-}off}$:

$z_{cut\text{-}off}$= (cut-off–$mean_{vit\ B12\ level}$) / $SD_{vit\ B12\ level}$

Step 4: Calculate the log(RR or OR) per SD increase in serum/plasma vitamin B12, $log(RR_{vit\ B12\ level})_{sd}$ (=standardized regression coefficient):

$log(RR_{vit\ B12\ level})_{sd}$ = $log(RR_{cut\text{-}off})$ /d_k, with d_k representing the difference in the mean values of a standard normal distribution above and below $z_{cut\text{-}off}$:

$$d_k = \frac{\phi(z_{cut-off})}{\Phi(z_{cut-off})\{1-\Phi(z_{cut-off})\}}$$

where ϕ and Φ are the standard normal density and distribution functions respectively.

Step 5: Calculate the β:
$\beta = \log(RR_{\text{vit B12 level}})_{sd} / SD_{\text{vit B12 level}}$

Transformation to calculate mean exposure or mean age of the total population when only exposure or age per subpopulation groups are available

Applied to studies of Eussen (2002) (27), Hooshmand (2010) (43), Kim (2008) (16), La Rue (1995) (28), Mooijaart (2005) (21), Morris (2006) (33), Nelson (2009) (32), Nurk (2005) (29):

Step 1: Calculate the combined mean:

$$\mu_x = \frac{\sum_i N_{xi}\mu_{xi}}{\sum N_{xi}}$$

where N_{xi} is the number of subjects in group i and μ_{xi} is the mean of group i.

Step 2: Calculate the combined standard deviation:

$$\sigma_x = \sqrt{\frac{\sum_i N_{xi}(\sigma_{xi}^{\ 2} + \mu_{xi}^{\ 2})}{\sum_i N_{xi}} - \mu_x^{\ 2}}$$

where N_{xi} is the number of subjects in group i, σ_{xi} is the standard deviation in group i, μ_{xi} is the mean of group I and μ_x is the combined mean.

Transformation to calculate mean exposure (m_x) and the standard deviation (s_x) of the population when only the median (med_x) and the interquartile range (IQR) are reported

Applied to study of Mooijaart 2005 (21):

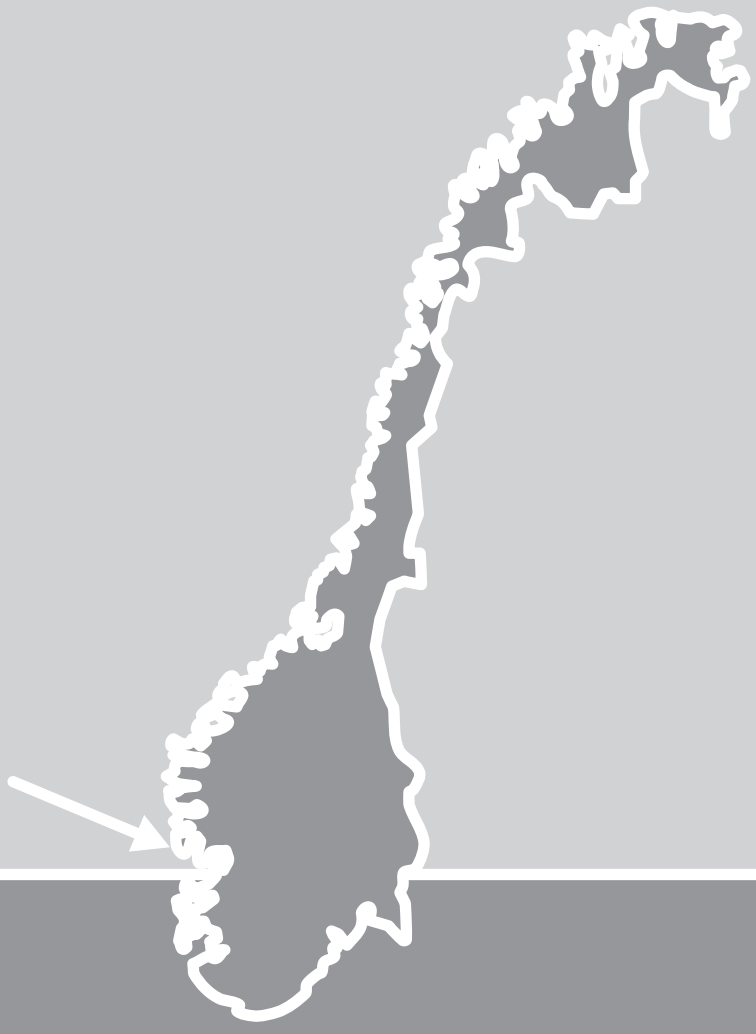
Step 1: $m_x = \ln(med_x)$

Step 2: To obtain an estimate of s_x from these quantities a search algorithm is employed to find the value of s_x for which the following equation holds

$$IQR_x = (med_x * e^{zs_x} - e^{-zs_x})$$

where z= 0.6745

chapter 6



Interactions between plasma concentrations of folate and markers for vitamin B12 status with cognitive performance in elderly people not exposed to folic acid fortification. The Hordaland Homocysteine Study

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ABSTRACT

Background: A combination of high folate with low vitamin B12 status has been associated with cognitive impairment in a population exposed to mandatory folic acid fortification of flour, but other studies yielded mixed results.

***Objective*:** To examine the interaction of folate and vitamin B12 markers in relation to cognitive performance in Norwegian elderly (n=2203, aged 72-74 y) who were not exposed to mandatory food fortification with folic acid.

Design: Plasma concentrations of folate and vitamin B12 markers (total vitamin B12, holotranscobalamin II (holoTC II) and methylmalonic acid (MMA)) were measured in 1992-1993 and 1997-1999. Cognitive performance was assessed in 1997-1999 by 6 cognitive tests from which a combined score was calculated with principal component analysis. Associations of folate, vitamin B12 markers and interactions between them in relation to cognitive performance were evaluated by quantile regression and ordinary least squares regression adjusted for gender, education, Apolipoprotein-E genotype, history of cardiovascular diseases or hypertension and creatinine.

Results: Cross-sectional analyses revealed a significant interaction between folate and plasma vitamin B12 in relation to cognitive performance (β (se), p-value) -0.058 (0.022), 0.009). When including holoTC II or MMA instead of plasma vitamin B12 in the model, interaction between folate and the vitamin B12 markers was not significant. In addition, we observed no significant interaction between folate and plasma vitamin B12 concentrations measured in 1992-1993 in relation to cognitive performance.

Conclusions: This large population based study in a population unexposed to mandatory folic acid fortification showed that low plasma vitamin B12 in combination with high folate was associated with better cognitive performance. However, these associations were not observed for sensitive markers of vitamin B12 status.

INTRODUCTION

Folate and vitamin B12 status have been positively associated with cognitive performance in cross-sectional (1-8) and prospective studies (9-18). Some early case reports observed accelerated neurologic deterioration in patients with pernicious anemia and severe vitamin B12 deficiency after treatment with folic acid (19, 20). These observations in combination with the known metabolic interrelation of folate and vitamin B12 suggest that the effects of one of these B-vitamins on cognitive performance might be modified by blood concentrations of the other B-vitamin. In line with this, cross-sectional analyses within the NHANES study, performed after the introduction of folic acid fortification of flour in the United States, revealed that high folate concentrations were associated with an increased risk of cognitive impairment in individuals with vitamin B12 deficiency (21, 22). It has been hypothesized that unmetabolized folic acid, which is likely to be present in individuals living in areas with folic acid fortification of food items (23), may mask or exacerbate metabolic and clinical consequences of vitamin B12 deficiency (21, 22, 24-29). Hematologic and neuropsychiatric disorders in vitamin B12 deficiency have been attributed to reduced function of the enzyme methionine synthase requiring vitamin B12 as cofactor and 5-methyltetrahydrofolate as substrate. This enzyme catalyzes the remethylation of homocysteine to methionine, which is the precursor of the universal methyldonor, S-adenosylmethionine (SAM). Lack of SAM and trapping folate as 5-methyltetrahydrofolate may compromise methylation reactions involving proteins, phospholipids, and neurotransmitters and cause insufficient folate needed for DNA synthesis and red blood cell maturation (30, 31).

The finding from NHANES that high folate concentrations were associated with an increased risk of cognitive impairment in individuals with vitamin B12 deficiency was not confirmed in other larger study populations not exposed (9) or exposed to folic acid fortification (32, 33). However, these previous studies used only one or two cognitive performance tests and measured either plasma vitamin B12 or holotranscobalamin II (holoTC II). It is therefore unclear whether the combination of high folate and low vitamin B12 status worsens certain aspects of cognitive performance. We investigated a combination of sensitive markers of folate and vitamin B12 status in relation to cognitive performance based on six cognitive tests in a large population-based study not exposed to mandatory food fortification with folic acid.

SUBJECTS AND METHODS

Study population

The study population consisted of residents of Bergen (Norway) born between 1925 and 1927, who participated both in the Hordaland Homocysteine Study in 1992-1993 and in the Hordaland Health Study (HUSK) in 1997-1999. A total of 2,841 elderly individuals were invited to participate in a sub study on cognitive tests in 1997-1999; 2,203 (77.5%) agreed. Details of this study are described elsewhere (34, 35). The Regional Committee for Medical Research Ethics of Western Norway approved the study and all participants provided written informed consent.

Assessment of cognitive performance

Cognitive performance was assessed at the study location by trained nurses and included six tests (36): a modified version of the Mini-Mental State Examination (m-MMSE; global cognition, maximum score=12) (37, 38), a modified version of the Digit Symbol test (m-DST; perceptual speed) (39), a short form of the Block Design (m-BD; visuospatial skills, maximum score=16) (39), the Kendrick Object Learning test (KOLT; episodic memory, maximum score=70) (40), an abridged version of the Controlled Oral Word Association test (COWAT; access to semantic memory) (41),and the Trail Making Test-part A (TMT-A; executive function) (42). For all tests, a higher score indicates a better performance, except the TMT-A where the test score is the time needed to complete the test and thus a shorter time used indicated a better performance.

Other covariates

Both in 1992-1993 and 1997-1999, participants underwent a brief health examination including measurements of height and weight. In addition, information on cardiovascular risk factors and lifestyle factors including smoking status (current smokers, ex-smokers or never smokers), consumption of coffee (0-1, 1-4 or more than 5 cups a day), and alcohol use (number of glasses per week), was collected via self-administered questionnaires as previously described (43). History of cardiovascular diseases (CVD) was based on self-reported information on history of myocardial infarction, angina pectoris and stroke as recorded both in 1992-1993 and 1997–1999, and on history of thrombosis and phlebitis as recorded in 1992-1993. Seventy-nine % of self-reported CVD cases were

validated with hospitalization records used in an earlier study (44), whereas the remaining 21% of CVD cases were presumably less severe and did not require hospitalization or occurred before 1992. A history of hypertension was defined as current or previous use of antihypertensive drugs and was based on self-reported data collected in 1997-1999.
Diabetes was based on self-reported information collected in 1997-1999. Depression score was assessed in 1997-1999 by a 7-item subscale for depression from the Hospital Anxiety and Depression Scale (HADS-D) (45). Educational level was classified as no primary school, primary school (≤9 y), vocational secondary school (10-12 y), theoretical secondary school (10-12 y), college or university <4 y, and college or university ≥4 y.

Plasma measurements

Non-fasting EDTA blood samples were collected for analyses of plasma markers for folate and vitamin B12 status. The EDTA samples were kept at 4°C until centrifugation. Samples collected in 1992-1993 were stored at -20°C for up to 10 years, whereas samples collected in 1997-1999 were stored at -80°C for up to 12 months before analyses. Plasma concentrations of folate and vitamin B12 were determined by microbiological assays (46, 47). A recent study showed folate degradation during storage (48). We therefore measured folate as pABG equivalents in 200 randomly selected samples collected in 1992-1993 and 1997-1999 by a method designed to recover degraded folate (48). Based on the results of these additional analyses we corrected for folate degradation during storage by using separate correction factors for the samples collected at baseline (corrected folate concentration '92-'93=5.3373+1.4045*folate concentration measured in '92-'93) and those collected at follow-up (corrected folate concentration '97-'99= 8.0512+1.1012*folate concentration measured in '97-'99).
Plasma concentrations of methylmalonic acid (MMA), an inverse marker for vitamin B12 status (49, 50), were determined by a modified gas chromatography–mass spectrometry method based on ethylchloroformate derivatization (51) and plasma concentrations of holoTC II were analyzed by microbiological assays (52). These indicators of vitamin B12 status were only measured in the samples collected in 1997-1999.
Serum creatinine levels were analyzed in the samples from 1997-1999 by a modification of a liquid chromatography-mass spectrometry (LC-MS/MS) procedure (53). Apolipoprotein-E (ApoE) genotypes (0, 1 or 2 APOE-ε4 alleles) were determined using a one-stage polymerase chain reaction

method (54) and methylenetetrahydrofolate reductase (MTHFR) genotyping (677C→T) was performed by a real-time polymerase chain reaction (55).

Statistical analyses

Plasma concentrations of folate and vitamin B12 measured in 1992-1993 and 1997-1999 were compared with a paired sample t-test. Relations between the different markers of vitamin B12 status measured in 1997-1999 were evaluated with Spearman correlation tests.

Principal component analysis (PCA) was used to create a summary score for cognitive performance that accounted for the correlations between the different cognitive performance tests and, thereby, maximized the explained variance. The number of components to be retained was determined according to 2 criteria: eigenvalues > 1 and by Cattles's Scree plot (plot of the total variance related to each component). For comparison of cognitive performance on the individual tests across quartiles of the cognitive performance components created with PCA, univariate analysis of variance was used.

In order to investigate single associations and interactions of folate and markers for vitamin B12 status in relation to cognitive performance, multivariate quantile regression and ordinary least squares (OLS) regression was used including the cognitive performance components extracted with PCA as the dependent variable. Single associations were assessed with regression models including a single marker for B-vitamin status as independent variable, whereas interactions were assessed with models including the combination of folate, a marker for vitamin B12 status and their interaction as independent variables. The quantile regression technique was used to provide distribution-free tests of whether the associations of folate, vitamin B12 markers and their interaction vary along the cognitive performance distribution. Plasma concentrations of folate and markers of vitamin B12 status were expressed as standardized z-scores to provide comparable associations per 1-SD increase.

All analyses were adjusted for the covariates gender, education level, history of CVD/hypertension ApoE genotype, and creatinine. These covariates were strong predictors for cognitive performance or associated with both B-vitamin levels and cognitive performance as demonstrated with analysis of variance or Pearson correlation coefficients. Body Mass Index (weight (kg)/ height (m^2)), smoking status, consumption of coffee, alcohol use, MTHFR genotype, diabetes and depression score were associated with either plasma

markers for folate or vitamin B12 or with cognitive performance, but adjusting for these biological and lifestyle factors did not markedly change the results of the analysis.
Descriptive analyses and PCA were performed using SAS 9.2 and regression analyses were performed with packages quantreg and mice of R version 2.13.1. P-values <0.05 were considered statistically significant.

RESULTS

Characteristics of the study population

Characteristics of the study population in 1997-1999 are presented in Table 1. The mean age of the participants was 72.5 years and 44.9% were men. Fifty-one percent had a history of cardiovascular disease such as myocardial infarction, angina pectoris, stroke, thrombosis, phlebitis or a history of hypertension. Furthermore, 17 % of the participants suffered from depression (HADS-D score ≥8), and 14 % were current smokers. Plasma folate concentrations (median (5th -95th percentile) measured in 1992-1993 were 12.5 (8.7-20.9) nmol/l after correction for folate degradation during storage, and lower than concentrations measured in 1997-1999, which were 15.8 (12.0-34.0) nmol/l (P for difference <0.0001). Plasma vitamin B12 concentrations in 1992-1993 were 338 (196-595) pmol/l and comparable with concentrations measured in 1997-1999 being 339 (192-651) pmol/l (P for difference =0.1246). In 1997-1999, 38.5% of the participants had low vitamin B12 status defined as plasma vitamin B12 concentrations <150 pmol/l or MMA> 210 nmol/l (56) and 0.5% had folate concentrations >59 nmol/l. Spearman correlations between plasma concentrations in '92-'93 and '97-'99 were 0.41 (P<0.0001) for folate and 0.63 (P<0.0001) for vitamin B12. Plasma vitamin B12 correlated significantly with holoTC II (Spearman r= 0.66, P<0.0001) and with MMA (Spearman r=-0.20, P<0.0001) and holoTC II also correlated significantly with MMA (Spearman r= -0.24, P<0.0001).

Table 1 Characteristics of the study population in '97-'99

Characteristic	n[a]	Number of subjects (%), mean (range) or mean/median (p5, p95)
Age, y	2203	72.5 (71.4-74.3)
Male sex	2203	990 (44.9)
Education	2024	
No primary school		149 (7.4)
Primary school (≤9 y)		648 (32.0)
vocational secondary school (10-12 y)		607 (30.0)
theoretical secondary school (10-12 y)		238 (11.8)
college or university <4 y		215 (10.6)
college or university ≥4 y		167 (8.3)
History of CVD or hypertension[b]	2073	1050 (50.7)
Diabetes[c]	2170	145 (6.7)
APoE-genotype	2187	
0 APoE-ε4 alleles		1485 (67.8)
1 APoE-ε4 allele[d]		633 (28.9)
2 APoE-ε4 alleles		69 (3.2)
MTHFR C677T status	2202	
CC		1104 (50.1)
CT		915 (41.6)
TT		183 (8.3)
Depression	1999	
HADS-D score normal (<8)		1536 (76.8)
HADS-D score depressed (≥8)		463 (16.7)
Smoking status	2203	
Smokers		310 (14.1)
ex-smokers		943 (42.8)
never smokers		950 (43.1)
Daily coffee consumption	2146	
0-1 cup		156 (7.3)
1-4 cup(s)		1656 (77.2)
≥5 cups		334 (15.6)
Alcohol consumption, glasses per week	1848	2 (0-37)
Plasma vitamin B12, pmol/l	2194	392/339 (192-651)
Plasma folate, nmol/l[e]	2186	18.0/15.8 (12.0-34.0)
Plasma MMA, µmol/	2192	0.22/0.19 (0.12-0.36)
Plasma holoTC II, pmol/l	2041	101/90 (43-192)
Creatinine, mmol/l	2202	93/91 (72-120)
m-MMSE	2181	11.5/12 (10-12)
m-DST	2188	10.2/9 (5-18)
m-BD	2186	15.0/16 (10-16)
KOLT	2197	35.2/35 (23-48)
COWAT	2193	15.1/15 (7-25)
TMT-a	2193	57.7/44 (28-124)

CVD:Cardiovascular diseases, HADS-D: Hospital Anxiety and Depression Scale—depression subscale, ApoE: apolipoprotein E, MTHFR: Methylenetetrahydrofolate reductase, m-MMSE: modified version of the Mini-Mental State Examination, m-DST: modified version of the Digit Symbol test, m-BD: short form of the Block Design, KOLT: Kendrick Object Learning Test, COWAT: abridged version of the Controlled Oral Word Association test, TMT-a: Trail Making Test part A

a The sample numbers may vary across the different variables due to different numbers of missing data

b based on self-reported CVD (myocardial infarction, angina pectoris, stroke, thrombosis and phlebitis) or hypertension at baseline or follow-up

c-based on self-reported diabetes

d E2E4 and E3E4 genotypes

e Folate concentrations are corrected for degradation

Cognitive performance factors

Median (5th-95th percentile) scores for the 6 cognitive performance tests are presented in Table 1. Based on m-MMSE scores ≤ 10 (36), 36% of the study population suffered from cognitive impairment. Eigenvalues and Cattel's scree plot revealed that one component derived by PCA should be retained, explaining 40.5% of the total variance, whereas factors 2-4 explained less than 14.6% each. The factor loading matrix is presented in Table 2. The first component strongly correlated with m-MMSE (r= 0.61), m-BD (r=0.55), m-DST (r=0.72), KOLT (r=0.59), COWAT (r=0.63), TMT-a (r=-0.70) and is further referred to as "overall cognitive performance". Overall cognitive performance scores ranged from -4.80 to 2.49 with mean of 0 and SD of 1. Across increasing quartiles of overall cognitive performance, subjects performed better on each individual cognitive tests (For all tests P for difference <0.0001, data not shown).

Table 2 Factor loading matrix from principal components analysis

	Factor 1	Factor 2	Factor 3	Factor 4
m-MMSE	0.61	0.01	0.66	0.30
m-BD	0.55	-0.68	0.16	-0.19
m-DST	0.72	-0.01	-0.38	0.12
KOLT	0.59	0.46	0.15	-0.65
COWAT	0.63	0.41	-0.10	0.39
TMT-A	-0.70	0.22	0.35	0.05
Eigenvalue	2.43	0.88	0.77	0.72
% Variance explained	40.5	14.6	12.8	12.0

m-MMSE: modified version of the Mini-Mental State Examination, m-DST: modified version of the Digit Symbol test, m-BD: short form of the Block Design, KOLT: Kendrick Object Learning Test, COWAT: abridged version of the Controlled Oral Word Association test, TMT-a: Trail Making Test part A
missing, n=47

Cross-sectional associations of plasma concentrations of folate and vitamin B12 markers with overall cognitive performance assessed in 1997-1999

Figure 1 shows no overall association of plasma vitamin B12, a significant positive association of folate, and a significant negative interaction term between folate and plasma vitamin B12 on the overall cognitive performance

distribution. The associations of gender, education, history of CVD/hypertension and ApoE4 status with overall cognitive performance were as expected. Notably, the associations between folate, education, history of CVD/hypertension, and ApoE4 and overall cognitive performance were asymmetric with strongest effects in the lowest ranges of the overall cognitive performance scores. When including holoTC II or MMA instead of plasma vitamin B12 in the model, the quantile regression plots showed non-significant associations of holoTC II and MMA, a non-significant interaction between folate and the vitamin B12 markers, but similar associations of the other covariates (data not shown).

Quantile regression estimates for the associations of both B-vitamins and most covariates were included in the 95 percent confidence intervals of the OLS regression estimates along the distribution of overall cognitive performance, suggesting that the OLS estimates are appropriate to represent the associations with overall cognitive performance. Table 3 presents the multivariate adjusted OLS regression estimates for the single associations of plasma folate and markers of vitamin B12 on the overall cognitive performance score, as well as the interaction between plasma folate and markers of vitamin B12 in relation to the overall cognitive performance score. In agreement with Figure 1, folate was positively associated, plasma vitamin B12 was not associated, and their interaction term was negatively associated with the overall cognitive performance score. The markers MMA and HoloTC did not show significant associations with the overall cognitive performance factor, nor were any of the interactions with folate significant (Table 3)

Prospective associations of plasma concentrations of folate and plasma vitamin B12 in 1992-1993 with cognitive performance assessed in 1997-1999

We observed no significant associations (β (se), p-value) of folate (0.019 (0.031), p=0.540) or plasma vitamin B12 (0.020 (0.033) p=0.541) measured in 1992-1993 along the distribution of overall cognitive performance. In addition, the interaction of folate and plasma vitamin B12 measured 4-y years prior to measurement of overall cognitive performance was not significant (0.024 (0.028), p=0.394) (data not shown).

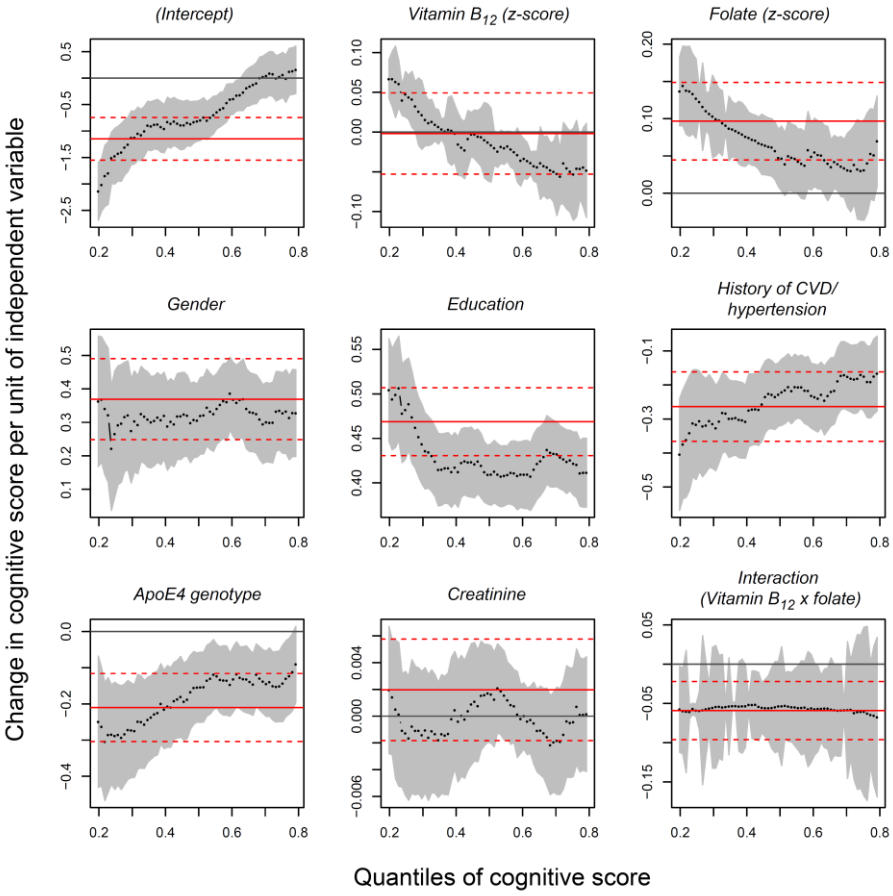

Figure 1 Changes in cognitive score according to plasma vitamin B12, folate and other determinants by quantile regression.

The black points represent quantile regression fits, dark shaded gray zones represent the 95% pointwise confidence intervals for the estimates, the black horizontal lines indicates no (zero) changes in cognition. An upward or downward slope indicates the highest or lowest response at the upper or lower tail, respectively, of the distribution of the cognitive scores, whereas a horizontal graph below or above zero indicates similar effects through the whole distribution. The horizontal red solid lines represent the ordinary least-squares estimates of the conditional mean effects, and the red dotted lines represent the conventional 95% confidence intervals for the least-squares estimates. Plasma vitamin B12 and folate were given as z-scores, gender categorized as (1) men and (2) women, education as (0) no primary school, (1) primary school (≤9 y), (2) vocational secondary school (10-12 y), (3) theoretical secondary school (10-12 y), (4) college or university <4 y, and (5) college or university ≥4 y, history of CVD/hypertension as (1) yes, (0) no, ApoE4 genotype as (0) 0, (1) 1, or (2) 2 ApoE-ε4 alleles and creatinine was given as µmol/L. CVD=cardiovascular diseases.

Table 3a Cross-sectional associations between plasma concentrations of single B-vitamins and overall cognitive performance measured in '97-'99[a]

	B-vitamins measured in 1997-1999			
	Folate	Plasma vitamin B12	HoloTC-II	MMA
N	1848	1854	1726	1852
Intercept	-1.178 (0.246) <0.001[b]	-1.203 (0.246) <0.001	-1.152 (0.247) <0.001	-1.213 (0.247) <0.001
B-vitamin[c]	0.081 (0.031) 0.009	-0.002 (0.031) 0.950	0.060 (0.031) 0.055	-0.013 (0.031) 0.666
Gender (women)	0.369 (0.074) <0.001	0.395 (0.073) <0.001	0.377 (0.074) <0.001	0.397 (0.073) <0.001
Education level	0.464 (0.023) <0.001	0.467 (0.023) <0.001	0.469 (0.023) <0.001	0.468 (0.023) <0.001
ApoE genotype	-0.236 (0.062) <0.001	-0.236 (0.062) <0.001	-0.238 (0.062) <0.001	-0.235 (0.063) <0.001
History of CVD/hypertension	-0.215 (0.057) <0.001	-0.217 (0.058) <0.001	-0.218 (0.057) <0.001	-0.216 (0.058) <0.001
Creatinine	0.002 (0.002) 0.339	0.002 (0.002) 0.331	0.002 (0.002) 0.445	0.002 (0.002) 0.316

CVD=cardiovascular diseases
a-Ordinary Least Squares regression coefficients adjusted for gender, education, ApoE4-status, history of CVD, and creatinine
b-β (se), p-value all such values
c-Standardized b-vitamin concentrations (z-scores)

Table 3b Cross-sectional associations of plasma concentrations of folate, markers for vitamin B12 status and their interaction in relation to overall cognitive performance measured in '97-'99[a]

	Marker of vitamin B12 status measured in 1997-1999		
	Plasma vitamin B12	HoloTC-II	MMA
N	1848	1721	1845
Intercept	-1.099 (0.243), <0.001[b]	-1.087 (0.244), <0.001	-0.861 (0.233), <0.001
B12 marker[c]	0.000 (0.031), 0.997	0.031 (0.031), 0.320	-0.012 (0.015), 0.401
Folate[c]	0.097 (0.031), 0.002	0.090 (0.031), 0.004	0.036 (0.013), 0.007
B12*folate[cd]	-0.058 (0.022), 0.009	-0.041 (0.027), 0.131	-0.005 (0.033), 0.880
Gender (women)	0.362 (0.073), <0.001	0.355 (0.073), <0.001	0.325 (0.066), <0.001
Education level	0.477 (0.023), <0.001	0.476 (0.023), <0.001	0.408 (0.020), <0.001
History of CVD/hypertension	-0.257 (0.062), <0.001	-0.262 (0.062), <0.001	-0.226 (0.057), <0.001
ApoE genotype	-0.223 (0.057), <0.001	-0.221 (0.057), <0.001	-0.145 (0.060), 0.015
creatinine	0.001 (0.002), 0.566	0.001 (0.002), 0.580	0.002 (0.002), 0.375

CVD=cardiovascular diseases

a-Ordinary Least Squares regression coefficients adjusted for gender, education, ApoE4-status, history of CVD/hypertension, and creatinine

b-β (se), p-value all such values

c-Standardized B-vitamin concentrations (z-scores)

d-The coefficient for the product term indicates how the association between folate status and the cognitive performance score changes when the concentration of the marker for vitamin B12 status increases

DISCUSSION

Main findings

This population based study investigated the hypothesis that high folate concentrations in combination with low plasma vitamin B12 concentrations increased risk for cognitive impairment, and was conducted in a population which was not exposed to mandatory fortification of food items with folic acid. The study, which included 2203 elderly people aged 72-74 y, revealed a significant negative interaction between folate and plasma vitamin B12 concentrations in relation to cognitive performance. However, interactions between folate and other markers of vitamin B12 status, holoTC II or MMA, in relation to overall cognitive performance were not significant.

The significant negative interaction between folate and plasma vitamin B12 indicates that the linear association between folate and overall cognitive performance changes for different concentration of plasma vitamin B12 and vice versa. The observed association for the interaction-term suggests that, among elderly with plasma vitamin B12 concentrations 2 standard deviations above the mean, the significant positive association between folate and overall cognitive performance changes from β=0.097 to β=0.097+2*(-0.058)=-0.019, and becomes negative. In contrast, among elderly with plasma vitamin B12 concentrations 2 standard deviations below the mean, the association between folate and overall cognitive performance changes from β=0.097 to β=0.097-2*(-0.058)=0.213, so becomes even stronger.

Our findings are not in line with the results from the NHANES study (n=1459, mean age=70 y), which showed that low vitamin B12 status in combination with elevated folate concentrations (n=278) was associated with cognitive impairment. One explanation for the discrepant findings between the NHANES and the current study is that the NHANES study has been conducted in an area of mandatory folic acid fortification leading to frequent exposure of unmetabolized folic acid. As a consequence, high folate concentrations (>59 nmol/L) were present in 20.7% of the NHANES study population (n=1459), compared to less than 1% ('92-'93 and '97-'99) in the present study. The prevalence of low plasma vitamin B12 concentrations (<148 pmol/l or MMA>210 nmol/l) was 25% in NHANES, and approximately 38.5% in our study population.

However, the NHANES study also showed that normal vitamin B12 status in combination with elevated folate concentrations was associated with protection against cognitive impairment as compared to normal

concentrations of both B-vitamins (OR (95% CI): 0.4 (0.2, 0.9)) (21). This is in line with our finding that folate concentrations were positively associated with overall cognitive performance and these results are also supported by previous observations that low folate concentrations were associated with a higher risk of cognitive impairment (6, 11, 15, 17).

Metabolic and clinical effects of a combination of high folate and low vitamin B12 status

Some (21, 22, 32, 57), but not all (33) studies have shown that elderly individuals with high folate and low vitamin B12 status have a higher prevalence of anemia, and higher concentrations of total homocysteine and MMA. As such, it has been proposed that high folate concentrations may exacerbate negative consequences of vitamin B12 deficiency. However, it is possible that subjects with a combination of low vitamin B12 and high folate in the studies decribed above suffered from severe vitamin B12 deficiency due to disorders that affected vitamin B12 absorption, such as pernicious anemia (58, 59). A recent study in healthy young adults without any medical conditions that could induce anemia or affect folate or vitamin B12 absorption did not observe any adverse effects of high folate concentrations on biochemical abnormalities related to vitamin B12 deficiency (60). In agreement with these findings, the current study did not show that the positive associations between folate concentrations and cognitive performance was negatively affected by decreasing vitamin B12 status, as was also true for two cross-sectional studies in populations exposed to folic acid fortification (32, 33). In these latter two studies, high folate concentrations occurred in 39% (cut-off >45.3 nmol/l) (32) and 5% (Cut-off >60 nmol/l) (33).

Both observational population based studies – whether or not exposed to folic acid fortification, and intervention studies show inconclusive results for the effects of B-vitamins on cognition. A recent meta-analysis (61) summarised the results from trials on the effects of folic acid supplementation on cognitive performance, and did not show an effect of folic acid on the prevention of age-related cognitive decline within 3 years of the start of treatment (61). Evidence from trials on the effects of vitamin B12 on the cognitive function was previously judged insufficient (62). In one trial including 233 elderly with mild cognitive impairment, 2 years of treatment with a combination of B-vitamins (0.8 mg folic acid, 0.5 mg

vitamin B12 and 20 mg vitamin B6 per day) showed beneficial effects on global cognitive performance, episodic memory and semantic memory among participants with elevated homocysteine concentrations (63). Other trials including 128 to 2009 participants did not show any effects of combined B-vitamin treatment on cognitive performance (64-69). However, one study suggested that supplementation reduced the risk of cognitive decline in participants with low dietary B vitamin intake at baseline (66). Taken findings from observational and intervention studies together, the efficacy of B-vitamins on cognition may depend on B-vitamin status and cognitive performance at baseline, the dosage of B-vitamins, and the duration of exposure to supplements.

Strengths and limitations

Data on cognitive performance and markers for vitamin B12 status (MMA and holoTC II) were only available for 1997-1999. As such, we were not able to investigate whether combined effects of folate and these sensitive vitamin B12 markers would affect future cognitive performance. Furthermore, folate degradation is likely to occur when stored for long periods (48). Even though we corrected plasma folate concentrations measured in 1992-1993 and in 1997-1999 for folate degradation by measuring PABA-Glu (70), concentrations measured in 1992-1993 were still lower than concentrations measured in 1997-1999. This may indicate either improved folate status over time or incomplete correction for folate degradation.

A major strength of our study is the use of an extensive cognitive test battery covering global cognition, perceptual speed, visuo-spatial skills, episodic memory, access to semantic memory and executive function, whereas the majority of previous studies assessed cognition by global cognition or only one cognitive domain. The cognitive performance component as derived by PCA provides a robust measure for cognitive performance. As previously shown, the use of composite scores reduce the risk of measurement errors, reduce the total amount of data and thereby reduce chance findings (71, 72).

In addition, we had the opportunity to include makers for vitamin B12 status that are considered more sensitive markers compared to plasma vitamin B12 concentrations, namely MMA and holoTC II, although none of these markers are considered as a gold standard (73, 74). For diagnostic tools in epidemiologic settings, vitamin B12 in combination with holoTC II or MMA is

recommended (75). The utility of these markers of vitamin B12 status in relation to cognitive performance has been evaluated in a limited number of observational studies. So far, elevated concentrations of MMA were significantly associated with a decreased cognitive performance (3, 5, 9, 16, 67), and for holoTC II both significant (2, 9) and non-significant positive associations with cognitive performance have been observed (5). In the current study, these associations could not be confirmed. Finally, we were able to adjust our analyses for a large panel of known risk factors, yielding reliable results.

In conclusion, this large study population not exposed to mandatory food fortification with folic acid did not confirm the previous finding that a combination of high folate and low plasma vitamin B12 was associated with decreased cognitive performance.

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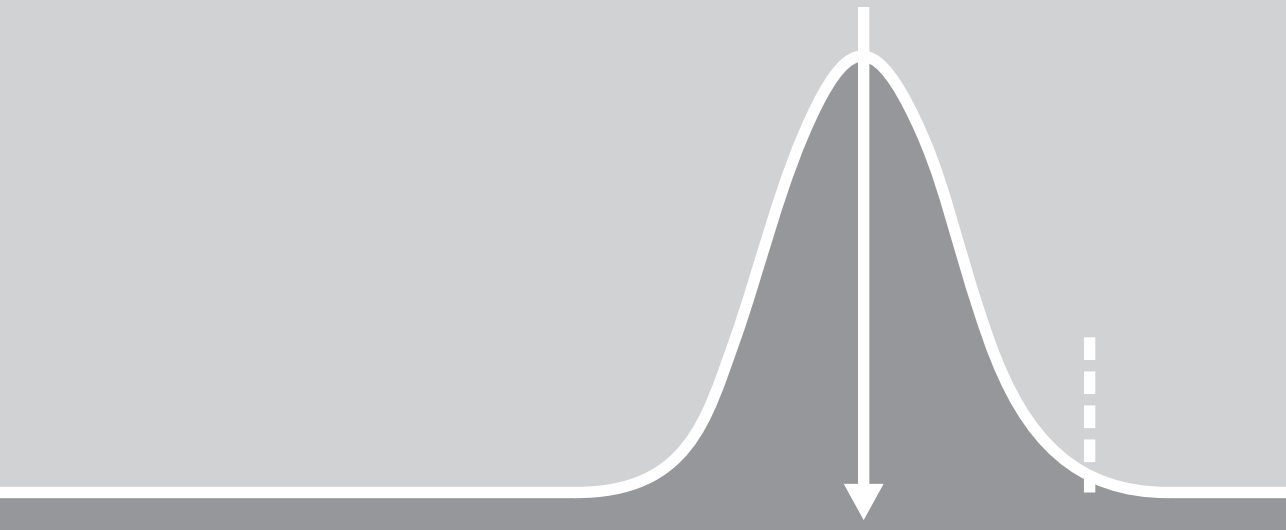
chapter 7



General Discussion

The aim of this PhD-thesis is to contribute to standardization of the process to derive evidence-based, transparent and harmonized micronutrient recommendations across Europe. First the need for harmonization is substantiated by a descriptive study on the variation in published micronutrient recommendations across Europe. In addition methodological factors were identified that should be considered for alignment of recommended intakes across Europe. Secondly, the evidence-base for establishing recommended vitamin B12 intakes was summarized in two systematic reviews. One review focused on requirements for the compensation of daily obligatory losses (factorial approach) and the other review evaluated the relation of vitamin B12 intake and status with cognitive performance (dose-response approach). Whether interactions between folate and vitamin B12 on cognitive performance should be considered for establishing recommended vitamin B12 intakes was evaluated in a large cohort study. In this final chapter, our main findings are summarized and complemented with information on dose-response data for concentration markers and other health endpoints that originated from the EURRECA NoE or from the literature. Pros and cons of different approaches that may be used for estimating vitamin B12 requirements will be discussed and gaps in current knowledge will be highlighted.

MAIN FINDINGS

In Europe, variation in recommended intakes for adults and elderly on vitamin B12 and other selected micronutrients mainly resulted from differences in the following issues (Chapter 2 and 3):

a)- the selection of health indicators and criteria for adequacy,
b)- assumptions regarding inter-individual variation in requirements,
c)- assumed bioavailability factors, and
d)- the selection and interpretation of evidence on requirements.

To harmonize the process of setting micronutrient recommendations, standardized methods are needed to address each of these issues.

In general there are two approaches for setting recommended intakes: the factorial approach and the dose-response approach. With the factorial approach, requirements are estimated as the mean intake needed to compensate daily losses assuming a specified level of bioavailability from the usual diet. A systematic review on daily losses of vitamin B12 in adults and elderly showed that the rate of loss was 0.13% of total body stores per day (95% confidence interval =0.10; 0.15) as estimated with a pooled analysis

of five studies including 52 subjects. Applying estimates of body stores among apparently healthy individuals (2-3 mg), absolute amounts of vitamin B12 lost per day will most likely range between 2.6 and 3.9 µg per day (Chapter 4).
Absorption of vitamin B12 is dependent on the ingested dose and food source and varies substantially between individuals. However, the overall relation between intake of vitamin B12 and the absorbed amount could be described by a linear regression equation including the natural logarithm of both intake and the amount absorbed. Based on the regression equation and assuming that mean dietary intakes of vitamin B12 in European countries (3.5-9.3 µg per day) are equally divided over three meals, absorption ranged between 29-37% . These absorption estimates are lower than the 50% that is commonly assumed for estimating vitamin B12 requirements (Chapter 4). Evidence on daily vitamin B12 losses and bioavailability can only be derived from relatively old studies published between 1958 and 1991 and can hardly be updated because this requires invasive methods or the use of isotopes that do not comply with current ethical standards. In view of the above, establishing recommended vitamin B12 intakes with the factorial approach can be based on evidence on the rate of loss (a relatively accurate and precise estimate) and bioavailability (large variability and uncertainty), together with an estimate of the required total body stores (crude estimate). However the available evidence is rather scarce and resulting estimates having considerable uncertainty . The commonly used coefficient of variation of 10-20% seems too low to cover uncertainty in all these underlying assumptions and may be at least 24% (Chapter 4).

The dose-response approach for setting recommended intakes is based on associations between intake and physiological or clinical health outcomes. For vitamin B12, traditionally maintenance of an adequate hematological status, as measured by stable hemoglobin levels, normal mean cell volume and normal reticulocyte response, was used as a health indicator for setting recommendations in some countries. These hematological indices may change in case of vitamin B12 depletion. However, rather than merely focusing on the prevention of symptoms of severe deficiency, the prevention of chronic diseases or longterm outcomes such as cognitive performance might also be considered as health outcomes for setting recommended intakes. A systematic review indicated that limited dose-response evidence

from 2 randomized controlled trials and 21 prospective cohort studies does not show assocations of vitamin B12 intake and status with dementia, global cognitive function or domain-specific cognitive function (Chapter 5).
Furthermore, in a population of 2203 Norwegian elderly people, cross-sectional and prospective analyses revealed no associations between vitamin B12 and cognitive performance and we did not find evidence for interaction of vitamin B12 and folate on cognitive performance (Chapter 6). Taking the review and the latter analysis together, the available evidence on the relation between vitamin B12 and cognitive performance is yet not convincing and thereby limits its use as an outcome for estimating vitamin B12 requirements.

The methods used in this PhD-thesis to collate, select and interpret the evidence on vitamin B12 requirements of adults and elderly were systematic, objective and transparent and can be applied to other micronutrients as well. Thereby, the approach outlined in this thesis contributes to the harmonization of the process for setting micronutrient recommendations.

VARIATION IN MICRONUTRIENT RECOMMENDATIONS

As shown in chapter 2 and 3 of this thesis, published micronutrient recommendations for adults and elderly vary widely between European countries, for some nutrients even up to two-fold (folate). Although there may be small physiological differences between people across Europe, these do not justify the observed variation in recommendations. Factors that have been shown to contribute to the variation include the use of different concepts, definition of population groups, different approaches, selection of health outcomes, differences in the evidence-base, different assumptions and expert judgment (1-3). The relevance of addressing this variation is linked to the two main applications of micronutrient recommendations, assessing the adequacy and planning of micronutrient intakes. Both applications stress the need for transparency regarding the concept of the recommendation (ANR+2SD or AI) as they are applied in a different manner. Moreover the selection of different health outcomes for estimating requirements may result in different values for the ANR, SD or AI and may thereby influence the probability of inadequate intake for an individual or the prevalence of inadequate intakes in a population. Roman Vinas et al. (2011) evaluated intakes of the micronutrients studied in chapter 3 across Europe using the ANRs provided by the Nordic countries. Among men and women

aged 19 years and older, the prevalence of inadequate intakes below the ANR were 0-40% for vitamin B12 (ANR=1.4 µg), 10-91% for folate (ANR=200 µg), 0-21% for iron (ANR=6 mg for men and 7 mg for women) and 1-31% for zinc (ANR=6.4 mg for men and 5.7 mg for women) (4). In general, applying higher or lower ANRs as provided by other European countries will substantially affect these prevalence estimates, although probably not that markedly for vitamin B12 and iron as mean intakes for both these micronutrient were well above the recommended intakes.

DEFINITION OF VITAMIN B12 REQUIREMENTS

Figure 1 shows the different approaches and health outcomes that have been used up to now for estimating vitamin B12 requirements of adults and elderly people (maintenance of body stores and haematological status), the health outcomes that were evaluated in this thesis (cognitive performance), and in addition the health outcomes that were evaluated in the context of the EURRECA project (bone health). The relation between vitamin B12 intake and status was also quantified as vitamin B12 status is considered as an intermediate in the relation between intake and health outcomes.

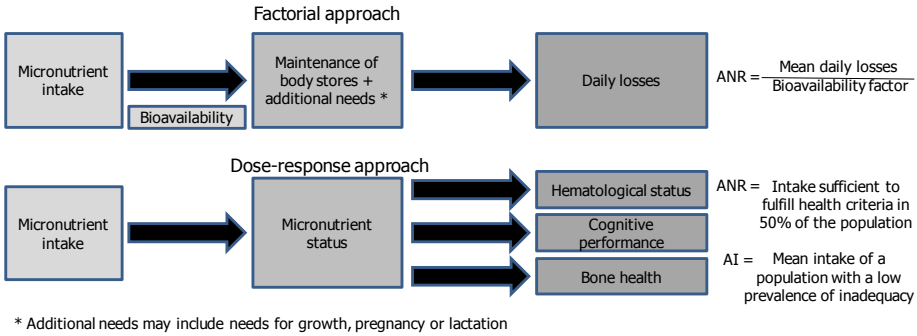


Figure 1. Schematic overview of approaches that may be used for estimating vitamin B12 requirements of adults and elderly

Factorial approach

The factorial approach is used to establish an ANR of vitamin B12 in the Netherlands. For defining an ANR based on the maintenance of body stores it would be necessary (by definition) to establish what body stores suffice to prevent deficiency or defined suboptimal function in 50% of the population. Such data cannot be obtained from healthy individuals for ethical reasons, therefore the ANR was based on stores from patients suffering from

malabsorption with normal hematological status and low serum vitamin B12, i.e. 525 µg (5). Strictly speaking this number is not reflecting an ANR, but rather defines minimum requirements for intake. It is not clear what stores will be sufficient for the general population to maintain health. Most estimates of body stores in apparently healthy people ranged between 2 and 3 mg, but whether it is beneficial to maintain stores larger than 0.5 mg is not clear. Based on estimates of body stores in apparently healthy people (2-3 mg), a rate of loss of 0.13% of stores per day and assuming a bioavailability of 29-37% (ignoring the uncertainty in the bioavailability and between subject variability), requirements of apparently healthy individuals would range between 7.0 and 13.4 µg/day. These numbers indicate the daily intake necessary to maintain body stores in apparently healthy populations and therefore reflect a range of adequate intakes rather than an ANR. To be able to define appropriate body stores for estimating vitamin B12 requirements, more data are needed on the predictive value of body stores for health, e.g. normal hematological status.

Dose-response approach: Maintenance of hematological status

The ANR based on the maintenance of hematological status, as proposed by IOM (6), was based on one single study including 7 subjects that were not able to absorb vitamin B12 from foods due to pernicious anemia (PA) (7). The daily parenteral dose of vitamin B12 needed to maintain stable haemoglobin, normal mean cell volume and normal reticulocyte response in half of the subjects was used as the basis for estimating requirements taking into account the additional loss that only occurs in PA patients. In line with this study, more recent non-placebo-controlled trials showed (large) increases in hemoglobin concentrations or hematocrit after intramuscular or oral vitamin B12 administration in patients with PA or age-related food-vitamin B12 malabsorption (8-11). However, the selection of hematological status as criterion for adequacy may be debatable for several reasons. Although the biological role of vitamin B12 in blood cell formation is well-defined (12-14) it has been estimated that 19–28% of patients with PA do not have anemia, and 17–33% have a normal mean cell volume (15, 16). Secondly, potentially irreversible neurologic disorders due to vitamin B12 deficiency may occur in the absence of anemia or an elevated mean cell volume (17-19). It is even suggested that the occurrence of neurological complications is inversely correlated with the degree of anemia; patients

who are less anemic show more prominent neurological complications and vice versa (20, 21).

Dose-response approach: Vitamin B12 and cognitive function

Based on the results of our systematic review we concluded that current evidence on the relation between vitamin B12 intake or status and cognitive function does not suffice for being considered in deriving vitamin B12 recommendations. However, based on our results and following the conclusions from previous consensus meetings, some issues need to be considered in future studies to assess associations between vitamin B12 and cognitive performance including the selection of study populations and cognitive outcomes (22-27).

For the selection of study populations for intervention studies, it needs to be considered that when selecting study subjects with cognitive impairment, the duration of cognitive symptoms may affect the response to treatment as brain damage may have already occurred. Therefore, trials may better be aimed at prevention of cognitive decline than at treatment, and selecting study participants at middle age instead of old age may be most appropriate for long-term interventions aiming at the prevention of cognitive impairment (23).

Normal cognitive aging, so not including dementia or mild cognitive impairment, shows a large inter-individual variation and nutrition is one of the factors that may contribute to this variation. Furthermore, cognitive test performance is subject to temporal fluctuations due to various factors such as tiredness, attention, glucose levels, and depression or to demographic factors such as age, education, and sex. Power to detect significant effects of treatment will therefore require large study populations. For cohort studies it is very important to evaluate a broad range of population characteristics for their potential of being a confounding factor.

Because the impact of vitamin B12 on the brain and on cognitive performance is still not fully understood, it is important to use a comprehensive range of cognitive performance tests that adequately differentiate between different cognitive domains to evaluate how vitamin B12 intake or status differentially affects different cognitive domains. Standardization of methods to assess cognitive function and diagnostic criteria for cognitive disorders could increase comparability across studies.

In addition, brain imaging techniques may be used to assess whether the structure and function of the brain is affected by vitamin B12 intake or

status. Previous studies showed that brain atrophy and damage to the white matter was associated with serum/plasma vitamin B12, holoTC II and MMA over the normal range (28, 29). Furthermore, evidence from a single trial showed that 2 year supplementation with a combination of vitamin B6, vitamin B12 and folate, reduced the rate of brain loss in 271 elderly people with mild cognitive impairment (30). Whether the observed effects were due to the combination of B-vitamins, or that one B-vitamin was more important should be further identified.

Dose-response approach: Vitamin B12 and bone health

The relation of vitamin B12 intake and status with bone health was not studied in this thesis, but a systematic review was performed in collaboration with EURRECA colleagues using the same methodology as described for cognitive performance (31). Vitamin B12 may influence bone health through its potential role in bone metabolism and therefore may be a risk factor for osteoporosis (15, 32, 33). The latter review addressed three outcomes of bone health: fractures, bone mineral density (BMD) and bone turnover markers and included studies published through February 2009. Dose response evidence from 2 randomized controlled trials (RCTs) and 17 observational studies on the relation of vitamin B12 intake and status with indicators of bone health is summarized in Table 1. Evidence on associations between intake and fractures (n=1), BMD (n=4) and bone turnover markers (n=2) was limited and yielded no or inconclusive results. A meta-analysis of 4 cohort studies including 7532 elderly subjects with 3 to 12.6 years of follow-up showed a modest decrease in risk of fractures of 4% per 50 pmol/l increase in serum/plasma vitamin B12 concentrations, which was borderline significant (RR=0.96, 95% CI = 0.92-1.00, I^2=0.0%). Out of nine cross-sectional and cohort studies on serum/plasma vitamin B12 concentrations in relation to BMD, 4 studies including 3031 adults and elderly observed that higher serum/plasma vitamin B12 concentrations were associated with improved BMD, whereas 5 other studies including 7428 subjects did not observe any associations.

Due to a large variability in the presentation of data no meta-analysis on this association could be performed.

Table 1 Main findings of a systematic literature review on vitamin B12 intake or status in relation to indicators of bone health

Design References	Number of studies	year	Exposure	Outcome	N total, sex	Main results
Cohort (39)	1	2008	Intake	Fractures	1800 360 cases Women	Risk for fractures does not change with increasing quartiles.
Cohort (34, 40-42)	4	2005-2008	Status	Fractures	7539 (702-4761) 458 cases Mixed	RR per 50 pmol/l= 0.96, 95% CI= 0.92-1.00
Cross-sectional (39, 43-45)	4	2004-2008	Intake	BMD	10114 (1241-5304) Women (3 studies) Mixed (1 study)	Inconsistent data: Two studies showed significant correlations between intake and BMD in a specific subpopulation (MTHFR CC/TT polymorphism) or at a specific timepoint. Two studies found no significant regression coefficients
7 cross-sectional, 2 cohorts (46-54)	9	2003-2008	Status	BMD	10459 (83-5329) Women (5 studies) Mixed (4 studies)	Inconsistent data: Four studies found positive associations, but different for men and women or high versus low vitamin B12 status. Five studies did not observe an association.
RCT's (55, 56)	2	2007	Intake	Bone turnover markers	182 (47-135) Mixed	No significant effects of respectively two and one year combined B-vitamin supplementation on blood levels of bone turnover markers.
Cohort (34)	1	2005	Status	Bone turnover markers	1253 Mixed	Only an association in women, not in men.

RR, Relative risk; 95% CI, 95% confidence interval; BMD, bone mineral density; RCT, Randomized Controlled Trial

Evidence on the association between serum/plasma vitamin B12 and bone turnover markers was limited to one study, which observed significantly higher concentrations of bone turnover markers in women (n=289) with normal as compared to low serum/plasma vitamin B12 concentrations ($p<0.05$), but not in men (n=268) (35). Overall, the evidence-base is suggestive of an association between plasma/serum vitamin B12 concentrations and bone health, although data on BMD are not unambiguous and data on bone turnover marker is limited. Evidence from prospective cohort studies and RCTs is needed to confirm the suggested association. Moreover, data on the functional markers of vitamin B12 status, MMA and holoTC II was very limited, and no conclusion about their associations with bone health could be drawn.

Dose-response approach: Vitamin B12 intake and markers for vitamin B12 status

The relation between vitamin B12 intake via diet, enriched foods or supplements, and different markers of vitamin B12 status (serum/plasma concentrations of vitamin B12, MMA and holoTC II) in adults and elderly was also systematically reviewed by EURRECA colleagues (35). Main results of studies published through January 2009 are summarized in Table 2. For 37 RCTs (n=3398) and 19 observational studies (n=12570), regression coefficients between intake and serum/plasma vitamin B12 were estimated for each individual study, based on the assumption of a linear relation on the double log-scale (natural logarithm of intake versus natural logarithm of status). These regression coefficients were combined in a meta-analysis to estimate the dose-response relation between vitamin B12 intake and serum/plasma vitamin B12 concentrations as $\log_e(y) = 0.16*\log_e(x)+5.31$. This relation indicates that a doubling of vitamin B12 intake increases serum/plasma vitamin B12 concentrations by $2^{0.16}=12\%$. When only including studies including elderly or RCTs, the percentage increase in serum/plasma vitamin B12 was slightly more. In total 9 RCTs were identified that addressed vitamin B12 intake in relation to methylmalonic acid (MMA) concentrations. A pooled analysis revealed that a doubling in vitamin B12 intake resulted in a 7% decrease in MMA concentrations. Three RCTs provided data on the relation between vitamin B12 intake and holotranscobalamin II (holoTC II). Each study showed that vitamin B12 supplementation either through capsules or enriched food products

Table 2 Main findings of a systematic literature review on the relation between vitamin B12 intake and markers of vitamin B12 status

Design References	Publication year	N total (range)	Intervention	Dose (µg/d)	duration (weeks)	Outcome	Main results
37 RCTs (57-84)	1985-2009	3398 (21-217)	24 supplementation 13 diet/enriched food	2.1–1000	4-104 weeks (median 12 weeks)	Serum/plasma vitamin B12	13% increase in status for every doubling of intake
19 observational (cross-sectional data) (85-100)	1984-2009	12570 (64-2156)		0.7-10.5		Serum/plasma vitamin B12	6% increase in status for every doubling of intake 12% increase in status for every doubling of intake
9 RCT's (34, 61, 64, 68, 69, 81)	2001-2009	850 (38-178)	5 diet/enriched food 4 supplements	2.5-987	12-24 weeks	Serum/plasma MMA	7% decrease in status for every doubling of intake
3 RCTs (64, 81)	2006-2008	350 (103-142)	Supplements	9.6-987	12-24 weeks	Serum/plasma Holo-TC II	Inadequate data

RCT, randomized controlled trial; MMA, methylmalonic acid; HoloTC II, holotranscobalamin II

significantly increased the concentrations of holoTC II in serum or plasma, but the number of RCTs was too small and the heterogeneity in study characteristics was too large to justify a meta-analysis.
The meta-analyses on dose-response relations between vitamin B12 intake and markers for vitamin B12 status showed a large between-study heterogeneity varying from 76-98%. Several factors that might explain the observed heterogeneity were considered by stratified analyses, including mean age of the study population, study design, percentage women in the study, duration of the trials and dose and form of vitamin B12 provided. However, these factors did not significantly explain the between-study heterogeneity.
Based on the dose-response relationship between vitamin B12 intake and serum/plasma vitamin B12 concentrations as described above, an average intake of 0.2 µg, 0.9 µg, 5.6 µg or 11.7 µg would be sufficient to achieve plasma concentrations of 150 pmol/l, 200 pmol/l, 258 pmol/l or 300 pmol/l respectively. These plasma concentrations of vitamin B12 are generally accepted as cut-off for clinical practice (150 pmol/l and 200 pmol/l), or suggested as cut-off for subnormal vitamin B12 status (258 pmol/l and 300 pmol/l) (36-38). Estimates of the average intake amounts needed to achieve these plasma concentrations can be denoted as plasma/serum based ANRs.

Integration of data collated with the dose-response approach

Within the scope of the EURRECA network, a bivariate normal model was used to describe the relation between vitamin B12 intake and status by incorporating the variability between individuals for both intake and status measures. The stochastic model was based on the RCTs and observational studies identified for serum/plasma vitamin B12 concentrations as described above. The other markers of vitamin B12 status were not considered as data were limited.
First, the variation in requirements is derived under the assumptions that all measurements of intake (x) and plasma/serum vitamin B12 (y) are error-free, and that a cut-off value of status defines exactly if individual requirements are met. Furthermore the joint distribution of $\log_e$ intake and $\log_e$ plasma/serum vitamin B12 concentrations is assumed to be bivariate normal with means (μ_X, μ_Y), standard deviations (σ_X, σ_Y) and correlation ρ, implying a linear dose-response relation on these scales. As the ANR is the intake sufficient for 50% of the population, an individual with an intake equal to the ANR has a probability of 50% that the intake is sufficient to

reach the cut-off for plasma/serum vitamin B12. Using this probability, the ANR may be derived based on the conditional distribution of plasma/serum vitamin B12 given an intake equal to the ANR. Subsequently the recommended intake may be derived under the assumption of parallel individual lines as the intake at which the probability of reaching plasma/serum vitamin B12 equal to or less than the cut-off is 2.5%.
The ANR and recommended intake derived with the methods described here is shown in Table 3.
The practicability of this stochastic method for deriving the ANR and recommended intake of vitamin B12 depends, as any other model, on the assumptions made. Some of these assumptions, e.g. the absence of measurement error, are unlikely to be met. Therefore, the model should be further developed to deal with these limitations. However, a major advantage of the stochastic method is that it allows the integration of different forms of data (e.g. mean or median, SD or confidence interval, regression-coefficients or correlations, log scale or normal scale) on the relation between intake and status of vitamin B12 that is widely available from different types of studies (RCTs and observational). Furthermore, the model can be extended to a trivariate model, also including health outcomes in addition to intake and status. However, so far this does not make sense for vitamin B12, as the evidence for a dose-response relation with cognitive, bone and other health outcomes is still inconclusive.

Methodological considerations systematic review and meta-analyses

The systematic review methodology as used in this thesis for summarizing evidence on vitamin B12 requirements has several strengths and limitations. The main strength of the methodology is its rigorous and transparent nature that minimizes bias, which is important when used as a basis for decision making processes by authorities in charge of developing evidence-based recommendations and policies. Prior to conducting the systematic review, research questions to be addressed by the review and a description of each step of the review procedure including search strategies, in- and exclusion criteria, data extraction and data synthesis methods were specified in a protocol. As new data become available this information can facilitate the updating process.

Table 3 Overview of vitamin B12 requirements derived with different approaches

Health outcome	Approach	Evidence-base (Number of studies and subjects)	Recommendation type	Recommendation (µg/day)
Current recommendations				
Minimal body stores	Factorial	7 experimental, n= 3-16	ANR + 20% or 40%	2.4 or 2.8
Hematological status	Dose-response	1experimental, n=7	ANR + 20%	2.4
Recommendations based on information reviewed within this thesis and in EURRECA				
Normal body stores	Factorial -	13 experimental, n=135	AI	6.8 - 13
Bone health	Dose-response	2 RCT's, 17 observational, n=28294		Insufficient data
Cognitive performance	Dose-response	2 RCT's, 19 observational, n=13915		Insufficient data
Serum/plasma vitamin B12, cut-off 150 pmol/L	Dose-response	37 RCT's, 19 observational, n=15918	ANR + 20%	0.2
	Stochastic		ANR	0.1
Serum/plasma vitamin B12, cut-off 200 pmol/L	Dose-response	37 RCT's, 19 observational, n=15918	ANR + 20%	1.1
	Stochastic		ANR	0.4
Serum/plasma vitamin B12, cut-off 258 pmol/L	Dose-response	37 RCT's, 19 observational, n=15918	ANR + 20%	6.7
	Stochastic		ANR	1.9
Serum/plasma vitamin B12, cut-off 300 pmol/L	Dose-response	37 RCT's, 19 observational, n=15918	ANR + 20%	14.0
	Stochastic		ANR	5.0
Maximum concentrations of markers of vitamin B12 status (serum/plasma vitamin B12, holoTC II, MMA)	Dose-response	5 observational, n=2982	AI	4-10

RCT, randomized controlled trial; holoTC II, holotranscobalamin II; MMA, methylmalonic acid; ANR, average nutrient requirement; AI, adequate intake

In addition, to ensure complete retrieval of all potentially relevant papers, a multi-database search was conducted in MEDLINE, EMBASE and COCHRANE. Although the largest proportion of papers finally included in the reviews was identified with MEDLINE, a small number of studies originated from the other databases. To ensure objectivity and standardization of the process, at the beginning of each step of the review process, a quality control took place by duplication of 10% of the work by two independent reviewers. Each review presented the available body of literature on the specific topic of the review and concurrently illustrated the knowledge gaps and associated research needs.

Where possible, data from different studies were aggregated by means of meta-analyses as this can increase statistical power and provide answers that no single study can give. Several limitations were encountered here. At first, studies included in our review did not always address our research question, and as a consequence the data needed could not be extracted. Therefore we contacted the study authors to obtain additional information or transformations of the original reported data were performed using validated methodology (101). Unfortunately not all authors responded to our request which limited the number of studies that could be included in the meta-analyses.

Secondly, meta-analyses are limited by the quality and availability of data. We addressed the quality of the different studies included in our review according to proposed methodology by the Cochrane collaboration. Nevertheless, we noticed that information needed to judge upon the quality of a study is often not reported clearly in the papers, for example, the methods used for sequence generation or allocation concealment in RCTs or variables tested for potential confounding (observational studies). This identifies the need for improvement in quality and nature of reporting, by following the CONSORT guidelines when reporting RCTs (102,103) or STROBE guidelines when reporting observational studies (104).

Third, a common concern in meta-analyses is statistical heterogeneity between studies. In the meta-analyses performed for bone health and cognitive performance the heterogeneity between studies was low or moderate as the number of included studies was small and most studies showed results in a similar direction. However, for the intake-status meta-analyses large heterogeneity was observed which could not be explained by study characteristics and is therefore likely to be related to a lack of standardization of methods for measuring intake and status and to a true

heterogeneity between study populations. This heterogeneity could be addressed by an individual participants data meta-analysis, however this is a major undertaking in terms of time, costs, and collaboration, and an inability to include individual participant data from all relevant studies may introduce selection bias.

FROM REQUIREMENTS TO RECOMMENDATIONS

When the available data on vitamin B12 requirements allow the estimation of ANR, we need an estimate of SD in order to derive recommended intakes. This SD should cover the inter-individual variation and uncertainties in the assumptions underlying the ANR, such as body stores and bioavailability. So far, no CV could be estimated for vitamin B12 requirements and therefore the usual CV of 10-20% is used for deriving recommended vitamin B12 intakes. This usual CV is based on estimates of inter-individual variation in protein requirements, i.e. 12.5%, and energy requirements, i.e. 10% or 20% depending on the methods used (105-107).

Two factors that may influence inter-individual variation in requirements between individuals are polymorphisms, and interactions between nutrients. If these factors are considered for estimating a CV, this actually implies that the recommendations should be made specific according to these factors. This is only useful when polymorphisms or nutrient-nutrient interactions substantially affect micronutrient requirements and knowledge on polymorphism is available for the target population to which the NIVs are applied (108). For vitamin B12 several polymorphisms (GIF, CUBN, AMN, TCII, TCIIR) were identified in genes encoding proteins involved in vitamin B12 metabolism, that may evoke vitamin B12 deficiency (109). However such deficiency may not be cured by increasing intake and is therefore not relevant here. Furthermore no micronutrients or food components have been identified so far that influence the requirement for vitamin B12 (105).

RECOMMENDATIONS FOR NUTRIENT RECOMMENDATION SETTING BODIES

An objective of this thesis was to illustrate standardized, transparent and objective review methods that can be used to summarize and evaluate the evidence-base for setting recommendations, using vitamin B12 as a case micronutrient. Table 3 summarizes the implications for recommendations on

vitamin B12 intake based on all the evidence on vitamin B12 requirements gathered within the context of this thesis.
As shown in Table 3, recommended intakes of vitamin B12 for maintenance of normal body stores are similar to recommendations for the prevention of subclinical deficiencies based on the estimated dose-response relation between intake and serum/plasma vitamin B12 (cut-off:258 pmol/l or 300 pmol/l), namely 6.7-14 µg per day. These results are also supported by observations from five studies that showed that markers for vitamin B12 status (plasma vitamin B12, MMA and holoTC II) leveled off at daily intakes between 4 and 10 µg per day (93, 96, 110-112).
Recommendations based on the prevention of deficiencies (maintenance of minimal stores or hematologic status, serum/plasma vitamin B12 ≤150 or 200 pmol/l) resulted in much lower values. However, it must be noted that the estimated dose-response relation was based on apparently healthy study populations with normal to high vitamin B12 intakes. Data on associations between intake and serum/plasma vitamin B12 concentrations from populations with marginal vitamin B12 intakes was not available. Therefore the estimated dose-response relation may be less appropriate to describe the relation between vitamin B12 intake and status in vitamin B12 deficiency.

This thesis illustrated that, at this point in time, evidence is not consistent that vitamin B12 intakes or status may be associated with long term health outcomes including cognitive function and bone health.
If it had been possible to estimate a dose-response relation between vitamin B12 status and cognitive function and bone health, it may have been possible to derive an optimal range of plasma concentrations for markers of vitamin B12 status specifically for each separate health outcome.
In that case, the most appropriate health outcome for estimating the ANR could be selected based on the highest intake required or the robustness of the dose-response relation. Another possibility would be to weigh the health outcomes in terms of disease burden (DALYs) and/or costs for society. However, selecting and weighing the health outcomes and choosing between policy alternatives is the responsibility of policy makers that requires expert deliberations in addition to scientific evidence, especially when uncertainties exist.

CONCLUDING REMARKS

This thesis illustrated that evidence underlying current ANRs of vitamin B12 (maintenance of minimal stores or hematological status) is old and has large uncertainties**,** whereas evidence on long term health outcomes in relation to vitamin B12 intake or status is yet very limited and not suitable for setting recommendations. The relation between vitamin B12 intake and markers of vitamin B12 status seem the best alternative and stochastic methods may provide a good opportunity to integrate dose-response data from different study types. However ways to define recommendations based on these dose-response data should be further developed.

Intakes needed to maintain normal body stores or prevent subclinical deficiency may be up to 5 times higher than current recommendations, but a missing piece of information is the amount of vitamin B12 intake associated with observable health benefits beyond the prevention of signs and symptoms of deficiency.

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et cetera

Summary in Dutch, Acknowledgements, About the author

SUMMARY IN DUTCH (SAMENVATTING)

In de meeste Europese landen worden aanbevelingen opgesteld voor de hoeveelheden micronutriënten (voedingsstoffen) die iemand via de dagelijkse voeding zou moeten innemen. Deze aanbevolen hoeveelheden geven het niveau van inname aan dat toereikend is om te voorzien in de behoefte van bijna alle individuen in de algemene gezonde populatie. Ze dienen als basis voor voedingsbeleid op (inter)nationaal en regionaal niveau, voedingsvoorlichting en voedingsrichtlijnen. Er bestaat geen fysiologische reden waarom verschillende Europese landen verschillende hoeveelheden zouden moeten aanbevelen. Desondanks bestaat er toch een grote variatie in de aanbevolen hoeveelheden tussen Europese landen doordat verschillende landen op verschillende wijze aanbevolen hoeveelheden afleiden. Begin 2007 is het EURRECA (EURopean RECommendations Aligned) Netwerk opgericht met als doel de methoden voor het afleiden van aanbevolen hoeveelheden voor micronutriënten binnen Europa te harmoniseren. Hierbij wordt speciale aandacht gegeven aan aanbevelingen voor kwetsbare populatiegroepen zoals oudere mensen.
Het doel van dit proefschrift, geschreven in de context van EURRECA, is tweeledig:

1. Het bestuderen van de variatie in de huidige aanbevelingen binnen Europa en helder krijgen welke verschillen in methodologische aspecten het meest van belang zijn voor het harmoniseren van aanbevolen hoeveelheden in Europa.
2. Het demonstreren van transparante gestandaardiseerde review methoden die gebruikt kunnen worden om het wetenschappelijke bewijs dat relevant is voor het afleiden van aanbevelingen samen te vatten en te evalueren. Hierbij wordt het micronutriënt vitamine B12 als voorbeeld gehanteerd.

Hoofdstuk 2 geeft een overzicht van de huidige aanbevelingen voor micronutriënten in Europa. Daarnaast wordt per land informatie gegeven over de aanbevolen hoeveelheden en de daarvoor gehanteerde concepten en definities. Verder laat dit hoofdstuk de variatie in Europese aanbevelingen zien voor vitamine A en vitamine D en worden mogelijke verklaringen voor deze variatie besproken door de verschillende methodes te vergelijken, die gebruikt zijn om deze aanbevelingen af te leiden. De informatie over de gebruikte methodes is verzameld op basis van de achtergrondinformatie uit de aanbevelingsrapporten en met een vragenlijst die is ingevuld door

vooraanstaande informanten werkzaam op het gebied van aanbevelingen. Uit deze studie blijkt dat de gehanteerde concepten en definities voor het uitdrukken van aanbevelingen in principe gelijk waren tussen de verschillende Europese landen, maar dat de terminologie tussen landen zeer verschillend was (bijvoorbeeld "aanbevolen nutrient inname" versus "referentie inname van de populatie").
De variatie in aanbevolen hoeveelheden voor vitamine A en vitamine D blijkt samen te hangen met de volgende factoren: verschillen in aannames, verschillen in geselecteerde gezondheidsuitkomsten en verschillen in typeswetenschappelijk bewijs dat gebruikt is om de behoefte te schatten. De beschikbare achtergrondinformatie in de rapporten was te beperkt om de kwantitatieve bijdrage van deze verklarende factoren te kunnen bepalen. Verschillen in concepten en verschillen in definities van bevolkingsgroepen (kinderen, adolescenten, volwassenen en ouderen) zijn minder van belang. Deze bevindingen geven aan dat er richtlijnen nodig zijn om binnen Europa 'evidence-based' aanbevelingen af te leiden.

Belangrijke aandachtspunten voor de harmonisatie van methodes voor het afleiden van aanbevelingen voor de inname van micronutriënten zijn geïdentificeerd in Hoofdstuk 3 door gedetailleerde informatie te zoeken waarmee de variatie in de huidige aanbevelingen kan worden verklaard. Eerst zijn de verschillen in aanbevolen hoeveelheden voor foliumzuur, vitamine B12, ijzer en zink voor volwassenen geïnventariseerd door aanbevelingen uit negen Europese en niet-Europese landen te vergelijken. Vervolgens zijn de aannames en methoden vergeleken die gebruikt zijn om tot de aanbevelingen te komen, alsook de gezondheidsuitkomsten die gebruikt zijn om de behoefte te schatten en de wetenschappelijke studies die hiervoor geraadpleegd zijn. Resultaten van deze vergelijkingen laten zien dat de variatie in aanbevelingen vooral gerelateerd is aan verschillen in de geraadpleegde wetenschappelijke studies (foliumzuur en zink), de geselecteerde gezondheidsuitkomsten (vitamine B12) en aannames ten aanzien van variatie in de behoefte tussen personen (vitamine B12), referentie gewichten van de populatie (ijzer) en biobeschikbaarheid (ijzer en zink). Deze resultaten laten zien dat voor het harmoniseren van methodes om aanbevolen hoeveelheden voor foliumzuur, vitamine B12, ijzer en zink af te leiden binnen Europa, gestandaardiseerde methoden nodig zijn voor a)-de selectie van gezondheidsuitkomstmaten op basis waarvan de behoefte geschat wordt, b)-de definitie van biomarker concentraties die

corresponderen met een adequate gezondheid, c)-het maken van veronderstellingen over de tussen-persoons variatie in behoefte, d)-het vaststellen van de biobeschikbaarheidsfactor, en e)-het selecteren en interpreteren van wetenschappelijk bewijs over behoefte.

Eén van de methodes om de behoefte voor een micronutriënt te schatten is de factoriële methode. Met deze methode worden alle dagelijkse obligate verliezen waarvoor gecompenseerd moet worden via de voedselinname bij elkaar opgeteld en gecorrigeerd voor de biobeschikbaarheid vanuit de voeding. Vitamine B12 aanbevelingen voor volwassenen en ouderen die zijn opgesteld met behulp van de factoriële methode zijn gebaseerd op een beperkt aantal studies die voornamelijk zijn gepubliceerd in de jaren 60 van de vorige eeuw. Hoofdstuk 4 beschrijft een systematische literatuur review over dagelijkse vitamine B12 verliezen en biobeschikbaarheid van vitamine B12 uit verschillende soorten voedingsmiddelen met schattingen van de variatie tussen personen. Deze review laat zien dat dagelijkse vitamine B12 verliezen in gezonde volwassenen en ouderen waarschijnlijk tussen de 2.6 en 3.9 µg per dag ligt, waarbij wordt verondersteld dat de totale lichaamsvoorraad van vitamine B12 tussen de 2 en 3 mg bedraagt.
Gebaseerd op gebruikelijke inname data en de relatie tussen vitamine B12 inname en de hoeveelheid vitamine B12 die geabsorbeerd wordt, kan een biobeschikbaarheid van vitamine B12 worden afgeleid tussen de 29 and 37% in plaats van 50% zoals meestal verondersteld wordt. Deze resultaten suggereren dat een vitamine B12 inname tussen 7.0 and 13.4 µg nodig is om te compenseren voor dagelijkse vitamine B12 verliezen in gezonde volwassenen en ouderen. Deze inname is 2.5 tot 5 maal hoger dan de inname die nodig is om verschijnselen van vitamine B12 tekort te voorkomen. De huidige vitamine B12 aanbeveling van 1.4-3.0 µg is mogelijk niet voldoende om een lichaamsvoorraad van 2-3 mg en optimale plasma concentraties van biomarkers voor vitamine B12 status te handhaven. Om goed te kunnen evalueren welke vitamine B12 status en lichaamsvoorraad in verband staan met waarneembare gezondheidsvoordelen zijn er meer gegevens nodig over de relatie tussen plasma concentraties van biomarkers van vitamine B12 status, de lichaamsvoorraad van vitamine B12 en gezondheidsuitkomsten op lange termijn.

Om te bepalen of in de toekomst de relatie tussen vitamine B12 en cognitief functioneren moeten worden meegenomen bij het afleiden van vitamine B12

aanbevelingen voor volwassenen en ouderen, is in Hoofdstuk 5 een systematische literatuur review beschreven van gerandomiseerde voedingsproeven (RCTs) en prospectieve cohortstudies die de relatie tussen vitamine B12 en cognitief functioneren bestuderen. De twee geïndentificeerde RCTs en 19 cohort studies lieten geen of een inconsistent verband zien tussen enerzijds vitamine B12 inname of status en anderzijds dementie, ziekte van Alzheimer, algemeen cognitief functioneren of domein-specifiek cognitief functioneren. Dit betekent dat het huidige wetenschappelijke bewijs voor de relatie tussen vitamine B12 en cognitief functioneren te gering is om mee te nemen bij het afleiden van aanbevolen hoeveelheden vitamine B12. Om deze relatie beter te onderzoeken zal toekomstig onderzoek zich moeten richten op meer gevoelige biomarkers voor vitamine B12 status en cognitieve functie. Dit zijn bijvoorbeeld methylmalonzuur (MMA) en holotranscobalamine II (holoTC II) voor vitamin B12 status, en de mate van vermindering van hersenweefsel (hersen atrofie) bij voorkeur in combinatie met domein-specifieke testen als cognitieve uitkomstmaat.

In 2007 toonde een studie in de Verenigde Staten dat een lage vitamine B12 status in combinatie met een hoge foliumzuur inname door consumptie van foliumzuur-verrijkte meelproducten mogelijk een cognitieve achteruitgang in een ouderen-populatie veroorzaakt. In Hoofdstuk 6 is onderzocht of zulke interacties ook aanwezig zijn in een populatie die niet is blootgesteld aan voedingsmiddelen verrijkt met foliumzuur. Hierbij is gebruik gemaakt van gegevens van 2203 Noorse ouderen verzameld binnen de Hordaland homocysteïne studie in de regio Bergen. Cross-sectionele analyses lieten een significante negatieve interactie zien tussen plasma concentraties van foliumzuur en vitamine B12 in relatie tot cognitieve prestatie. Dit resultaat suggereert dat onder ouderen met een lage vitamine B12 status, de positieve associatie tussen foliumzuur en cognitieve prestatie juist sterker wordt. Echter, wanneer holoTC II of MMA in plaats van plasma concentraties van vitamine B12 in het model worden opgenomen, is de interactie tussen foliumzuur en de marker van vitamine B12 status in relatie tot cognitieve prestaties niet significant. De resultaten van deze studie geven in lijn met Hoofdstuk 5 aan dat de relatie tussen vitamine B12 en cognitief functioneren momenteel onzeker is en daardoor niet geschikt als gezondheidsuitkomstmaat om de vitamine B12 behoefte te schatten.

In het laatste hoofdstuk van dit proefschrift (Hoofdstuk 7), worden de belangrijkste bevindingen samengevat en aangevuld met bewijs over dosis-respons gegevens voor de relatie tussen vitamine B12 inname of status en gezondheidsuitkomsten die zijn bestudeerd binnen het EURRECA Netwerk (hematologische kenmerken en botgezondheid). Daarnaast is de relatie beschreven tussen inname van vitamine B12 en biomarkers van vitamine B12 status. Voor- en nadelen van de verschillende methoden die gebruikt kunnen worden om vitamine B12 behoeftes te schatten worden bediscussieerd en hiaten in de huidige kennis worden benadrukt.
De belangrijkste conclusie van dit proefschrift is dat het bewijs op grond waarvan de huidige aanbevolen hoeveelheden voor vitamine B12 zijn afgeleid (handhaven van minimale lichaamsvoorraden of hematologische kenmerken) oud is en wordt omgeven door grote onzekerheden, terwijl bewijs over gezondheidsuitkomsten in relatie tot vitamine B12 inname of status (cognitief functioneren, bot gezondheid) tot nu toe zeer beperkt is en niet geschikt als basis voor het opstellen van vitamine B12 aanbevelingen. Het beste alternatief is om op basis van de relatie tussen vitamine B12 inname en status de inname af te leiden die correspondeert met een vitamine B12 status die in het algemeen als gezond kan worden beschouwd. De statistische methode hiervoor dient echter nog verder te worden ontwikkeld.

Tot slot laten de berekeningen in dit proefschrift zien dat innames die nodig zijn om normale lichaamsvoorraden van vitamine B12 te handhaven mogelijk tot 5 maal hoger kunnen liggen dan de huidige vitamine B12 aanbevelingen die gericht zijn op het voorkomen van signalen en symptomen van subklinisch deficiëntie/tekort. Doordat gegevens over het verband tussen de lichaamsvoorraad van vitamine B12 en waarneembare effecten op de gezondheid ontbreken, blijft het helaas onzeker of deze hogere vitamine B12 aanbeveling daadwerkelijk gezondheidsvoordeel oplevert.

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ABOUT THE AUTHOR

Curriculum Vitae

Esmée Lucinda Doets was born in Koog aan de Zaan, the Netherlands on the 20th of December, 1982. After completing secondary school at "Bertrand Russel College" in Krommenie, she started the Bachelor program "Nutrition and Health" at Wageningen University and afterwards enrolled in the Master program. She completed a Master's thesis at the community health service in Amersfoort, the Netherlands, where she developed guidelines for the evaluation of the primary prevention of obesity in a defined area in Amersfoort (B.Slim moving more, eating healthy). For her second thesis she performed a quality evaluation and update of the food composition table of Mali. During an internship in Potchefstroom, South Africa, she investigated the relation between nutritional status and HIV status in children aged 0-5y at a day-care centre in the black community. In September 2007, Esmée obtained her Master's degree and afterwards directly started with a PhD-project at the Division of Human Nutrition of Wageningen University. The PhD-project was part of the EU funded Network of Excellence EURRECA - European Recommendations Aligned, and the results are described in this thesis. During her PhD-project, she critically reviewed the variation in current micronutrient recommendations in Europe and conducted two systematic reviews with meta-analysis. In collaboration with the University of Bergen, she performed observational data analyses.

Esmée joined the educational program of the graduate school VLAG and attended several (international) project meetings and conferences in the field of nutrition, public health and ageing. She was involved in teaching at the BSc and MSc level and chaired the PhD-committee of the Division of Human Nutrition. Since March 2012, she is employed as post-doctoral fellow at the Division of Human Nutrition where she is involved in the EU funded SMILING project. Within this project Esmée is leading a work package targeted at evaluating quality and updating food composition tables of South East Asian countries.

List of publications

Original research papers

Doets EL, van Wijngaarden JP, Szczecinska A, Dullemeijer C, Souverein OW, Dhonukshe-Rutten RAM, Cavelaars AEJM, van 't Veer P, Brzozowska A, de Groot LCPGM. Vitamin B12 intake and status and cognitive function in elderly people: a systematic review with meta-analyses. *Epidemiol Rev* (accepted with modifications).

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Doets EL, Cavelaars AE, Dhonukshe-Rutten RA, van 't Veer P, de Groot LC. Explaining the variability in recommended intakes of folate, vitamin B12, iron and zinc for adults and elderly people. *Public Health Nutr* 2012; 15 (5): 906-915

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Cavelaars AEJM, Kadvan A, **Doets EL**, Tepsić J, Novaković R, Dhonukshe-Rutten R, Renkema M, Glibetić M, Bucchini L, Matthys C, Smith R, van't Veer P, de Groot CPGM, Gurinović M. Nutri-RecQuest: a web-based search engine on current micronutrient recommendations. *Eur J Clin Nutr* 2010; 64 (Suppl 2): S43-7.

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Submitted papers

Doets EL, in 't Veld PH, Szczecinska A, Dhonukshe-Rutten RAM, Cavelaars AEJM, van 't Veer P, Brzozowska A, de Groot LCPGM. Systematic review on daily vitamin B12 losses and bioavailability for deriving recommendations on vitamin B12 intake with the factorial approach. *Submitted to Ann Nutr Metab.*

Van Wijngaarden JP, **Doets EL**, Szczecińska A, Dhonukshe-Rutten RAM, Souverein OW, Duffy ME, Dullemeijer C, Cavelaars AEJM, Pietruszka B, van 't Veer P, Brzozowska A, de Groot CPGM. Vitamin B12, folate, homocysteine and bone health 1 in adults and elderly people: a systematic review with meta-analyses. *Submitted to J Nutr Metab.*

Dullemeijer C, Souverein OW, **Doets EL**, van der Voet H, van Wijngaarden JP, de Boer WJ, Plada M, Dhonukshe-Rutten RAM, in `t Veld PH, Cavelaars AEJM, de Groot LCPGM, van `t Veer P. Systematic review with dose-response meta-analyses between vitamin B12 intake and EURRECA's prioritized biomarkers of vitamin B12 including randomized controlled trials and observational studies in adults and elderly. S*ubmitted to Am J Clin Nutr*.

Abstracts

Doets EL, Cavelaars AEJM, Dhonukshe-Rutten RAM, van 't Veer P, de Groot CPGM. Explaining the variability in recommended intake levels of folate, vitamin B12, iron and zinc for adults and elderly people. *Eur J Public Health* 2010; 20 (suppl 1): S124

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Doets EL, Dhonukshe-Rutten RAM, de Groot LCPGM. Differences and similarities in European recommendations on a subset of micronutrient of concern for elderly. 5th European congress on Nutrition and Health in the Elderly people, Warsaw, Poland, 2008

Overview of completed training activities

Discipline specific courses and activities

- "The Nottingham systematic review course", 2008 (Nottingham, United Kingdom)
- VLAG course "Evidence-based nutrition: From requirements to recommendations and policies", 2008 (Warsaw, Poland)
- EURRECA workshop: "Reviews for requirements", 2008 (Norwich, United Kingdom)
- EURRECA workshop: "Alignment methodology for reviews and meta-analyses", 2010 (Wageningen, The Netherlands)
- 2nd Integrating meeting EURRECA (oral presentation), 2008 (Sveti Stefan, Republic of Montenegro)
- 3rd Integrating meeting EURRECA (oral presentation), 2009 (Barcelona, Spain)
- 4th Integrating meeting EURRECA (oral presentation), 2010 (Copenhagen, Denmark)
- Congress on Nutrition and Health in Elderly People, "Successful aging through diet and healthy lifestyle" (oral presentation), 2008 (Warsaw, Poland)
- Wageningen Nutritional Sciences Forum "Too much-too little" (poster presentation), 2009 (Arnhem, The Netherlands)
- Nutrition Society Annual Summer Meeting (poster presentation), 2009 (Surrey, United Kingdom)
- Annual meeting NWO nutrition, 2009 (Deurne, The Netherlands)
- Workshop: "Towards an adequate intake of micronutrients: what do we need? "(The Hague, The Netherlands)
- VoedingNederland, 2010 (Ede, The Netherlands)
- 2nd World Congress of Public Health Nutrition (oral and poster presentation), 2010 (Porto, Portugal)
- 3rd Annual European Public Health Conference (2 oral presentations)

General courses and activities

- EURRECA training course, 2008 (Cork, Ireland)
- VLAG-PhD introduction week, 2008 (Bilthoven, NL)
- WGS course "Writing and Presenting a scientific paper", 2009 (Wageningen, The Nethelands)
- NIHES course "Principles of Epidemiology: Data Analysis", 2010 (Rotterdam, The Netherlands)

- VLAG masterclass "Linear and logistic regression", 2010 (Wageningen, The Netherlands)

Optional courses and activities

- MSc-course "Analytical Epidemiology", 2010 (Wageningen, The Netherlands)
- PhD study tour in Denmark, Sweden and Finland, 2009
- Literature group "Old's mobiles"and "Journal Club", 2007-2009
- Preparation research proposals and research presentations, 2007-2012

COLOPHON

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Approaches for setting micronutrient recommendations

a case study of vitamin B12 for adults and elderly people

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