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GENERAL INTRODUCTION

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Nutrition and Ageing

We live in an ageing society and thus the number of older people is increasing rapidly around the world (1). The ageing process makes people more prone to certain health conditions and many older people experience multiple health problems. Furthermore, the percentage of people with chronic diseases increases with age. What is more, due to an increase in life expectancy, the increasing prevalence of chronic health problems result in an equally increasing loss of healthy life years (2). Moreover, with aging accumulation of DNA damage and alterations in epi-genetic and cellular mechanisms occur that affect the trajectory of healthy ageing (3). Nutrients and nutritional status could also influence these epi-genetic and cellular mechanisms (4). Hence, cumulative effects of ageing and inadequate nutritional status result in a further increased risk of negative health outcomes.

Accordingly, nutritional status and body composition changes as a result of ageing, and there are many factors that influence nutritional status during ageing. These include gender, genetic variation in individuals, physical functional status (such as physical activity, strength and endurance), socio- economic status, cognition, nutrition and medical health status, such as chronic or acute health conditions and the use of medication (**figure 1**) (5).

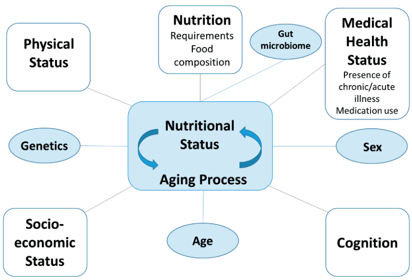

Figure 1. Factors that influence nutritional status as people age (5)

Furthermore, an age-related set of clinical syndromes characterized as “frailty”, results in an increased risk of worse health outcomes, such as falls, disability, hospitalization and mortality (6). It is possible that improving nutritional status in older people could help to inhibit the ageing process and reverse frailty (7). Poor nutritional status of the ageing population is caused partly by low intake of macro- and micronutrients (**figure 2**), in particular dietary protein intake, which is besides associated with frailty (8). Community-dwelling older adults face health problems, and micronutrient deficiencies (deficiencies in vitamins and minerals) are similarly more common among this group due to inadequate diet, and age-related changes in the absorption, distribution, metabolism and/or excretion of nutrients (9). A common approach to measure vitamin deficiencies is measuring dietary intake or biomarkers in blood, urine or tissue (**figure 2**).

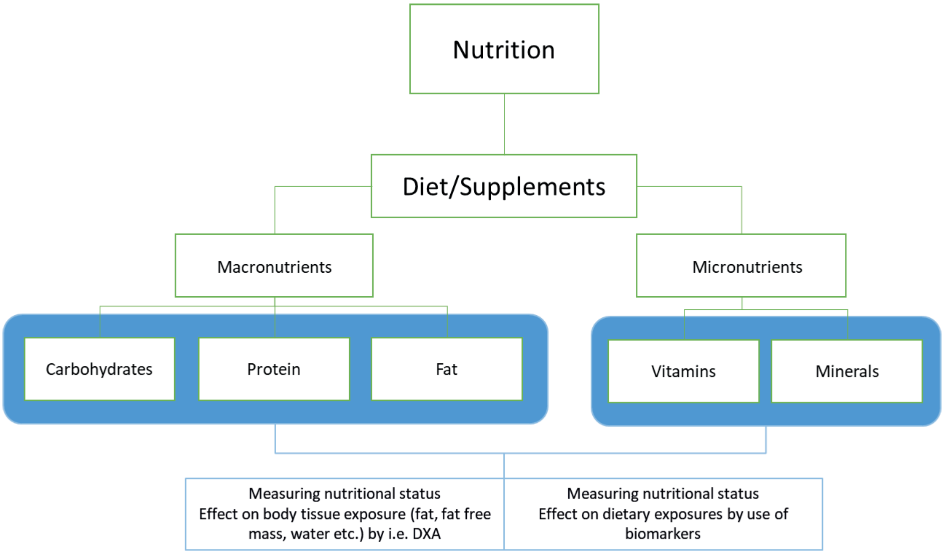

Figure 2. Nutrition and measuring nutritional status, effect on body tissue and dietary exposures

In the Netherlands older adults regularly use dietary supplements to prevent vitamin deficiencies (10). The dietary supplements that are most frequently used by older adults are multi-vitamins including B-vitamins, vitamin D and calcium (11). The global use of dietary supplements, such as vitamins and minerals has become a routine part of many people's lives, including many older people. For example, in the US the national Health and Nutrition Examination Survey showed that in 2011-2014, 70% of the older population used dietary supplements, and in the Netherlands around 45% of this part of the population used dietary supplements in 2010-2012 (12, 13). It has been shown that the use of supplements in the Netherlands is increasing with age (11). Yet, the role of dietary supplements on health, especially in the elderly, is not completely understood. Dietary supplements could play a role in optimizing health, however it could further be harmful and have negative effects on health (14).

Role of Genetic Factors in nutritional status

Genetic variation in individuals may play a role in nutrition and could result in differences in nutritional processes such as absorption, food metabolism, micronutrient metabolism and gut microbiome composition. Moreover, the genetic factors affect food preference(15, 16). An approach used in genetic research to associate specific genetic variations with particular diseases is called genome-wide association studies (GWAS) (17). GWAS helps us to increase our understanding of the genetic variation and diseases. With GWAS we are able to improve our knowledge of subtle differences between individuals, including behavioural characteristics and health (18). For example, different Loci for serum calcium concentrations were identified with GWAS (19). These Loci then could be used as a combined genetic score, like polygenetic risk score (PRS). Additionally, PRS can be applied as effect-modifier in association analysis or in Mendelian Randomisation studies to understand causality in the association between micronutrients (e.g. calcium) and disease (e.g. cancer). By using genetic variations as natural experiments, MR studies provides evidence about assumed causal relationships between a modifiable risk factor and the outcome of interest (20).

MICRONUTRIENTS AND HEALTH

Due to inadequate intake of nutrients and changes in absorption in our digestive system, micronutrient deficiencies, like vitamin D deficiency, are common in older persons (9).

Concomitant with age-related changes in body composition, the incidence of obesity increases too among older populations (21). These changes in body composition are further associated with lower micronutrient status, such as in the case of vitamin D, vitamin B12 and folic acid, for example (22, 23). So, in the older populations, changes in body composition as well as micronutrient deficiencies are more common. However, the association between intake and circulating levels of these micronutrients and body composition in older adults is unclear.

The commonly used measure of body composition, BMI (as weight in kg divided by square height in meters) may underestimate the prevalence of body composition among older adults. Ageing is associated with height loss due to changes in the bones, joints and muscles loss (24), therefore, in studies involving older people measuring fat mass and fat-free mass provides a better insight into the actual body composition and the prevalence of obesity. However, for a better insight into body composition, especially in an ageing population, other measures than BMI are needed. For example, the DXA scan (or other alternatives such as Bod Pod) that measure the body fat and fat free mass, is considered a better alternative (**figure 2**) (25).

Vitamin deficiencies appear to play a role in several chronic and common diseases in older persons. Community-dwelling older persons are at risk of vitamin B12 and vitamin D deficiency However, the role of vitamin B12 and folic acid in common diseases in older adults, such as osteoporosis, cancer and cardiovascular disorders remains unclear. It is possible that hyperhomocysteinemia is an important factor in this relationship.

Homocysteine concentration is negatively associated with vitamin B12 and folate concentrations, as depicted below, and an effective method to reduce homocysteine concentration is the provision of B-vitamins (26, 27). The association between hyperhomocysteinemia and the risk of cardiovascular disease and fractures has been observed consistently (28, 29) and showed that elevated homocysteine level appears to be a strong predictor of ischemic heart disease, stroke and fractures. Consequently, observational studies have shown an association between B-vitamin intake (dietary and supplements) and lower risks of fractures and cardiovascular disease, however with conflicting results that may be explained by bias such as confounding. Thus, to assess the effect of B-vitamin intake (dietary and supplements) on the risk of fractures and cardiovascular disease, randomized controlled trials are needed. Several intervention trials have been performed to investigate the effect of treating these common conditions with B-vitamins, but again with conflicting results (30, 31). Due to few RCT's, in 2008 the B-PROOF (B-Vitamins for the PRevention Of Osteoporotic Fractures) trial was designed to investigate the effect of B-vitamins on different outcomes in an older population using a multicentre RCT design (more details below). After 2-3 years of supplementation with vitamin B12 and folic acid, a reduced risk of osteoporotic fractures was observed in only a subgroup of compliant persons aged 80 years and over (32). In addition, the B-vitamin intervention was observed to have no effect on the overall incidence of coronary heart disease, with the exception of a significantly reduced risk of cerebrovascular events among females (33). Unexpectedly, we found as well a higher self-reported incidence of cancer in the intervention group compared to the control group (32). In view of the relative short duration of the B-PROOF intervention, it was additionally speculated if longer follow up would reveal more consistent effects.

Thus, additional research questions arose following the B-PROOF trial: what is the long-term effect of the B-PROOF intervention on cancer risk? What is the long-term effect of the intervention on osteoporotic fractures and cardiovascular diseases? Other more general questions included: what is the effect of micronutrients supplementation (i.e. B-vitamins, vitamin D and calcium) on age related disease in the general population? Who benefits from taking supplements? What are the risks of excessive and over the counter micronutrient- and dietary supplements? In this thesis, we aimed to address these questions. In order to answer them, some pathophysiological background is needed first, which we will further explain in the next section, by starting with vitamin B12, folate, calcium and vitamin D and their potential influence on ageing and related-diseases.

B-VITAMINS IN ONE-CARBON METABOLISM

Vitamin B12 & Folate

Vitamin B12 (cobalamin) is water-soluble and naturally present in products derived from animals, such as meat, milk, cheese, fish, and eggs. Together with folic acid, vitamin B12 plays a role as an important co-factor in the one-carbon metabolism. Cells require one-carbon units for DNA synthesis and methylation (**figure 3**). In the methionine cycle, vitamin B12 is involved in the synthesis of methionine from homocysteine (both amino-acids) by two intermediates, namely S-adenosylmethionine (SAM) and S-adenosylhomocystine (SAH). Homocysteine is an amino acid that is not available from food but is made after the demethylation of methionine, which is an essential amino-acid available from food (34). So, methionine and homocysteine are necessary for DNA synthesis by the methyl group released when SAM converts to SAH (35).

Vitamin B12 in food is attached to animal protein (haptocorin) and is released from this protein by gastric acid and pepsin in the stomach to free vitamin B12. Vitamin B12 is absorbed in the intestine and in the small intestine vitamin B12 binds with the intrinsic factor (IF) (which is produced in the stomach) and absorption is facilitated by mucosa in the small intestine. It is transported to other tissues via the blood. Vitamin B12 that is not used immediately is stored in the liver (36). The daily recommended intake of vitamin B12 for adults is 2.8 micrograms and currently no limited upper intake level has been defined for vitamin B12 (37).

Folate is a water-soluble B-vitamin too, which is naturally present in grains and green leafy vegetables. The bioavailability of natural folate is lower than in its synthetic form, folic acid, which is used in supplements (38). The metabolism of folate starts in the intestine in its bounded form. It is absorbed in the jejunum, and the hydrolysed folate attached to methyl groups is delivered to the liver and other body cells via the blood. The methyl group is in the one-carbon metabolism removed from 5-methyl-THF in the synthase reaction. The enzyme 5-methyl-THF together with vitamin B12 as co-factor is needed before homocysteine can be converted into methionine, which serves as a methyl group donor through transformation to SAM, and can be used to methylate DNA, for example (**figure 3**) (38). In the Netherlands, the daily recommended intake of folate for adults is 300 micrograms and 400 micrograms for pregnant women in order to prevent major birth defects in the baby's brain and spine, like neural tube defects. The European Food Safety Authority (EFSA) specifies an upper limit on the intake of folic acid from supplements of 1,000 micrograms per day, because vitamin B12 deficiency is not detected if the intake of folic acid is too high (39).

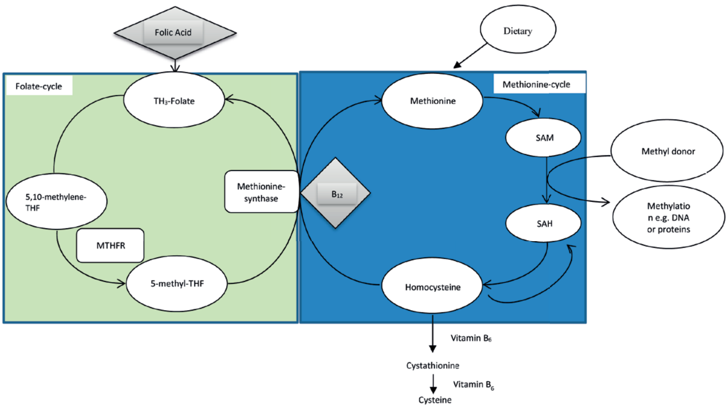

Figure 3. One-carbon metabolism

Calcium & Vitamin D

Calcium is a mineral needed in cellular metabolism and is, together with phosphate, incorporated in hydroxyapatite which is crucial for the structure and maintenance of the bones and teeth (40). Calcium plays a key role in the optimal functioning of bone, tooth formation, muscles and nerves, blood clotting, the transport of other minerals and hormone excretion (41). Furthermore, it plays a role in cell signalling and fluid balance (42).

As a dietary component, calcium is naturally present in milk, dairy products, vegetables, nuts and legumes. It is absorbed in the gut with the aid of a controlling hormone, 1.25-Dihydroxyvitmin D3 (1.25(OH2)D3), the active form of vitamin D (43).

Most calcium in the body is deposited in the skeleton and less than 1% is found in the blood, soft tissues and extracellular fluid (41). Circulating calcium in serum exists in 3 forms, whereby 5-15% is complexed calcium bound to anions, 30-50% is bound to albumin, and the remaining circulates as free ionized calcium (Ca2+) (44).

The Health Council of the Netherlands recommends 950 milligrams of calcium daily for men aged between 25-69 years and women aged 25-50 years. The daily recommended intake for persons older than 70 years is 1,200 milligrams daily; for women aged 51-69 years it is 1,100 milligrams. Pregnant women and lactating women, need 1,000 milligrams of calcium daily (45). The upper limit on intake from food and supplements is 2,500 milligrams daily, due to the elevated risk of kidney stones, renal insufficiency, vascular and soft issue calcification and hypercalciuria (45).

Vitamin D is a fat-soluble vitamin that is naturally present in fat fish, and in lower amounts in meat and eggs. Additionally, it is fortified in margarines. Sunlight is the most important source of vitamin D (3), which enables the synthesis of 1.25(OH2) D3 in the skin, produced by 7-hydrocholesterol through exposure to ultraviolet B. In the liver, 25-hydroxylation of vitamin D results in the formation of 25-hydroxyvitamin D (25(OH)D), which is considered a more longer term reliable measure of vitamin D status, reflecting vitamin D reservoirs in persons with normal kidney function (45).

As mentioned, vitamin D is important for calcium homeostasis (46). The daily recommended intake of vitamin D is 10 micrograms for adults and 20 micrograms a day for everyone aged above 70 years. The upper daily limit for vitamin D is 100 micrograms a day. High intake of vitamin D is not that common in healthy people, however, it can result in symptoms of malaise, drowsiness, loss of appetite and obstipation (45).

Diet and medication: Diuretics as an example

In addition to the increased use of dietary supplementation, effects of use of medications and polypharmacy on nutritional status are as well a growing concern among older population (47). A frequently prescribed medication group to treat heart failure and hypertension, especially in older people is diuretics (48, 49). A study in 2015 showed that between 33 and 47% of older people aged 50-90 years or older used diuretics. The potential adverse effects of diuretics may have serious impact on older persons. Recently, recognition of the relevance of food-drug interactions in clinical practice has further been growing (40). Interestingly, it has been widely documented that diuretics can have various effects on bone health (50-52). Thiazide diuretics have been shown to have a protective effect in protecting bone mass and in decreasing the risk of fractures (51). In contrast, loop diuretics may have a negative impact on bone turnover by increasing urinary calcium excretion (53). Calcium and vitamin D have been hypothesized to play a role in the association between the use of diuretics and bone health (54). Thus, knowledge of the food-drug interaction regarding diuretics and the use of vitamin D and calcium supplementation in relation to the effects on bone health may be therefore relevant.

STUDY POPULATIONS AND METHOLOGY

For this thesis, the data from the B-PROOF trial and the Rotterdam study was used. For the initial B-PROOF study, the inclusion criteria were, age >65 years, a homocysteine level >12 µmol/L and <50 µmol/L and creatinine level <150 µmol/L. Initially 2,919 older persons participated. For the second follow-up study after 5-7 years, 1,298 participants responded to the extended studies follow-up questionnaire. Details on the design of the B-PROOF can be found elsewhere (55) and details of the follow-up study are provided in **chapter 3.2**. Exposures from B-PROOF trial studied in this thesis were serum vitamin B12 level, holotranscobalamin (HoloTC), methylmalonic acid (MMA), folic acid, vitamin D and the intervention (folic acid and vitamin B12 supplements). Furthermore, the outcomes assessed in this thesis were body composition (fat mass and fat free mass measured by DXA-scan), cancer (from national cancer registry), fractures (self-reported and verified by the GP's), cardiovascular and cerebrovascular diseases (self-reported).

The Rotterdam Study (RS) is a population-based prospective cohort that has been going on since 1990 and includes participants aged 40 years and over. The study focuses on the most common diseases in this age category, such as cardiovascular, locomotor, endocrine, neurological and respiratory diseases. There are various examination cycles in the RS. For this thesis, we included the participants from RS I, RS II and RS III. The rationale, design and the diagram of the cycles of the study is described elsewhere (56). Exposures from RS studied in this thesis were diuretics use (thiazide and loop diuretics prescriptions) and calcium (intake by FFQ, levels and supplements). Outcomes assessed in this thesis were bone mineral density (BMD) and the trabecular bone score (TBS, measured by DXA-scan) and cancer (from national cancer registry).

AIMS AND OUTLINE OF THIS THESIS

An important societal development of concern is the increasing use of the dietary supplementation due to growing interest in the role of micronutrients in optimising health and preventing certain diseases (14). Supplements of B-vitamins, calcium and vitamin D are now commonly used among older adults (11). However, there are a number of questions with regard to the effects of these vitamins on age-related changes in body composition, fracture risk, and the prevalence of cardiovascular disorders and cancer. Thus, the overall aim of this thesis is to study the role of micronutrients and body composition and the long-term effect of micronutrients on common negative health outcomes such as fractures, cancer and cardiovascular events in the older population **(Figure 4)**. Further objectives of this thesis are to study the role of drug-micronutrients interaction on bone health, specifically diuretics, and to use genetic variation involved in regulating calcium metabolism as a tool to understand causality in the association between micronutrients (e.g. calcium) and cancer (colorectal).

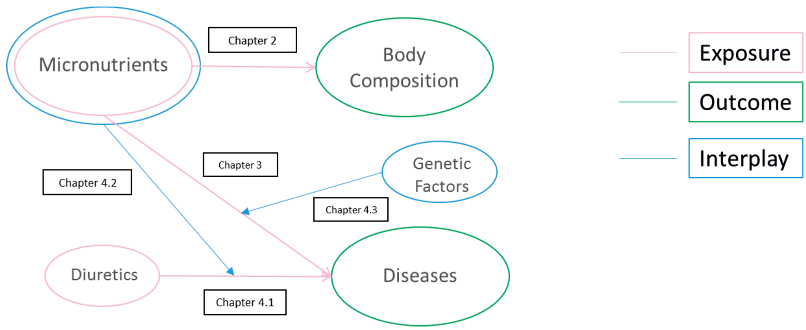

Figure 4. Overview of the topics of the several chapters in this thesis and their relationships

Chapter 2 focuses on the associations between vitamin D, B-vitamins and body composition using data from the B-PROOF study. In **Chapter 2.1**, the associations between vitamin D and BMI and fat mass are described. **Chapter 2.2** presents the observational and experimental evidence from the B-PROOF study on folic acid, vitamin B-12 and body compositions.

Chapter 3 focuses on the long-term effect of micronutrients on disease outcomes. **Chapter 3.1** shows the long-term effect of the folic acid and vitamin B12 intervention on the risk of cancer. The results concerning the long-term effect of folic acid and vitamin B12 intervention on the B-PROOF's primary and secondary outcome- osteoporotic fractures and cardiovascular disease- are presented in **Chapter 3.2**.

Chapter 4 focuses on the interplay between micronutrient intake and levels, genetic variation and drug use in relation to colorectal cancer and bone health, respectively. **Chapter 4.1** presents the impact of thiazide diuretics on bone mineral density and trabecular bone score in the Rotterdam study. **Chapter 4.2** examines whether vitamin D level and dietary calcium intake modified the association between loop diuretics and bone health. **Chapter 4.3** describes the associations between dietary calcium intake, calcium level and calcium supplementation and colorectal cancer and the interaction with genetic variation. In **Chapter 5**, the overall findings of this thesis are discussed regarding their implications, including some recommendations for future studies.

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