GENERAL DISCUSSION & SUMMARY

GENERAL DISCUSSION

As the usage of dietary supplements grows worldwide, especially among older people, the objective of this thesis was to understand more about the role of micronutrients in health and diseases in the older population. The micronutrients that were the main topic of this thesis were folic acid, vitamin B12, vitamin D and calcium because they are widely used as dietary supplements, and have pleiotropic effects on several age-related diseases.

The association between vitamin D and body composition was studied, as well as the effect of folic acid and vitamin B12 on body composition (**Chapter 2**). The long-term effects of folic acid and vitamin B12 supplementation (the B-PROOF study) on common age-related diseases as such as cancer, fractures and cardiovascular diseases was assessed (**Chapter 3**). Lastly, the role of micronutrients on bone health and colorectal cancer in interactions with diuretics and genetic variation on bone health and colorectal cancer, respectively was assessed (**Chapter 4**). This chapter provides an overview and discussion of the main findings of this thesis, followed by an outline of the clinical and public health implications and suggestions for potential further research.

MAIN FINDINGS AND COMPARISONS WITH RECENT STUDIES

Micronutrients and body composition

The prevalence of micronutrient deficiencies is increased in people with obesity, which is expressed as having a BMI >30. However, obesity is a multifaceted physiological phenomenon leading to changes in body composition and is sometimes difficult to assess in an ageing population. Therefore, BMI as a measure may underestimate the prevalence of obesity among older adults. In studies involving this population group, measuring fat mass and fat-free mass as aspects of this changing body composition provides a better insight into the actual body composition and the prevalence of obesity. Therefore, the association between micronutrients and body composition (by measuring body fat percentage next to BMI) in an older population was assessed. We found that a higher BMI and higher body fat percentage were both significantly associated with lower serum 25(OH)D levels. In this study we confirmed that overweight older persons and those with a higher percentage of body fat were at risk of vitamin

D insufficiency (**Chapter 2.1**). This finding corresponds with other studies involving various populations (1, 2).

This association can be due to a decreased bioavailability of vitamin D due to its deposition in adipose tissue (3). But to assess the causality and the direction of the association between the level of serum 25(OH)D and the change in BMI and fat percentage, one can use vitamin D supplementation studies and Mendelian Randomization studies. Mendelian randomisation studies suggested that vitamin D is causally related to markers of obesity, e.g. adiponectin (4). In the B-PROOF intervention trial with B vitamin supplements, all participants were also given vitamin D supplementation, therefore whether vitamin D supplementation might change body composition could not be assessed. Neither did we have any information on serum 25(OH)D levels after the intervention. However, a recent RCT showed that vitamin D supplementation had no effect on body composition (5). Yet, a systematic review and meta-analysis of RCTs showed that obesity reduced the effect of vitamin D supplementation on vitamin D level (6, 7). In addition, evidence from RCTs has also shown that optimal vitamin D supplementation may benefit obesity-related disorders (3). Altogether these results suggest that increase in overweight and fat mass seems to reduce serum 25(OH)D level and that obese people may need a higher doses of vitamin D to achieve the normal range of 25(OH)D compared to non-obese people. Further RCTs on the role of vitamin D supplementation in obesity-related disorders are needed (see implications and future research).

Another aspect that was evaluated in this thesis, was the effect of folic acid and vitamin B12 supplementation on body composition. In **chapter 2.2** we observed that higher serum folate was associated with a lower BMI, and a higher vitamin B12 status was associated with a higher Fat Mass Index (FMI) from observational data, which was partly in line with previous studies (8). However, this association was not confirmed as causal, because the intervention with folic acid and vitamin B12 supplementation in the B-PROOF study had a null-effect on body composition, suggesting that B-vitamins may not have a role in the aetiology of obesity or changes in body composition in older individuals. Neither was the causal role of a lower vitamin B status in obesity supported in a Mendelian randomisation study of genetic determinants of vitamin B12 (9). However, a relatively strong association has been observed between the pleiotropic Fucosyltransferase 2 (*FUT2)* gene variant rs602662 and serum vitamin B12 (9). FUT2 is thought to be involved in interactions between host factors and gut microbiome composition, which is involved in production of vitamin B12. Therefore, it can be hypothesized that differences in the gut microbiome composition might lead to the observed associations with obesity and body composition, and the concomitant

vitamin B12 changes might be a by-stander effect (10). Other recent studies support the causal role of obesity on vitamin B12 status by finding an association between *FTO* gene variants (which are associated with obesity (11)), and serum vitamin B12 levels (12). On the basis of these findings it can be concluded that obesity may influence B-vitamin status, but not the other way around.

Long-term effect of micronutrients on cancer

In the B-PROOF trial, the combination of vitamin B12 and folic acid supplementation was observed to be associated with an elevated risk of cancer, and especially colorectal cancer (**Chapter 3.1**). Previous studies on the association between folic acid and vitamin B12 supplementation and cancer risk were inconclusive, with some studies showing a null-effect or even a reduced risk of cancer after supplementation (13). The proposed underlying mechanism is that the carbon group of folic acid supplementation contribute to cell proliferation through purine nucleotide synthesis that accelerate the process of cell growth or by the provision of methylgroup donors to influence the process of DNA methylation and expression of genes that may be involved in carcinogenesis. Indeed, in a follow-up study of the B-PROOF study, when assessing genome wide methylation patterns on 450.000 CpG sites in genomic DNA of circulating blood cells using the Illumina array, variations in DNA-methylation were observed in several genes involved in carcinogenesis (14) in the intervention group.

One explanation for the opposing outcomes of several trials compared to our findings is that most studies on the effects of B vitamins on cancer have examined the effect of folic acid supplementation only, rather than in combination with vitamin B12 supplementation. Since vitamin B12 also plays a role in the one-carbon mechanism, it might also play a role in the aetiology of cancer. The VITamins and Lifestyle cohort-study (VITAL), assessed the association of vitamin B12 supplementation and showed a higher risk of lung cancer in men between 50 and 76 years with a higher use of vitamin B12 supplements (15).

However, studies on the association between cancer on the one hand and dietary intake or plasma levels of these B vitamins on the other hand, have shown conflicting results. Results from observational studies have suggested that the intake of these micronutrients individually is associated chiefly with a decreased risk of several cancers (16-18). However, studies of plasma levels of folic acid and vitamin B12 did not find an association with an increased risk of several cancers (19, 20). For example, in a study of subjects in UK primary care, having higher plasma B12 levels was found to be associated with an increased one-year cancer risk compared to normal B12 levels (21).

In a nested case-control study including a Mendelian randomisation approach based on 8 genetic variants for circulating vitamin B12, an increased risk of lung cancer was found to be associated with an increase in circulating vitamin B12 concentrations suggesting a causal effect of B vitamins on cancer risk (22).

Taken the results of previous studies, the B-PROOF study and RCT together, it seems that folic acid and/or vitamin B12 supplements, due to higher dose and higher bio-availability, might potentially increase the risk of cancer in older persons (such as the participants in the B-PROOF study) (23). This could be due to individual effect of folic acid or vitamin-B12, or due to an interactive effect of both folic acid and vitamin-B12 combined, and that this effects could be due to changes in DNA methylation patterns.

Effects of B vitamins on fractures, CVD and stroke

With regard to the findings of the B-PROOF trial during a prolonged follow-up period on the main pre-specified outcome, no effect on fracture risk was observed, and neither an effect on cardiovascular disease was seen (**Chapter 3.2**). Similar to B-PROOF, another long-term RCT by Stone *et al.* found that vitamin B12 and folic acid supplementation had no effect on the risk of fractures in women (24). One of the limitations of B-PROOF could be the limited power to detect any effect of the B-vitamin supplementation due to relatively low incidence of events in the limited follow period. We therefore extended the follow-up period (of 2-3 years) of the initial B-PROOF trial to 6-9 years to capture more events, and evaluated the long-term effect of the intervention. A questionnaire was sent to the participants with informed consent for contacting in the future. In this extended study of the B-PROOF trial, however, only 90 new fractures were captured. We hypothesize that this may be an underestimation due to high loss to follow-up, mainly due to the high morbidity and mortality rates in these older adults (with a median age of 76 years when contacted in this extended follow-up study). During the second follow-up questionnaire, extra information was documented on the non-responders shown in the flow-chart below (**Figure 1**). In contrast with B-PROOF extended follow-up, a recent study showed that higher intake of vitamin B12 (≥ 30 vs < 5 µg, diet and supplements) in a longer follow-up period (of 20.9 years), was associated with increased fracture risk (25). This study documented 2304 cases with hip fracture of 75 864 included in women nurse health study with lower intake of vitamin B12 than B-PROOF study. Nevertheless as this study has a cohort design of the study, residual confounding may be present and thus randomized controlled trials with a larger follow-up are needed to establish adverse effects in the long run.

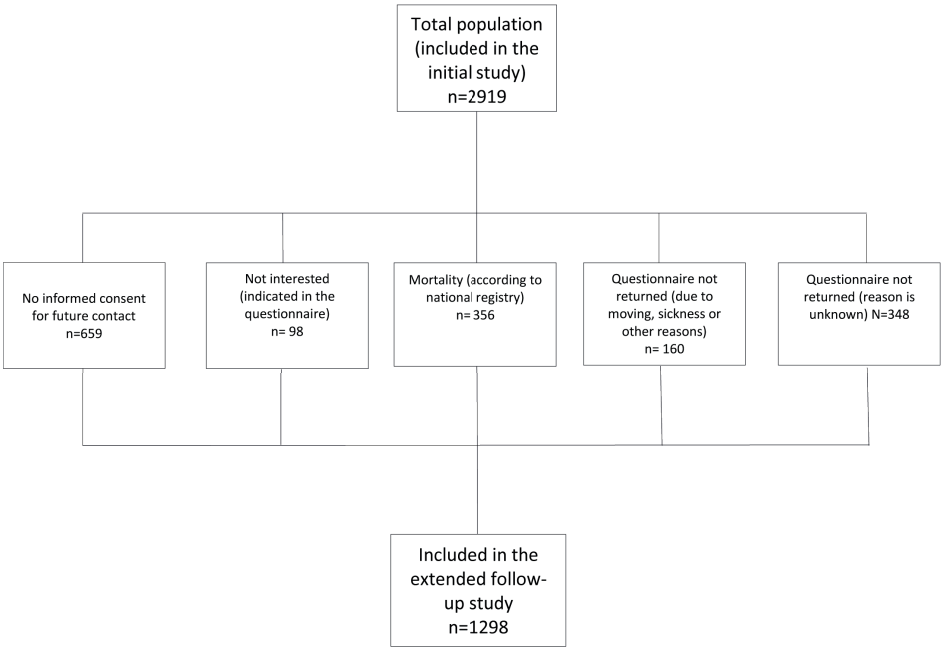

Figure 1. Flow-chart showing an overview of reasons for non-response of participants in the secondary analysis of the B-PROOF trial (Chapter 3.2).

In the B-PROOF trial, which selected and included participants with elevated homocysteine levels, B-vitamin supplementation was observed to may have been beneficial in reducing fractures, but only in individuals with high total homocysteine concentrations **(Chapter 3.2)**. This suggests that in subgroups of older persons or those with a higher risk of a deficiency of B-vitamins, supplementation may decrease the risk of fracture. However, it was a subgroup analysis and because of the low number of cases (n= respectively 16, 31, 23) in the stratified analysis of different levels of homocysteine levels (≤ 13.2, 13.2-15.1 and ≥15.1 mmol/l), these findings should be interpreted with caution. Similarly, in the study by Stone *et al.*, the number of participants was too low to study the effects of B-vitamin supplementation among women with deficiency in the Women's Antioxidant and Folic Acid Cardiovascular Study (WAFACS) (24). Thus, replication is needed to test the effect of B-vitamin supplementation especially in the deficient individuals. Potentially there is benefit of supplementation and the effect of B-vitamins on fracture risk in this deficient older persons.

With regard to the long-term follow-up effect of B-vitamin intervention in the B-PROOF trial on CVD and stroke, no effect was observed from analysis of the self-reported data. Systematic reviews on homocysteine-lowering with B-vitamins, showed only a

decrease in stroke risk and not in the risk of other cardiovascular diseases, identical as the outcomes of the initial B-PROOF study (26, 27).

Interplay between micronutrients, Single Nucleotide Polymorphisms (SNPs) and drugs on disease

Diuretics and bone health

In the Rotterdam Study (RS), a positive association was observed between thiazide use (past and current) and bone mineral density at the lumbar spine (LS-BMD) but not with lumbar spine trabecular bone score (LS-TBS), a DXA-derived measure of bone micro-architecture and quality. This relation was observed in the general population and in particular in participants older than 65 years, compared to participants younger than 65 years (**Chapter 4.1**). In line with this observation, other studies showed a lower fracture risk with the use of thiazide diuretics (TDs) (28). This is further supported by another study among women that showed that TD users had a slower annual change in femoral neck and spine BMD compared to non-users, which suggests that BMD loss was lower in TD users, confirming the results observed in RS (29). Thus, the possible protective effects in relation to fracture risk could be explained by an increase in BMD, which increased with the dosage and duration of thiazide use, without improving bone microarchitecture. The increased BMD in TD users may be explained by estimated decrease in osteocalcin level, a marker of osteoblast activity, bone formation, and bone turnover (30) the expression of which might be influenced by TD use (**Chapter 4.1**).

In contrast to thiazide diuretics, loop diuretics (LDs) may adversely affect bone health by inhibiting calcium reuptake. Indeed, in the Rotterdam Study, TBS was found to be only decreased if LDs were used in the past and LDs seem to be weakly associated with higher BMD when used for a period of 121-365 days, compared to never users, but the results were not consistent (**Chapter 4.2**). The potentially harmful effect of LDs on bone were more firmly confirmed in other studies (31-33). Because of the common use of LDs among older persons and the high prevalence of osteopenia and osteoporosis, we investigated the effect-modification of vitamin D and calcium on the association between LDs and bone health. Yet, in this study, no consistent associations were found between LD use and bone outcomes after stratification by serum 25(OH)D levels. Even though the analyses were adjusted for body fat mass, residual confounding may have biased the association between serum 25(OH)D levels and bone health caused by other co-morbidities, such as other measures of body composition, physical activity, lifestyle and health status.

Calcium and cancer

In **chapter 4.3**, we studied the association between the intake of calcium (dietary and supplements), serum calcium level, and colorectal cancer (CRC). In this study, an association was only found for a higher calcium intake (≥ 1,485 mg/day), compared to an average calcium intake (1,100-1,485 mg/day), which suggests that normal intake of dietary calcium is not associated with higher risk of CRC. This association was also not seen between calcium supplement use and CRC, and neither between calcium levels and CRC risk. In addition, we found no statistically significant linear trend between dietary calcium intake and CRC, perhaps suggesting a threshold effect. From previous studies, calcium intake from dairy products may be associated with reduced CRC risk while the association for non-dairy calcium intake and CRC risk is still inconclusive (34).

The different results from this and previous studies may be explained by differences in bioavailability of calcium (35). After adjusting serum calcium levels for albumin in a subgroup, circulating serum calcium levels were observed to be positively associated with an increased risk of CRC. This supports the observation of the relation between calcium dietary intake with increased CRC risk. However, these observations do not necessarily indicate a causal relationship. Calcium is partly (around 40%) bound to albumin (36) and it is known that calcium serum level is under close homeostatic control (under normal circumstances), and disturbed calcium homeostasis is often found in diseases including cancer (37). Studying genetic determinants of calcium concentrations, as a stable proxy, could give more insight into calcium homeostasis. This is possible because through GWAS several such genetic determinants have been found (38), which are combined into a so-called polygenic risk score, or polygenetic risk score (PRS), which can explain a certain percentage of the variance in calcium levels in the population. Therefore, we also studied the interaction between calcium intake, levels and supplementation and a PRS of calcium concentrations, in relation to colorectal cancer risk (**Chapter 4.3**). Indeed, the associations between dietary calcium intake or serum calcium levels and CRC, were modified by the calcium PRS, with a lower CRC risk for subjects with a lower PRS, compared to subjects with a higher PRS. Taken together, results of the calcium PRS analysis suggest a relationship between circulating calcium and risk of cancer, but further replication in other studies with different population are needed.

METHODOLOGICAL AND ETHICAL CONSIDERATIONS

Influence by a case of scientific misconduct?

For this thesis specifically the methodological and ethical considerations relevant to the B-PROOF trial concern two complexities: data integrity in the community of science, and secondary post-hoc analyses as an approach for analysis of the unexpected adverse events, which we will discuss in this section.

The design of the B-PROOF study was based on findings of earlier observational studies on the association between homocysteine levels and osteoporotic fractures, including those in the Dutch study populations of the Rotterdam Study and LASA (39-41). Interestingly, an intervention trial appeared, soon after these initial epidemiological association studies, to show that B-vitamin supplementation had a beneficial effect on fracture risk, which was the study by Sato *et. al.* (42). Many other trials, including the B-PROOF trial, have since been carried oud to investigate the effect of B-vitamin supplementation on bone health, but have not been able to replicate the results of the RCT by Sato *et al.* Therefore, around 10 years after publication of Sato's RCT, concern was expressed regarding the integrity and scientific validity of Sato's study (43). In the meantime, due to the null-findings of the primary results of the B-PROOF trial and concerns regarding the possible adverse effect of B-vitamin supplementation on cancer risk, the researchers in the B-PROOF trial were interested in conducting a meta-analysis including Sato's trial. However, the authors did not respond to this opportunity despite numerous attempts to get in touch with them. At the same time, a study to evaluate the validity of the results reported by the *Journal of the American Medical Association* had begun, and as a result, the article by Sato *et al.* was retracted along with 21 (of 33) of his other articles due to scientific misconduct and concerns about data integrity (44). Unfortunately, the results published by Sato *et al.* had -by that time- already resulted in several large-scale intervention trials being conducted, based in part on the spurious results of Sato's work.

The question therefore arises whether the B-proof study would have been conducted if the study by Sato *et al.* had not been published. Because of the positive results of other observational studies on the association between homocysteine level and osteoporotic fractures and the limited evidence from RCTs, the B-PROOF trial was designed to study the effect of homocysteine-lowering intervention through B-vitamin supplementation on osteoporotic fractures irrespective of Sato *et al.* Thus, most likely the B-PROOF study would still have been designed and conducted given the

order of events and available evidence at that time. Nevertheless, the power analysis of the B-PROOF trial was based on Sato's publications, and if the study by Sato *et al.* had not been published, the power analysis based on the observational studies would have resulted in recruitment of a larger number of study participants and/or a longer follow-up.

Importantly, the question also arises if unnecessary harm could have been prevented if the Sato *et al.* study was not published. The dosage of the B-vitamin intervention of the initial B-PROOF trial was chosen also based on safety considerations, since there were already concerns regarding the adverse effects of pharmacological doses of vitamin B supplementation. Furthermore, individuals with a five-year history of cancer were excluded from the study, and if any participant reported a cancer diagnosis during the study, they were advised to discontinue the supplementation, while follow-up of the other outcomes continued. Participants were advised to discontinue supplementation when reached high dosage of folic acid and vitamin B12 (beyond recommended daily intake) when combined with the intervention. Moreover, half way through the study, we compared the number of incident cancer cases with the incidence of cancer in the general population, without de-blinding the intervention, and observed these figures to be similar at that point in time. As mentioned, at the end of the B-PROOF trial and de-blinding, there were further concerns about the possible adverse effect of folic acid and or vitamin B-12 supplementation on cancer risk based in results from the B-PROOF trial. This was studied further in a secondary analysis, by linking the B-PROOF data to data of the national cancer registration. Subsequently, the concerns were confirmed by indeed showing an adverse effect for these B-vitamins on incidence of cancer overall, and colorectal cancers in particular. So, taken together it is unlikely that unnecessary harm was inflicted on B-PROOF subjects by including the Sato *et al.* study, and all required precautions were taken to prevent such harm from happening.

Thus, even without the RCT conducted by Sato *et al.*, the B-PROOF trial would have been designed and performed, however, probably with a different power calculation.

Lessons learned

Because we cannot turn back time, we can learn a great deal from this, one of science's major scandals, which by now has been widely publicized. This was however 7 years after B-PROOF was initiated.

Negative results are important

First of all, the number of publications is increasing, but the number of retracted papers is also increasing. Currently, the pressure to publish positive and novel results in science is high due to pressure on citation numbers in order to secure prizes and funding (45). Previous work has also shown that statistically significant results are more likely to be published than papers with null results. This could feed scientific misconduct, as was the case with Sato's RCT, and potential publication bias (*i.e.*, leading to distortion of the scientific literature and misleads health professionals, and policymakers in their decision-making (46)). It is therefore essential to be able to publish the negative results, too. A change in mind-set is needed when it comes to accepting negative papers, such as the follow-up B-PROOF manuscript with its null-findings on the risk of fractures and cardiovascular disease.

Secondary analyses are informative

A second methodological consideration in this thesis was the secondary *post hoc* analysis of the long-term effect of primary and secondary outcomes as well as the adverse events. Most of the studies focusing on the effect of B-vitamins on the relevant health outcomes (fracture or cancer) are secondary analyses alongside other outcomes (such as cardiovascular disease), which are the primary outcome. Selection bias and sample size calculations are some examples of issues that need to be taken into account with respect to interpreting these findings. Being aware of this, in future studies, issues of selection- and information bias and confounding can be dealt with in the design.

For the analysis of the long-term effect of the intervention on primary and secondary outcomes and the adverse events in the extension of the B-PROOF study, participants with informed consent for future studies from the B-PROOF trial were selected. Luckily as there were no differences between the intervention and the control group in the selected population, the randomisation was still intact. Another issue to address in secondary analyses is sample size. However, for studies on the unexpected adverse effects of supplementation such as our initial B-PROOF trial, or studies involving rare diseases as an outcome, it is maybe difficult to predict the correct sample size in advance, but assumptions are possible. It is important to report clearly that the study is a secondary analysis or an extended follow-up. Also, the methods of the secondary analysis and the number of participants which were included in the analysis derived from the primary trial should be reported. The recommendation of Hopewell *et al.* (2013) (47) is adapted from the 2010 CONSORT Statement, and states which information should be included in these reports in order to enable readers to evaluate the

validity and reliability of the results of the secondary analysis, and ultimately to use these to assess the scientific implications. Possible biases should also be mentioned, as well as their potential effects. In the secondary analysis of the B-PROOF in **chapter 3.1**, all these items are mentioned.

Longer follow up is beneficial

Moreover, a post-trial follow-up of a randomized controlled trial can provide valuable information about the long-term effect of the intervention on the primary outcomes of the trial, especially in this age category (48). Most of the RCTs are designed as short-term trials, but a longer follow-up time could help to detect persistent effects in the years following the intervention and identify the latency-effect of the intervention. We expected the primary outcomes of the B-PROOF trial to detect the persistent effects on fracture years after the intervention, and to detect the latency-effect of the intervention on CVD as in the baseline and at the end of the first follow-up, a follow-up questionnaire was used for the post-trial follow-up. Unfortunately, given the older age group the prolonged follow-up period resulted in a high loss to follow-up, resulting in limitations of the generalizability of the results. In light of this knowledge, prolonged follow-up is preferably already taken up in the initial study design. For example through patient consent forms a route could be to secure access to routine health records in order to access data on detailed health information relating to these participants using diagnostic codes (e.g. ICD-10), including mortality data and the cause of death in follow-up participants, where relevant. Of course, valid informed consent at the baseline would need to be in accordance with the new privacy guidelines. In the Netherlands, the electronic patient record (EPD) is useful but sometimes difficult to obtain information source and at the time of data collection the use of the EPD as researcher would have let to unanticipated regulatory issues. Additionally, from the mortality data and the cause of death, a cause-specific mortality analysis can provide more information on the effect of the intervention, since for example several studies have indicated that cancer patients may have a higher risk of CVD mortality (49).

Better documentation of supplement use helps interpretation

The examination of the supplement use (over the counter or additional supplementation on top of the intervention) of the participants in the B-PROOF trial and the Rotterdam study was based on self-reported data, which could result in misclassification of supplement use due to insufficient recall problems. The use of supplementation also varies over the years. Another method of monitoring dietary supplement use

more truthfully, was used in the study by White *et al.*. In this study, researchers collected the supplements data by repeated questionnaires and then calculated an high validity and reliability compared with questionnaire at baseline, three months after, supplement inventory and to the biomarkers of the nutrients (50). In the future, better monitoring of the supplement use will be necessary (see below, implications and future research).

Dosage counts matter

Another point of discussion that needs mentioning, is the dosage of the intervention. For the B-BROOF study, a dosage of 500 µg/day for vitamin B12 and 400 µg/day for folic acid was chosen. For vitamin B12, the dosage was higher than the recommended daily intake (51). However, this dosage was chosen based on a dose-finding study which showed that a dose of 647 µg/day for four months is sufficient in an older population with mild deficiency to normalize vitamin B12 status (52). The majority of dietary supplements contains a high level of micronutrients. For some micronutrients, such as folic acid, there is an upper intake limit, but this is not yet the case for vitamin B12 (51). As some colleagues showed previously, dietary intake of vitamin B12 is significantly associated with vitamin B12 biomarkers (53). Furthermore, the association between total vitamin B12 intake (from diet and supplements) showed a stronger association with vitamin B12 biomarkers, probably due to the higher bioavailability and higher absorption of the free vitamin B12 from supplements (54).

Other micronutrients are also important

As we mentioned previously, other nutrients are also involved in one-carbon mechanism, such as choline and methionine, and these are also relevant to the study of the effect of micronutrients on health. For example, choline metabolism disruption could affect DNA methylation (55). Additionally, high methionine is associated with methionine/transmethylation metabolism, which could increase DNA damage and carcinogenesis (56). However, more studies on the restriction of methionine and the prevention of cancer are needed. Unfortunately, there is no data available on these nutrients, but it is important to evaluate these mechanisms. However, given the fact that the B-PROOF trial was randomized and no major differences in baseline characteristics were observed between the intervention and control group, we assume that the effect of the B-vitamin intervention is not explained by differences in the intake of other methyl donors. On the other hand, for the cross-sectional analysis of the B-PROOF trial and in The Rotterdam Study, other nutrients involved in one-carbon mechanism could have played a role. Unfortunately, these were not available.

IMPLICATIONS AND FUTURE RESEARCH

What determines deficiency

The most common deficiencies in Western Europe are iron, vitamin D, folate and vitamin B12 (57). These nutrient deficiencies are fairly easy to detect, diagnose with laboratory tests and treat with the appropriate supplements. However, subclinical deficiency is difficult to recognize and can still result in pathological changes, which may subsequently lead to clinical diseases. It is therefore important to recognize the 'at-risk' population and to treat them appropriately (for example by a dietician) in order to prevent clinical diseases due to the depletion of micronutrients. Community-dwelling older persons are at risk of vitamin B12 deficiency induced anaemia that may be masked by the use of high dosages of folic acid (58). These potential underdiagnoses that can cause neurological complications needs further investigation. General Practioners should therefore be alert to the correct diagnosis of vitamin B12 deficiency, such as for example by assessing serum methylmalonic acid analysis (MMA) (59).

As well as the method of measuring deficiencies, the cut-off for some biomarkers is also under debate. For example, for vitamin D the Endocrine Society (ES) uses the cut-off of 75 nmol/L (60) and the Institute of Medicine (IOM) uses a cut-off of 50 nmol/L (61) for 25(OH)D concentrations. Yet, also based on the studies presented here it is highly recommended that a different cut-off for deficiency should be used for each age group due to different nutritional needs in different stages of life, particularly among older age persons, and due to physiological variations, the pathological ageing process, the role of medication and how it influences nutritional biomarkers (62).

Body composition

Since the studies in this thesis demonstrated that body composition, and in particular obesity, may have an effect on micronutrient status, future studies should focus on the effect of changes in body composition and the mechanisms that underlie their potential effects on micronutrient status. For example, in a meta-analysis of studies that measure the changes in the body composition and changes in B-vitamin levels along with the interaction of the genes associated with body composition and B-vitamins. Furthermore, due to the possible risks of unnecessary supplementation with high doses of B-vitamins (and possibly vitamin D) (63), a meta-analysis is needed to examine the differences in vitamin D and B-vitamin status in an obese population and whether vitamin D influences obesity-associated health risks over the short and long

term. The prevention of obesity (especially in early life) could be an important public health strategy for reducing metabolic syndrome and obesity-associated health risks.

Aspects of micronutrient research

Certain other important questions remain unanswered, including the following. Is it the structure of B-vitamins that makes the difference in the effect on cancer (cells), or the amount of intake regardless of the source of the folate/folic acid and vitamin B12? Is there a cut-off point for these micronutrients, after which they cease to be preventive and start to be harmful? What is the effect of these supplements in deficient people? Future research is required to understand the potential beneficial and adverse effects of micronutrient supplementation in more detail. Ideally, randomized controlled trials would provide more insight into the negative effects of dietary supplements but, due to potential ethical issues, this is not possible. Analysing the potential harm in the relevant subgroups within a meta-analysis of all the trials that have measured the effect of B-vitamins and vitamin D and accurately addressing adverse event reports, would therefore be a better solution. It is necessary to report adverse events in randomized controlled trials like the B-PROOF study, but trials in Good Clinical Practice (GCP)-based pharmaceutical settings have stricter rules for monitoring of adverse effects. In the future, there should be better monitoring of the use of dietary supplementation and adverse effect in the general population, including all age groups, similar to the clinical trials used to test the (new) drugs. A mandatory improved report on adverse effects, drafted in accordance with the International Conference on the Harmonisation of Good Clinical Practice (ICH GCP) guidelines (64), should cover all adverse effects and analyse the risks of dietary supplements in randomized controlled trial studies. However, clinical trials are not always feasible (due to earlier mentioned potential ethical issues), and so a population-based cohort such as the Rotterdam Study, with improved repeated measurement of the use of dietary supplements by the participants (including the use of over-the-counter supplements), could also provide valuable information and facilitate the analysis of the adverse effects of micronutrients (from food and dietary supplements). This type of study (population-based cohort) may also have better generalizability to the community-dwelling population as a whole (65).

Disentangling cause and effect

However, it is important to note that, in population-based studies, the reliability of the results derived from association analysis, whereby the direction of the association is not certain, i.e. potential ‘reverse causation’, is not always adequate.

Moreover, residual confounding is a problem in observational studies, and therefore Mendelian randomization (MR) could provide a solution, because this method is less likely to be affected by reverse causation or confounding. This method provides evidence about assumed causal relationships between a modifiable risk factor and the outcomes of interest, by using genetic variations as natural experiments. In our case, the genetic variants could be that for homocysteine to support causal inferences regarding the effect of cardiovascular disease (modifiable risk factors) (66). It should be noted that this method depends on assumptions for a valid instrumental variable: the relevance assumption (the instrumental variables should associate with the risk factor of interest); the independence assumption (the instrumental variables should share no common cause with the outcome); and the exclusion restriction assumption (the instrumental variables should do not affect the outcome except through the risk factor). Together this means that pleiotropy, i.e., one gene and/or variant of a gene can have multiple biological functions, can be a problem here, and will need to be addressed in future MR studies.

The challenge of drug-nutrient interactions

In our aging society prevalence of medications use is high and continues to rise, with accompanying polypharmacy. Furthermore, older age is an important risk factor for nutritional status, thus also prevalence of poor nutritional status is rising. As such, recognition of the importance of food and drug interaction has been growing in clinical practice (67). Diuretics are frequently prescribed in the treatment of heart failure and hypertension (68, 69), and they have been shown to influence calcium homeostasis and bone metabolism. As mentioned previously, the association between the use of diuretics and trabecular bone score has not been investigated previously, and the possible changes in bone microarchitecture need more detailed research using more refined techniques, such as high-resolution peripheral quantitative computed tomography (HR-pQCT). Further studies are needed to investigate the association between loop diuretics and general bone health, especially trabecular bone score. Moreover, research and replication is needed on nutrient-drug interactions with bone health, using other biomarkers such as PTH and bone turnover markers. Nutrient-drug interactions are largely understudied. The knowledge about this subject is scarce and awareness of potential adverse effect of nutrient-drug interaction, and also training for clinicians is needed (67). Guidelines for the prevention of bone loss due to use of loop diuretics might also be helpful. Future research should also focus on the association between polypharmacy and nutritional status in the older population due to the higher use of medication among this group and the possible association with reduced intake of some nutrients and potential food-drug interactions (70).

An early recognition of (colorectal) cancer

Clinical trials are not always achievable, as mentioned before. Thus to assess the effect of the B-vitamin supplementation on cancer an early diagnosis of malignant neoplasm is appreciated. Biopsies are the standard methods for diagnosis of lesions (71, 72), which are often invasive methods for the patient. Currently, research into microRNA (miRNA) and cancer is being conducted and has shown that the expression of miRNAs is dysregulated in different tumours and miRNAs is suggested as a potential biomarker for diagnostic and also prognostic targets in cancer (73, 74). In the follow-up to B-PROOF, we could have measured such potential biomarkers in order to detect a variety of common cancers earlier; however, due to budget constraints we were unable to do this, so we linked our data to data of the national cancer registration for the diagnosis of cancer. In other studies, the role of the miRNAs has been shown in the diagnostic and treatment of colorectal cancer (75), which could be used to investigate the effect of B-vitamin supplementation on cancer risk in the future.

Is folic acid fortification universally good ?

As we know, fertile women are advised to take folic acid pre- and peri-conceptionally in order to reduce the risk of neural tube defects (76). For this reason, folic acid fortification (folic acid added to enriched grain products) has been introduced in some countries. Due to fortification, the National Health and Nutrition Examination Survey Data determined a higher folic acid intake, which exceeded the upper limit intake (77). Now that we suspect a possible adverse effect of excess folic acid and/or vitamin B12 intake among older persons (presumably with malignant cells), the question arises as to whether it is still appropriate to add folic acid to food, since the majority of the population does not need extra folic acid or other micronutrients. On the other hand, there may be arguments that the older population may also benefit from fortification (due to their higher likelihood of deficiencies because of inadequate diet and changes in the absorption and excretion of micronutrients (78)). A recent study evaluated the effect of voluntary fortification of vitamin B12 and folic acid, and showed that in older adults, fortification was not an effective manner of preventing deficiencies (79). The B-PROOF study evaluated the effect of folic acid and vitamin B12 supplementation, and this study provides a further insight into the potential adverse effect of these micronutrients in the older population and contributes to evaluation and decision-making around the fortification of food. The suggestion from work presented in this thesis is not to stimulate widespread use but to limit the advice to consume micronutrients (through supplements) to pre- and peri-conceptional women and others who need additional micronutrients, such as those with a proven

deficiency. However, although it is conceivable that deficient individuals may be more likely to benefit from supplementation (80), studies addressing the specific subpopulations who may or may not benefit from supplementation are scarce.

Again, the B-PROOF study showed an adverse effect of vitamin B12 (and/or folic acid) on cancer, especially on colorectal cancer, and another recent study showed a potential association between higher vitamin B12 level and higher mortality, which has to be confirmed in other studies (81). Nevertheless, the adverse effects of high vitamin B12 is the subject of debate (with respect to both intake and level), and therefore, it can be argued that setting a maximum upper intake level for vitamin B12 (from supplements) is preferable in order to avoid the excessive intake of this micronutrient.

CONCLUSION

To conclude, the community-dwelling older population with obesity or excess body weight is at increased risk of deficiency in several micronutrients including vitamin D, vitamin B12 and folate. Those with obesity may need a higher intake of micronutrients. However, considering the potentially adverse effects of B-vitamins and the risks with respect to health outcomes, including more acute life-threatening ones such as cancer, dietary supplements may not always be beneficial to health in this population. Consequently, I do not recommend using B-vitamin supplementation for older persons as a population group, and micronutrients should preferably be obtained from a balanced diet. On the basis of the findings of this thesis it is suggested that supplements are recommended in case of proven deficiency but, even then, not in excessive amounts. The optimum dosage for supplementation needs to be further investigated. Furthermore, the use of micronutrients should be monitored carefully and the potential risks and adverse effects of dietary supplements should be investigated thoroughly. Possible nutrient-drug interactions also need to be explored in greater depth.

Finally, due to the impact of "frailty", *i.e.*, the combined effect of several chronic disabling and degenerating phenomena in elderly, in a community-dwelling older population, future interventions should focus on addressing factors that may accelerate the ageing process and exacerbate nutritional status, as described in the introduction. Such factors include quality of nutrition and improving physical activity to prevent frailty in the ageing population. Good quality of nutrition includes simply following the official guidelines for healthy nutrition and an achievable, personalized physical activity/exercise regime that suits the daily life of the ageing population under the supervision of a dietician.

REFERENCES

1. Yanoff LB, Parikh SJ, Spitalnik A, Denkinger B, Sebring NG, Slaughter P, et al. The prevalence of hypovitaminosis D and secondary hyperparathyroidism in obese Black Americans. Clin Endocrinol (Oxf). 2006;64(5):523-9.
2. Gallagher JC, Yalamanchili V, Smith LM. The effect of vitamin D supplementation on serum 25(OH)D in thin and obese women. J Steroid Biochem Mol Biol. 2013;136:195-200.
3. Hypponen E, Boucher BJ. Adiposity, vitamin D requirements, and clinical implications for obesity-related metabolic abnormalities. Nutr Rev. 2018;76(9):678-92.
4. Husemoen LLN, Skaaby T, Martinussen T, Jørgensen T, Thuesen BH, Kistorp C, et al. Investigating the causal effect of vitamin D on serum adiponectin using a mendelian randomization approach. European Journal of Clinical Nutrition. 2014;68(2):189-95.
5. Karefylakis C, Sarnblad S, Ariander A, Ehlersson G, Rask E, Rask P. Effect of Vitamin D supplementation on body composition and cardiorespiratory fitness in overweight men-a randomized controlled trial. Endocrine. 2018;61(3):388-97.
6. de Oliveira LF, de Azevedo LG, da Mota Santana J, de Sales LPC, Pereira-Santos M. Obesity and overweight decreases the effect of vitamin D supplementation in adults: systematic review and meta-analysis of randomized controlled trials. Rev Endocr Metab Disord. 2019.
7. Walsh JS, Bowles S, Evans AL. Vitamin D in obesity. Curr Opin Endocrinol Diabetes Obes. 2017;24(6):389-94.
8. Kimmons JE, Blanck HM, Tohill BC, Zhang J, Khan LK. Associations between body mass index and the prevalence of low micronutrient levels among US adults. MedGenMed. 2006;8(4):59.
9. Allin KH, Friedrich N, Pietzner M, Grarup N, Thuesen BH, Linneberg A, et al. Genetic determinants of serum vitamin B12 and their relation to body mass index. Eur J Epidemiol. 2017;32(2):125-34.
10. Degnan PH, Taga ME, Goodman AL. Vitamin B12 as a modulator of gut microbial ecology. Cell Metab. 2014;20(5):769-78.
11. Loos RJ, Yeo GS. The bigger picture of FTO: the first GWAS-identified obesity gene. Nat Rev Endocrinol. 2014;10(1):51-61.
12. Surendran S, Jayashri R, Drysdale L, Bodhini D, Lakshmipriya N, Shanthi Rani CS, et al. Evidence for the association between FTO gene variants and vitamin B12 concentrations in an Asian Indian population. Genes Nutr. 2019;14:26.
13. Qin X, Cui Y, Shen L, Sun N, Zhang Y, Li J, et al. Folic acid supplementation and cancer risk: a meta-analysis of randomized controlled trials. Int J Cancer. 2013;133(5):1033-41.
14. Kok DE, Dhonukshe-Rutten RA, Lute C, Heil SG, Uitterlinden AG, van der Velde N, et al. The effects of long-term daily folic acid and vitamin B12 supplementation on genome-wide DNA methylation in elderly subjects. Clin Epigenetics. 2015;7:121.

15. Brasky TM, White E, Chen CL. Long-Term, Supplemental, One-Carbon Metabolism-Related Vitamin B Use in Relation to Lung Cancer Risk in the Vitamins and Lifestyle (VITAL) Cohort. J Clin Oncol. 2017;35(30):3440-8.

16. Liu Y, Wang X, Sun X, Lu S, Liu S. Vitamin intake and pancreatic cancer risk reduction: A meta-analysis of observational studies. Medicine (Baltimore). 2018;97(13):e0114.

17. Arthur RS, Kirsh VA, Rohan TE. Dietary B-Vitamin Intake and Risk of Breast, Endometrial, Ovarian and Colorectal Cancer among Canadians. Nutr Cancer. 2019;71(7):1067-77.

18. Ma JL, Zhao Y, Guo CY, Hu HT, Zheng L, Zhao EJ, et al. Dietary vitamin B intake and the risk of esophageal cancer: a meta-analysis. Cancer Manag Res. 2018;10:5395-410.

19. Essen A, Santaolalla A, Garmo H, Hammar N, Walldius G, Jungner I, et al. Baseline serum folate, vitamin B12 and the risk of prostate and breast cancer using data from the Swedish AMORIS cohort. Cancer Causes Control. 2019;30(6):603-15.

20. Matejcic M, de Batlle J, Ricci C, Biessy C, Perrier F, Huybrechts I, et al. Biomarkers of folate and vitamin B12 and breast cancer risk: report from the EPIC cohort. Int J Cancer. 2017;140(6):1246-59.

21. Arendt JFH, Sorensen HT, Horsfall LJ, Petersen I. Elevated Vitamin B12 Levels and Cancer Risk in UK Primary Care: A THIN Database Cohort Study. Cancer Epidemiol Biomarkers Prev. 2019;28(4):814-21.

22. Fanidi A, Carreras-Torres R, Larose TL, Yuan JM, Stevens VL, Weinstein SJ, et al. Is high vitamin B12 status a cause of lung cancer? Int J Cancer. 2019;145(6):1499-503.

23. Chen QW, Zhu XY, Li YY, Meng ZQ. Epigenetic regulation and cancer (review). Oncol Rep. 2014;31(2):523-32.

24. Stone KL, Lui LY, Christen WG, Troen AM, Bauer DC, Kado D, et al. Effect of Combination Folic Acid, Vitamin B6 , and Vitamin B12 Supplementation on Fracture Risk in Women: A Randomized, Controlled Trial. J Bone Miner Res. 2017;32(12):2331-8.

25. Meyer HE, Willett WC, Fung TT, Holvik K, Feskanich D. Association of High Intakes of Vitamins B6 and B12 From Food and Supplements With Risk of Hip Fracture Among Postmenopausal Women in the Nurses' Health Study. JAMA Netw Open. 2019;2(5):e193591.

26. Chrysant SG, Chrysant GS. The current status of homocysteine as a risk factor for cardiovascular disease: a mini review. Expert Rev Cardiovasc Ther. 2018;16(8):559-65.

27. Spence JD, Yi Q, Hankey GJ. B vitamins in stroke prevention: time to reconsider. Lancet Neurol. 2017;16(9):750-60.

28. Barzilay JI, Davis BR, Pressel SL, Ghosh A, Puttnam R, Margolis KL, et al. The Impact of Antihypertensive Medications on Bone Mineral Density and Fracture Risk. Curr Cardiol Rep. 2017;19(9):76.

29. Solomon DH, Ruppert K, Zhao Z, Lian YJ, Kuo IH, Greendale GA, et al. Bone mineral density changes among women initiating blood pressure lowering drugs: a SWAN cohort study. Osteoporos Int. 2016;27(3):1181-9.

30. Shetty S, Kapoor N, Bondu JD, Thomas N, Paul TV. Bone turnover markers: Emerging tool in the management of osteoporosis. Indian J Endocrinol Metab. 2016;20(6):846-52.

31. Rejnmark L, Vestergaard P, Mosekilde L. Fracture risk in patients treated with loop diuretics. J Intern Med. 2006;259(1):117-24.

32. Mazziotti G, Canalis E, Giustina A. Drug-induced osteoporosis: mechanisms and clinical implications. Am J Med. 2010;123(10):877-84.

33. Rejnmark L, Vestergaard P, Heickendorff L, Andreasen F, Mosekilde L. Loop diuretics increase bone turnover and decrease BMD in osteopenic postmenopausal women: results from a randomized controlled study with bumetanide. J Bone Miner Res. 2006;21(1):163-70.

34. Project CU. DIet, nutrition, physical activity and colorectal cancer. World Cancer Research Fund American Institute for Cancer Research, 2018.

35. Charles P. Calcium absorption and calcium bioavailability. J Intern Med. 1992;231(2):161-8.

36. Moore EW. Ionized calcium in normal serum, ultrafiltrates, and whole blood determined by ion-exchange electrodes. J Clin Invest. 1970;49(2):318-34.

37. Farfariello V, Iamshanova O, Germain E, Fliniaux I, Prevarskaya N. Calcium homeostasis in cancer: A focus on senescence. Biochim Biophys Acta. 2015;1853(9):1974-9.

38. O'Seaghdha CM, Wu H, Yang Q, Kapur K, Guessous I, Zuber AM, et al. Meta-analysis of genome-wide association studies identifies six new Loci for serum calcium concentrations. PLoS Genet. 2013;9(9):e1003796.

39. van Meurs JB, Dhonukshe-Rutten RA, Pluijm SM, van der Klift M, de Jonge R, Lindemans J, et al. Homocysteine levels and the risk of osteoporotic fracture. N Engl J Med. 2004;350(20):2033-41.

40. Cashman KD. Homocysteine and osteoporotic fracture risk: a potential role for B vitamins. Nutr Rev. 2005;63(1):29-36.

41. Dhonukshe-Rutten RA, Pluijm SM, de Groot LC, Lips P, Smit JH, van Staveren WA. Homocysteine and vitamin B12 status relate to bone turnover markers, broadband ultrasound attenuation, and fractures in healthy elderly people. J Bone Miner Res. 2005;20(6):921-9.

42. Sato Y, Honda Y, Iwamoto J, Kanoko T, Satoh K. Effect of folate and mecobalamin on hip fractures in patients with stroke: a randomized controlled trial. Jama. 2005;293(9):1082-8.

43. Bauchner H, Fontanarosa PB. Expression of Concern: Sato et al. Effect of folate and mecobalamin on hip fractures in patients with stroke: a randomized controlled trial. JAMA. 2005;293(9):1082-1088. Jama. 2015;313(19):1914.

44. Bolland MJ, Avenell A, Gamble GD, Grey A. Systematic review and statistical analysis of the integrity of 33 randomized controlled trials. Neurology. 2016;87(23):2391-402.

45. Sarewitz D. The pressure to publish pushes down quality. Nature. 2016;533(7602):147.

46. Sharma H, Verma S. Is positive publication bias really a bias, or an intentionally created discrimination toward negative results? Saudi J Anaesth. 2019;13(4):352-5.

47. Hopewell S, Collins GS, Hirst A, Kirtley S, Tajar A, Gerry S, et al. Reporting characteristics of non-primary publications of results of randomized trials: a cross-sectional review. Trials. 2013;14:240.

48. Llewellyn-Bennett R, Bowman L, Bulbulia R. Post-trial follow-up methodology in large randomized controlled trials: a systematic review protocol. Syst Rev. 2016;5(1):214.

49. Sturgeon KM, Deng L, Bluethmann SM, Zhou S, Trifiletti DM, Jiang C, et al. A population-based study of cardiovascular disease mortality risk in US cancer patients. Eur Heart J. 2019.

50. White E, Patterson RE, Kristal AR, Thornquist M, King I, Shattuck AL, et al. VITamins And Lifestyle cohort study: study design and characteristics of supplement users. Am J Epidemiol. 2004;159(1):83-93.

51. Netherlands HCot. Dietary reference intakes: vitamin B6, folate and vitamin B12. The Hague: Health Council of the Netherlands, 2003: 2003.

52. Eussen SJ, de Groot LC, Clarke R, Schneede J, Ueland PM, Hoefnagels WH, et al. Oral cyanocobalamin supplementation in older people with vitamin B12 deficiency: a dose-finding trial. Arch Intern Med. 2005;165(10):1167-72.

53. van Wijngaarden JP, Dhonukshe-Rutten RAM, Brouwer-Brolsma EM, Enneman AW, Swart KMA, van Dijk SC, et al. Vitamin B12 Intake and Related Biomarkers: Associations in a Dutch Elderly Population. J Nutr Health Aging. 2017;21(10):1268-76.

54. Dullemeijer C, Souverein OW, Doets EL, van der Voet H, van Wijngaarden JP, de Boer WJ, et al. Systematic review with dose-response meta-analyses between vitamin B-12 intake and European Micronutrient Recommendations Aligned's prioritized biomarkers of vitamin B-12 including randomized controlled trials and observational studies in adults and elderly persons. Am J Clin Nutr. 2013;97(2):390-402.

55. Zeisel S. Choline, Other Methyl-Donors and Epigenetics. Nutrients. 2017;9(5).

56. Martinez Y, Li X, Liu G, Bin P, Yan W, Mas D, et al. The role of methionine on metabolism, oxidative stress, and diseases. Amino Acids. 2017;49(12):2091-8.

57. Shenkin A. Micronutrients in health and disease. Postgrad Med J. 2006;82(971):559-67.

58. Koehler KM, Pareo-Tubbeh SL, Romero LJ, Baumgartner RN, Garry PJ. Folate nutrition and older adults: challenges and opportunities. J Am Diet Assoc. 1997;97(2):167-73.

59. Green R. Vitamin B12 deficiency from the perspective of a practicing hematologist. Blood. 2017;129(19):2603-11.

60. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011;96(7):1911-30.

61. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab. 2011;96(1):53-8.

62. Raghavan R, Ashour FS, Bailey R. A Review of Cutoffs for Nutritional Biomarkers. Adv Nutr. 2016;7(1):112-20.

63. Bouillon R, Lips P, Bilezikian JP. Vitamin D supplementation and musculoskeletal health. Lancet Diabetes Endocrinol. 2019;7(2):85-6.

64. Agency EM. Guideline for good clinical practice E6(R2). UK: 2015 23 July 2015. Report No.

65. Thadhani R, Tonelli M. Cohort Studies: Marching Forward. Clinical Journal of the American Society of Nephrology. 2006;1(5):1117-23.

66. Davies NM, Holmes MV, Davey Smith G. Reading Mendelian randomisation studies: a guide, glossary, and checklist for clinicians. Bmj. 2018;362:k601.

67. Otles S, Senturk A. Food and drug interactions: a general review. Acta Sci Pol Technol Aliment. 2014;13(1):89-102.

68. Beaulieu MD, Dufresne L, LeBlanc D. Treating hypertension. Are the right drugs given to the right patients? Can Fam Physician. 1998;44:294-8, 301-2.

69. Sica DA, Gehr TWB, Frishman WH. Use of Diuretics in the Treatment of Heart Failure in Older Adults. Heart Fail Clin. 2017;13(3):503-12.

70. Heuberger RA, Caudell K. Polypharmacy and nutritional status in older adults: a cross-sectional study. Drugs Aging. 2011;28(4):315-23.

71. Schapiro M, Auslander MO, Schapiro MB. The electronic video endoscope: clinical experience with 1200 diagnostic and therapeutic cases in the community hospital. Gastrointest Endosc. 1987;33(2):63-8.

72. Sivak MV, Jr., Fleischer DE. Colonoscopy with a VideoEndoscope: preliminary experience. Gastrointest Endosc. 1984;30(1):1-5.

73. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435(7043):834-8.

74. Peng Y, Croce CM. The role of MicroRNAs in human cancer. Signal Transduct Target Ther. 2016;1:15004.

75. Mohammadi A, Mansoori B, Baradaran B. The role of microRNAs in colorectal cancer. Biomed Pharmacother. 2016;84:705-13.

76. Lumley J, Watson L, Watson M, Bower C. Periconceptional supplementation with folate and/or multivitamins for preventing neural tube defects. Cochrane Database Syst Rev. 2000(2):CD001056.

77. Wallace TC, Frankenfeld CL, Frei B, Shah AV, Yu CR, van Klinken BJ, et al. Multivitamin/Multimineral Supplement Use is Associated with Increased Micronutrient Intakes and Biomarkers and Decreased Prevalence of Inadequacies and Deficiencies in Middle-Aged and Older Adults in the United States. J Nutr Gerontol Geriatr. 2019;38(4):307-28.

78. ter Borg S, Verlaan S, Hemsworth J, Mijnarends DM, Schols JM, Luiking YC, et al. Micronutrient intakes and potential inadequacies of community-dwelling older adults: a systematic review. Br J Nutr. 2015;113(8):1195-206.

79. Laird EJ, O'Halloran AM, Carey D, O'Connor D, Kenny RA, Molloy AM. Voluntary fortification is ineffective to maintain the vitamin B12 and folate status of older Irish adults: evidence from the Irish Longitudinal Study on Ageing (TILDA). Br J Nutr. 2018;120(1):111-20.

80. Lips P, Eekhoff M, van Schoor N, Oosterwerff M, de Jongh R, Krul-Poel Y, et al. Vitamin D and type 2 diabetes. J Steroid Biochem Mol Biol. 2017;173:280-5.

81. Flores-Guerrero JL, Minovic I, Groothof D, Gruppen EG, Riphagen IJ, Kootstra-Ros J, et al. Association of Plasma Concentration of Vitamin B12 With All-Cause Mortality in the General Population in the Netherlands. JAMA Netw Open. 2020;3(1):e1919274.