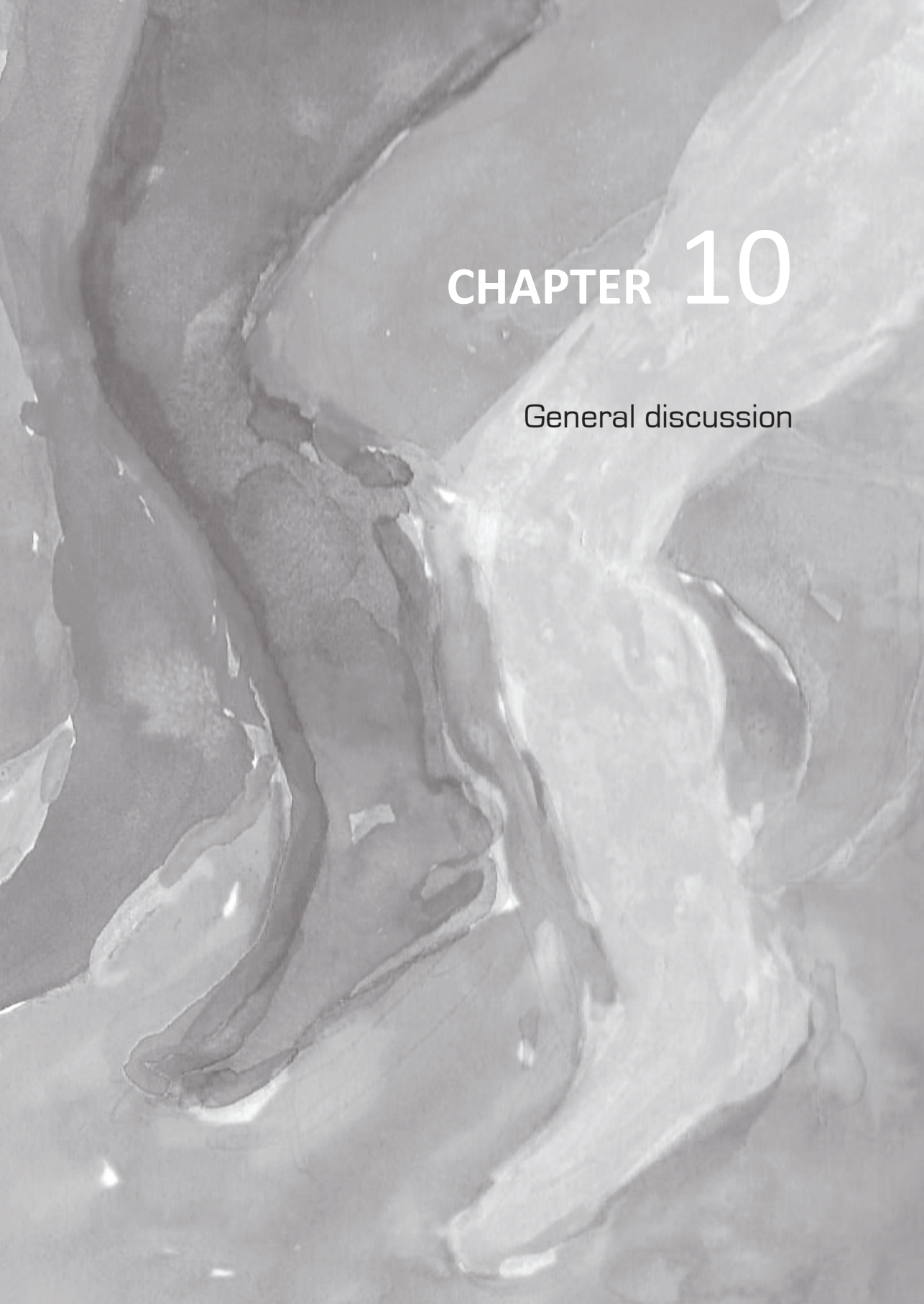

CHAPTER 10

General discussion

Introduction

This thesis aimed to examine the effect of homocysteine-lowering by vitamin B12 and folic acid supplementation on fracture incidence in older persons. Potential mechanisms at work were considered by 1) examining the association of homocysteine with different aspects of physical functioning and falling, and 2) by examining the effects of homocysteine-lowering therapy on those outcomes, as well as on bone characteristics, including bone mineral density and quantitative ultrasound parameters. In this final chapter, the results as described in this thesis will be reflected on, the methodological strengths and weaknesses will be discussed, and recommendations for future research and clinical and public health implications will be made.

Main findings and their interpretation

Table 1 describes the main findings of this thesis.

The effect of B-vitamin supplementation on osteoporotic fractures

In Chapter 7, 8, and 9 we reported the effects of homocysteine-lowering B-vitamin supplementation in elderly with mildly elevated homocysteine levels. The follow-up data from the B-PROOF study did not show an overall effect of supplementation of 500 µg vitamin B12 and 400 µg folic acid on the main outcome osteoporotic fracture incidence, compared to placebo. Results were similar for any type of fracture. However, among elderly persons >80 years who were compliant with the supplement, a reduced osteoporotic fracture incidence was observed in explorative analyses (Chapter 7).

When taking the results of previous intervention trials in consideration, the effect of B-vitamin supplementation on fractures remains inconclusive: in the total B-PROOF study sample the intervention was not effective concerning fracture prevention, and this finding was consistent with the results from the HOPE-2 and VITATOPS trials.[1,2] In contrast, evidence among Japanese stroke patients suggested a large preventative effect.[3] The differences in findings between the trials might be explained by differences in the study populations. Baseline homocysteine levels, for instance, might be an important factor. In the Japanese stroke study, median baseline homocysteine levels were substantially higher compared to the levels in the other trials, probably due to the inclusion of stroke patients who were aged >65 years. This is supported by the observed effects in the B-PROOF study among persons >80 years in the intervention group who were compliant with the intervention. Among those persons, baseline homocysteine levels were significantly higher than among persons <80 years. Moreover, in this subgroup, homocysteine levels in the

intervention group appeared to decrease more compared with the decrease in persons <80 years, especially when taking into account the changes over time as shown by the placebo group. If reducing homocysteine is an important mechanism, we would have expected larger reductions in fracture risk in subjects with higher baseline homocysteine levels. However, indications for such effects were not found, as the interaction of treatment with homocysteine levels was not significant. It should be noted that we did not examine the interaction of treatment with change in homocysteine, and that the effect could have been missed in case of a homocysteine threshold.

It should be noted that we widened the age and homocysteine ranges for the B-PROOF inclusion criteria during the recruitment period to ensure a sufficient number of participants. Originally, the age criterion was ≥70 years and the homocysteine criterion was ≥15 µmol/L.[4] Extended data analyses from observational studies suggested that the association between homocysteine and fractures was also present in the age category 65-70 years, and at lower homocysteine levels.[4,5] Therefore, the criteria were adjusted to ≥65 years and ≥12 µmol/L respectively. In retrospect, this decision might have affected our results. First, if, as speculated, homocysteine is indeed an important factor for the effect of the trial, lowering the cut-off of the criteria for both age and baseline homocysteine levels might have resulted in a reduced treatment effect. Second, the incidence of fracture among persons 65-70 years is lower than among persons >70 years.[6] We may had to have adjusted our sample size calculation accordingly and recruited more participants, but instead we maintained our target of 1,500 participants per treatment arm. A new sample size calculation, based on an incidence of 5% and a fracture risk reduction of 34%, results in 1,775 participants per treatment group. As could be expected, the total number of fractures was indeed somewhat lower than anticipated in the original sample calculation (observed rate of 5.1% in the placebo group vs expected rate of 6%).

Another power issue concerns the expected fracture risk reduction of 34%. This estimation of the risk reduction was based on 1) observational studies among free-living elderly people, that showed large relative risks (>2) for fractures in subjects with a high homocysteine level,[5] and 2) studies that showed an homocysteine lowering of approximately 30% due to B-vitamin supplementation.[7,8] Although the Japanese stroke study demonstrated a risk reduction of 75%,[3] such high-risk reductions might be unrealistic. In comparison to other nutritional interventions, a meta-analysis of vitamin D and calcium supplementation studies showed a risk reduction to fracture of 12% in older adults.[9] It may be concluded that our study was somewhat underpowered to obtain an effect on (osteoporotic) fractures.

The intervention study provided indications for a beneficial effect on osteoporotic fractures among persons >80 years. When secondary outcomes were considered, similar age-effects were found: with respect to physical performance, no overall effects were observed, but among compliant persons >80 years, evidence for a borderline significant

Table 1 Main findings of this thesis.

Chapter	Study	Study design	Determinant/ intervention	Outcome	Results
2	B-PROOF	Study protocol	-	-	-
3	LASA	Cross-sectional and longitudinal cohort study	Plasma Hcy, serum vitamin B12	Physical performance	Higher Hcy levels were associated with lower physical performance score (β=-0.93 for Q4 vs Q1) and decline in performance (β=-0.69 for Q4 vs Q1) in women. In men and for vitamin B12, the associations were less consistent.
4	LASA	Cross-sectional and longitudinal cohort study	Plasma Hcy	Muscle mass, handgrip strength, functional limitations, falling	In men, higher Hcy levels were associated with lower handgrip strength (β=-3.07 for Q4 vs Q1), and more functional limitations at baseline (β=1.15 for Q4 vs Q1). In women, higher homocysteine levels were associated with more functional limitations after 3 years (β=1.19 for Q4 vs Q1). No associations were observed for muscle mass and falling.
5	B-PROOF	Cross-sectional cohort study	Plasma Hcy, MTHFR polymorphisms	Physical performance, muscle mass, handgrip strength, postural sway	Higher Hcy levels were associated with lower handgrip strength (β=-1.14 for Q4 vs Q1) and physical performance score (Q3 vs Q1: β=-0.53 for Q3 vs Q1 and β=-0.94 for Q4 vs Q1) in women only. High plasma Hcy in the 677CC and 677CT genotypes, but not in the 677TT genotype, was associated with lower physical performance.
6	B-PROOF	Cross-sectional cohort study	Serum folate	Serum CRP	A significant U-shaped association was observed between serum folate and CRP in the total study sample, with optimal serum folate concentrations around 20 nmol/L. In elderly with CVD, higher folate concentrations were significantly associated with higher CRP concentrations (β=0.05). Below a threshold of approximately 17 nmol/L, a borderline significant inverse association between folate and CRP was observed in elderly without CVD (β=-0.12).

7	B-PROOF	RCT	500 µg vitamin B12, 400 µg folid acid, and 600 IU vitamin D3 vs 600 IU vitamin D3	Osteoporotic fractures, any type of fractures, cancer	Osteoporotic fracture risk was not significantly different between treatment groups (HR=0.84). Moreover, any type of fracture risk was not significantly different (HR=0.83). For persons >80 years, in the per-protocol analyses, the HR was 0.28. Supplementation was associated with an increased cancer risk (HR=1.55), that was more pronounced in persons >80 years (HR=3.68) and women (HR=2.34).
8	B-PROOF	RCT	500 µg vitamin B12, 400 µg folid acid, and 600 IU vitamin D3 vs 600 IU vitamin D3	Physical performance, handgrip strength, falling	The 2-yr decline in physical performance and handgrip strength did not differ between treatment groups. Time to first fall and the number of falls per participant were not significantly different (HR=1.0, and OR= 1.0, respectively). Per-protocol analyses identified a borderline significant beneficial effect of the treatment on physical performance in persons >80 years (difference in decline between groups=0.6).
9	B-PROOF	RCT	500 µg vitamin B12, 400 µg folid acid, and 600 IU vitamin D3 vs 600 IU vitamin D3	BMD, quanitative ultrasound	No statistically significant differences between the treatment groups were observed for follow-up FN-BMD, LS-BMD, SOS, and BUA. Per-protocol analyses revealed a small positive effect of the intervention on BUA at follow-up among persons >80 years (estimated marginal mean 64.4dB/MHz for the intervention group and 61.0 dB/MHz for the placebo group, p=0.04 for difference).

BMD= bone mineral density, BUA= broadband ultrasound attenuation, CRP= C-reactive protein, CVD= cardiovascular disease, FN-BMD= femoral neck bone mineral density, Hcy= homocysteine, HR= hazard ratio, LS-BMD= lumbar spine bone mineral density, MTHFR= methylenetetrahydrofolate reductase, OR= odds ratio, Q= quartile, RCT= randomized controlled trial, SOS= speed of sound.

beneficial effect was found that could be clinically relevant (Chapter 8). Similarly, a small beneficial effect on broadband ultrasound attenuation (BUA) was observed in the intervention group compared to placebo among compliant persons >80 years, while no effect was found in the total study sample (Chapter 9). Physical performance and BUA could theoretically be intermediates in the pathway of homocysteine and fractures in compliant persons >80 years. For physical performance, we explored this hypothesis by adding physical performance to the fracture model in compliant persons >80 years. After this adjustment, the hazard ratio for fractures did not change, indicating that physical performance cannot be considered a significant mediator. The number of fractures among participants with BUA data was insufficient to explore BUA as mediator. The intervention had neither an effect on handgrip strength and falling (Chapter 8), nor on bone mineral density (BMD) and speed of sound (SOS) (Chapter 9). Hence, those factors are unable to explain the observed treatment effect among persons >80 years.

Age effects of B-vitamin supplementation

Although only a small number of persons >80 years (N=398) were included, and the result among this subgroup should therefore be regarded as explorative, the consistency in age effects across the outcomes is intriguing and merits further elaboration. The observation that the lowering of homocysteine in the >80 years subgroup appeared to be more pronounced, might (partly) explain the findings. This is supported by the results obtained in the per-protocol compared to the intention-to-treat analyses. Moreover, the longitudinal decline after 2 years in the outcomes in persons >80 years was larger than in persons <80 years: in the placebo group the decline was -9.5% and -6.8% in persons >80 years vs +0.5% and -2.9% in persons <80 years for respectively physical performance and BUA, despite lower baseline levels in persons >80 years. With respect to fractures, the a priori risk to fracture is higher among persons >80 years.[6] The scope to improve among persons >80 years, and the absence of decline among persons <80 years might account for the observed age effects.

In this context, the distinction between chronological and biological age seems important. Because tissues age at different rates and because diseases vary among individuals, there is a large variability of aging.[10] The biological age estimates the functional status of an individual in reference to chronological peers on the basis of how well he or she functions in comparison with others of the same chronological age,[11] and is therefore a better indicator of the aging process than chronological age. Biological age may serve as an indicator of an individual's general health status, remaining healthy life span, and active life expectancy.[10] Several biomarkers of biological aging that have been proposed include changes in telomere length,[11-13] cross-linking of collagen,[14] glycosylated hemoglobin,[15] pulse wave velocity,[16] as well as sarcopenia.[17] Biological age was not taken into account in this

thesis, but the interaction of the B-vitamin treatment with biological age might be stronger than with chronological age.

Alternatively, the explanation might be more complex. In late-life, risk factors for health and disease might differ from risk factors in mid-life. For instance, among the oldest old, classical risk factors for cardiovascular mortality have been shown to loose predictive value.[18] Also the role of hypertension in the development of dementia is equivocal: in mid-life, hypertension is associated with an increased risk of cognitive decline and dementia, whereas in late-life hypertension it is suggested to be beneficial.[19] In analogy, risk factors for other health related outcomes might change as well in the oldest old, and therefore, the effect of interventions might be different among those persons. For instance, it could be that homocysteine is a causal factor for fractures in the oldest old, but not in younger persons.

Homocysteine in relation to physical function: Risk factor or risk marker?

Before the start of this thesis, limited evidence was available of the association between homocysteine and physical function.[20-22] Using observational data from two different cohorts, the body of evidence was further strengthened. In Chapter 3, 4, and 5 we described the association of homocysteine with several aspects of physical functioning in older persons. Cross-sectional and longitudinal data from the LASA study revealed that higher homocysteine levels were associated with lower physical performance scores and decline in physical performance in women (Chapter 3). The association was observed with chair stands and walking test as indicators of physical functioning, but not with balance. The associations in men and with vitamin B12 were less consistent, and an interaction effect with age was not observed (Chapter 3). When studying other aspects of physical functioning within the LASA (Chapter 4), we observed that higher homocysteine levels were associated with lower handgrip strength in men, and more functional limitations in both men and women. We did not observe associations of homocysteine with muscle mass, or falling (Chapter 4). The inverse association of homocysteine with different indicators of physical function in certain groups was also observed in data from the B-PROOF study (Chapter 5). Again, the association with physical performance was only observed in women. With respect to handgrip strength, in contrast to the LASA findings, a significant association was observed in woman only (Chapter 5). Associations of homocysteine with muscle mass and postural sway were not observed (Chapter 5).

For physical performance, the findings within the LASA and the B-PROOF study were consistent regarding gender differences, that is, an association was observed in women only. In both studies we had the possibility to control for confounding factors. Other observational studies also showed an inverse association between homocysteine and performance measures, including gait speed, but most studies did not differentiate

between men and women.[20-23] The study of Kuo et al. (2007) demonstrated an association between higher homocysteine concentrations and lower gait speed, and observed that gender did not modify this association.[21] No clear explanation exists for the gender differences as observed in this thesis.

In the same study of Kuo et al. (2007), the homocysteine-gait speed association could be largely explained by a lower quadriceps strength.[21] The suggested role of muscle strength was not consistent in our data with regard to handgrip strength. Handgrip strength is a good indicator of overall strength; it has been positively correlated with both upper-body and lower-extremity strength in older persons.[24,25]

Long-term stable homocysteine levels, as reflected by the MTHFR polymorphisms, were not associated with physical performance score or handgrip strength (Chapter 5). This was an indication that homocysteine might be non-causally related to these outcomes. It should be noted that homocysteine is also influenced by other genetic variants. A genetic risk score for hyperhomocysteinemia has been described recently.[26] The use of this risk score could result in a more accurate prediction.

More evidence for (non)causality was derived from the study described in Chapter 8 evaluating the effects of the intervention. We did not observe an effect of vitamin B12 and folic acid supplementation on handgrip strength. Concerning physical performance, one previous trial among older men and women did not report an effect of 500 µg vitamin B12, 800 µg folic acid, and 3 mg vitamin B6 vs placebo on movement performance, but the intervention period was only 4 months.[27] Our results suggest that the intervention might be beneficial in the oldest old, but this hypothesis warrants further investigation (Chapter 8).

All in all, inconsistent gender associations, the negative MTHFR polymorphism findings, as well as the lack of treatment effects suggest that homocysteine should be considered as marker for handgrip strength in older persons rather than a causal factor. For physical performance, the results remain inconclusive.

Potential risks of folic acid supplementation

Several detrimental effects of folic acid supplements are known or have become apparent in the B-PROOF study. First, previous studies showed that positive effects of folic acid might have indirect negative effects on health: high intake of folic acid might mask the symptoms of vitamin B12 deficiency, e.g. megaloblastic anemia, and might thereby delay the diagnosis of vitamin B12 deficiency which could lead to irreversible neurodegenerative changes.[28] For this reason, folic acid doses were relatively low, and combined with vitamin B12 supplementation in the B-PROOF study.

Second, we observed that higher levels of circulating folate were associated with high levels of inflammation, particularly in a pro-inflammatory state, such as occurs in patients with cardiovascular disease at a relatively high age (Chapter 6). The observed association

between folate and CRP might provide an explanation for the disappointing effects from previous B-vitamin supplementation trials on cardiovascular endpoints or inflammation,[29,30] which does not necessarily destabilize the homocysteine hypothesis in cardiovascular disease: B-vitamin supplementation, particularly folic acid, may stimulate low-grade inflammation independent of their homocysteine lowering effects.[31] This cross-sectional study should be regarded as hypothesis generating, and warrants further investigation.

Third, we unexpectedly observed a higher cancer incidence after supplementation with folic acid and vitamin B12 compared to placebo (Chapter 7). The difference in cancer incidence was observed in the total B-PROOF study sample, but was more pronounced in persons >80 years and in women. Although this finding only represents a small part of this thesis, the potential public health implications justify elaborating on this finding.

Adverse event: cancer

Because of the double-blind study design, we did not notice the difference in the number of cancer diagnoses between the intervention and placebo group during the study period. Adverse events were reported and documented, but the total number of cancer cases was consistent with our expectations: the incidence in the Dutch population was 3.9% in 2010/2011 (skin cancer excluded),[32] compared with 3.6% in the total B-PROOF sample. Only after the randomization codes were revealed, the difference between treatment groups became apparent. Earlier studies (individual studies as well as a meta- analyses) with higher doses of folic acid supplement use (0.5 to 40 mg) than applied in the B-PROOF study did not show evidence for a higher cancer risk associated with such supplementation, although the results were close to statistical significance.[33-35] Further analyses on the duration of treatment (2 to 7 years), or dose of folic acid (0.5 to 40 mg) did not indicate a trend in effects of folic acid on overall cancer incidence in one meta-analysis,[33] and the existence of prior adenoma did not increase the cancer risk.[33] In addition, because the applied doses in the B-PROOF study were even lower than the lowest dose that was previously used, the observed effect on cancer was very unexpected. Given the earlier studies and meta analyses, the most likely explanation for our results regarding cancer risk, may be a chance finding. Statistically, with the generally applied p-value of 0.05, 1 out of 20 studies will report a false positive result. Therefore, a false positive result in the B-PROOF study cannot be ruled out.

When the B-PROOF data were considered in even more detail, we found some indications that may support this suggestion for chance finding. Next to an interaction effect of treatment with age and sex, we observed a post-hoc interaction effect of study location. The cancer risk in the intervention group compared to placebo was higher for subjects located in Amsterdam and surroundings as compared to participants from Rotterdam or Wageningen. More women and persons >80 years were recruited in Amsterdam, which may explain this interaction. Some inconsistencies in cancer rates across study locations were

observed that might also point to chance (Table 2). In Wageningen, the cancer rates in both treatment groups were relatively low, which might be explained by the younger study sample. The cancer rates in Amsterdam and Rotterdam were similar in the intervention group, but interestingly, the cancer rate in placebo group was lower in Amsterdam than in Rotterdam. This suggests a preventative effect in the placebo group rather than a detrimental effect in the intervention group in the Amsterdam sample.

Table 2 Cancer rates per study location according to treatment group as derived from intention-to-treat analyses.

Study location	Placebo group		Intervention group	
	N of cases	Rate/100 person- years	N of cases	Rate/100 person- years
Wageningen (n=854)	8	0.8	13	1.4
Amsterdam (n=774)	8	1.0	19	2.4
Rotterdam (n=1,278)	26	2.0	31	2.4

Nevertheless, some indicative findings from epidemiological and pathophysiological studies suggest that the cancer risk increasing effect of vitamin B supplementation may be plausible. Two joint trials with prolonged follow-up showed that supplementation with folic acid and vitamin B12 had adverse effects on cancer,[36] although their study populations and doses were different from ours. The fact that the observed effects were somewhat stronger in the per-protocol analyses than in the intention-to-treat analyses is also indicative for a true effect.

It is suggested that folic acid may protect against the initiation of cancer, while it may enhance growth and progression of established neoplastic cells.[37] The fact that the difference in probability of cancer diagnosis between both treatment groups of the B-PROOF study appeared already shortly after the start of the intervention (Chapter 7) supports this hypothesis; new tumors do not develop in such a short time period. The B-PROOF study population is older than the examined populations in previous studies and meta-analyses. As cancer risk increases with age,[32] it is likely that more latent tumors are present in older adults as compared to younger adults, and therefore folic acid-induced tumor growth is more likely to occur in older adults than in younger adults. As a result, cancer growth could have been missed in the earlier studies among younger study populations.

In summary, the role of folate in cancer prevention and/or development is complex and inconclusive. Its role might depend on timing and dose of folate supplementation during carcinogenesis,[38,39] and further research is necessary, especially in the oldest-old.

Vitamin D supplementation

Both the B-PROOF intervention and placebo tablet contained 600 IU vitamin D. The rationale for the addition of vitamin D to the tablets was to ensure normal vitamin D status. International consensus regarding the optimal serum 25-hydroxyvitamin D level is lacking, and hence different cut-offs for vitamin D deficiency have been used in the literature. In general, a serum level <50 nmol/L is considered as deficient or insufficient.[40-42] Within the LASA sample, 48% of the persons >65 years had serum 25-hydroxyvitamin D levels <50 nmol/L.[43] In the B-PROOF study, 45% of the participants had levels <50 nmol/L.

Although the addition of vitamin D is justified and ethical in a sample of older persons among whom the prevalence of vitamin D deficiency is high, it complicates the interpretation of the results. Vitamin D has been linked to most of the outcomes that were examined in this thesis, and potential effects of vitamin D supplementation on the outcomes may have masked possible effects of vitamin B12 and folic acid. Findings from randomized controlled trials and subsequent meta-analyses demonstrated a reduction in hip fractures after vitamin D supplementation with co-administration of calcium. However, vitamin D without calcium did not have a preventative effect on fractures as indicated by meta-analyses,[44,45] although some individual studies indicated a positive effect.[46] A recent meta-analysis of vitamin D supplementation on BMD reported a small but significant increase in BMD of the femoral neck, but not of the total hip.[47] The authors concluded that such a localized effect could be artificial. In other meta-analyses, positive effects of vitamin D supplementation have been demonstrated on muscle function,[48] as well as on the risk of falling and rate of falls among elderly persons.[49] There is no evidence of any beneficial or adverse effect of vitamin D supplementation on cancer,[50] despite observed inverse associations of plasma levels of 25-hydroxyvitamin D with cancer.

Methodological considerations

Some methodological strengths and weaknesses should be taken into account in the interpretation of the results that are described in this thesis. The most important issues are described in this section.

Study designs

The use of the population-based LASA data in the association studies has major strengths. The longitudinal design of the LASA provided the opportunity to study changes over time, with the certainty that the exposure preceded the outcome. In our studies we applied follow-up durations of 3 years. Moreover, the data allowed us to include multiple potential confounders and effect modifiers.

The randomized placebo-controlled study design, such as the B-PROOF design, is regarded as the gold standard for clinical trials. Randomization minimizes selection bias and allocation bias, thereby balancing both known and unknown prognostic factors. Stratified randomization was applied to ensure good balance of the participant characteristics in both groups. Furthermore, all participants were blinded, as well as those assessing outcomes, and those performing the analyses. According to the nine-item Delphi list for quality assessment of a randomized controlled trial,[51] the B-PROOF trial appears to be a high quality randomized controlled trial. The possible issues regarding the age range of the study population and related power issues were already addressed in this General discussion.

Populations, sampling and response

LASA data were collected in a population-based sample, and thereby the external validity of the findings is high. Men and the oldest participants were oversampled to ensure that there would be reasonable numbers of very old men, even after long periods of follow-up.

In the B-PROOF study, participants were screened for elevated homocysteine levels. The limited range of homocysteine in the B-PROOF study might have reduced the power to show associations, and subsequently could also have led to an underestimation of the associations in the general population of older persons. Elevated homocysteine levels have been linked to several adverse health outcomes (cardiovascular disease, cognitive decline, osteoporosis), although causality has not yet been proven. On the one hand, it is therefore likely that the B-PROOF sample might be less healthy than the general population of older persons. On the other hand, persons who are willing to participate in randomized controlled trials are in general more healthy.

In cohort studies of older persons such as the LASA and the B-PROOF study, selective non-response and loss to follow-up among the more frail and unhealthy persons is an inevitable problem. Attrition in the LASA can be attributed for the largest part to mortality and to lesser extent to refusal, or other reasons.[52] Even though attrition due to mortality does not necessarily influence the representativeness of the sample because high mortality is characteristic of older populations, attrition due to mortality is related to specific sample characteristics. In the B-PROOF study, the main reason for attrition was that the intervention was considered too burdensome and therefore persons were no longer capable to participate. Attrition might have led to an underrepresentation of older persons with lower muscle mass and muscle strength, lower physical performance, and higher homocysteine concentrations, which could have resulted in an underestimation of the observed associations, or may have contributed to the non-significance of some associations.

In both studies, Dual-energy x-ray (DXA) assessments were performed in a subsample to measure BMD and body composition. Since persons had to be able to visit the study center, the subsample was not fully representative of the complete study population. The

somewhat selective sample does not only underestimate the examined associations, but also hampers definite conclusions about the absence of an effect of B-vitamin supplementation on BMD in persons >80 years (Chapter 9).

Measures

In the B-PROOF study, fractures were self-reported on a research calendar, the calendar data were verified at the follow-up interview, and all fractures were subsequently validated with the general practitioner or hospital. All fractures were considered osteoporotic, except for head, hand, finger, foot or toe fractures, fractures caused by traffic accidents, and fractures caused by cancer. It could be argued whether this is the best definition, and whether the distinction between osteoporotic fractures and non-osteoporotic fractures is even relevant. However, we did not observe different treatment effects for osteoporotic fractures and fractures of any kind.

Physical performance was measured with three objective tests: a walk test, a chair test, and a tandem stand. It should be noted that the limited responsiveness to detect a change in physical performance was a problem in our study: in the B-PROOF study, a ceiling effect was observed for the tandem test, since 67% of the participants were able to hold position for at least 10s at baseline. In comparison, in the LASA 1995 data collection wave this was even 76% of the participants.

Because of its power to reflect current and future health and functioning, physical performance measures are frequently used by clinicians and researchers to evaluate treatments, interventions, and associations. The observed changes might reflect measurement error instead of an actual change, or a real change that might not be clinically relevant. Therefore, it is important to know the clinical relevance of a change. Two studies on the minimal clinically important change (MCIC) of physical performance in older persons have been conducted.[53,54] In the first study, the meaningful change of different physical performance measures, including the Short Physical Performance Battery (SPPB), was determined.[54] A change score of -0.99 to -1.34 of the SPPB was considered to be clinically important in that study. In a second study a clinically important SPPB change score of -0.4 to -1.5 points was found.[53]

Different methods to establish the MCIC have been developed and evaluated.[55] In the anchor-based methods, an anchor, or external criterion, is used to assess what patients or their clinicians consider as an important change. The distribution based methods use distributional properties of a sample, for instance the standard errors of measurement. The combination of the two methods, the anchor-based MIC distribution method, is thought to overcome some of the limitations of both methods: e.g. the anchor-based methods fail to take into account the variability of the instrument, and/or the sample, whereas the distribution-based methods do not provide a good indication of the importance of the

observed change in themselves.[56] For the MCIC of physical performance, results derived from the integrated method of anchor-based MIC distribution are lacking.

We attempted to determine the MCIC of physical performance summary score (range 0-12) in the B-PROOF sample, using the anchor-based MIC distribution approach. In order to do so, we constructed an anchor that was hold against the distribution of the actual 2-year change in physical performance score. Unfortunately, we failed to establish the MCIC. The distributions of the performance change scores overlapped to a great extent between persons who judged themselves as being decreased in physical performance and persons who judged themselves as not being decreased (Figure 1).

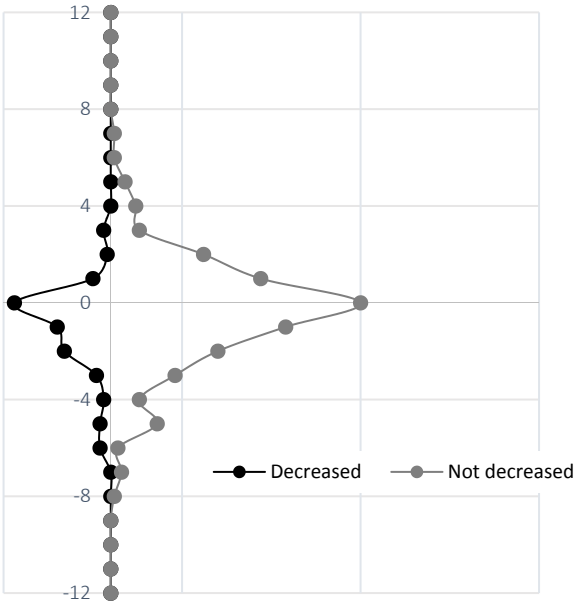

Figure 1 Distribution of physical performance change scores per anchor category (decreased versus not decreased).

In the evaluation of the results, it was noted that the anchor correlated better with the performance level itself (r: -0.53) than with the change score (r: -0.11). A 2-year period might be too long to recall, especially in a population of older persons. In addition, the lack of correlation with the change score can be explained by the lack of variation in the change score. Moreover, self-report and performance tests represent different constructs.[57-59] The development of an accurate MCIC deserves further attention.

Statistical analyses

The effects of the B-PROOF study were analyzed by both intention-to-treat and per-protocol analyses. Drop-outs might be treatment-dependent and thus selective, and could bias the results. Intention-to-treat analyses were done to avoid these bias effects of selective drop-outs. It provided information about the policy of the treatment. In contrast, per-protocol analyses were restricted to participants who fulfilled the protocol, and thereby provided information about biological effect.

In most chapters of this thesis, subgroup analyses are described. Multiple testing increases the risk of false positive findings.[60,61] In our studies, subgroup analyses were restricted to a limited number, they were pre-specified in the analysis plan, and applied when the interaction term reached significance.

Multiple imputation of missing data was used the analyses of Chapter 4. Compared to the complete case analyses as applied in the analyses of the other chapters, multiple imputation makes use of all available data, and accounts for the uncertainty within the imputed data. It is therefore more efficient and increases the power to show an association.

In the analyses of Chapter 8 we used ANCOVA to examine the effect of the B-PROOF intervention on a continuous outcome (BMD and quantitative ultrasound parameters), whereas in the analyses of Chapter 9 linear mixed models were applied (physical performance and handgrip strength). An important feature of linear mixed models, necessary for longitudinal analyses, is that the dependency of repeated observations within subjects is taken into account. Since only one follow-up measurement was performed, this was accomplished in ANCOVA by adjusting for baseline values. In contrast, subjects are included regardless of missing values in the linear mixed models only. Thus, subjects who are lost to follow-up were also included in these analyses. When the methods were exchanged, the results did not change.

Implications for future research

The studies described in this thesis contribute to the body of evidence regarding therapeutic homocysteine-lowering on fractures, but also suggest new important research questions, and hereafter I describe some suggestions for further research.

Additional research is necessary on the role of homocysteine and B-vitamins in bone. First, to gain more insight in the effect of our intervention on bone health, the performed analyses can be extended to other secondary endpoints. Within the B-PROOF study, results are awaited regarding the effect of B-vitamin supplementation on bone turnover markers, trabecular bone score, and vertebral fractures. Second, because the lack of a significant effect on fractures might be the result of a lack of power, a meta-analysis on B-vitamin supplementation and fractures would be useful, preferably with individual patient data. And third, as the effect of B-vitamin supplementation on fractures remains inconclusive so far,

Mendelian randomization will be a valuable alternative approach that might contribute to our understanding. Although Mendelian randomization also knows some limitations, its starting point is that the influence of confounding factors is minimized by the random segregation of alleles, and therefore allows for a randomized comparison.[62] In case a genotype induces differences in phenotype that are related to disease risk, the genotype can be used to create natural randomization.[62] This approach has been applied, for instance, for the MTHFR gene (genotype), homocysteine levels (phenoptype), and coronary heart disease (disease).[63] To date, no studies have been published that used such study design for fractures or BMD.

Intervention trials require considerable time and expense. If new B-vitamin intervention trials are intended in the future, special attention for adverse effects would be warranted. Although many participants would be needed, a 2x2 factorial design with folic acid and vitamin B12 would be appropriate to examine the individual contribution of vitamin B12 and folic acid supplementation, as well as their interaction on the outcomes. Regarding in- and exclusion criteria, it would be useful to include a more specific population. The B-PROOF study included persons ≥65 years and with homocysteine levels ≥12 µmol/L, thereby including approximately 50% of the persons initially screened for inclusion. More specific populations might benefit more from the intervention, for instance persons with a higher age, or persons with higher homocysteine levels.

In line with this, more research is needed to determine whether the described age effects can be attributed to B-vitamin supplementation. To understand what is happening in this age group, data from existing cohort studies and intervention studies can be used to study effect modification by age in association and intervention studies. To differentiate between chronological and biological age would be very interesting as well, and this could be done within the B-PROOF study. Also, future (updates of) meta-analyses should address potential age effects.

In the B-PROOF study, we observed both beneficial and detrimental effects of B-vitamin supplementation. The lag effects of the intervention should be studied to determine whether the effects persist after the intervention period. By studying the effect of the termination of supplement use, additional information will be provided about whether the effects can be attributed to B-vitamin use. In addition, future research should provide a harm-benefit analysis in order to determine the net effect of B-vitamin supplementation on health, to establish recommendations for clinical practice, and to estimate public health implications. The scope of this future analysis should not be limited to the outcomes as described in this thesis, i.e. bone health and cancer, but also include effects on cerebro- and cardiovascular health and cognitive functioning. An economic evaluation is also necessary.

Next to future studies on the net effect on health and costs, studies on the perception of the elderly are important as well. We got the impression that the B-PROOF participants were more concerned about a future hip fracture or stroke than about a cancer diagnosis.

The presumed effect of these outcomes on quality of life and independence (e.g. nursing home admission) as intuitively seen by individuals might play a critical role. Indeed, different diseases have been shown to have a different impact on quality of life.[64] The perception of the elderly in this respect needs to be subject of future studies.

Clinical and public health implications

Because of the observed beneficial and detrimental effects, conflicting findings in the literature and the subsequent need for further research, vitamin B12 and folic acid supplementation for older persons in general cannot and should not be recommended at this moment. Patients who receive folic acid or vitamin B12 on prescription on clinical grounds, for instance patients with anemia, or patients with rheumatoid arthritis or cancer using methotrexate, should continue to use the supplements in consultation with their physician.

Supplements that contain vitamin B12 and/or folic acid, such as multivitamins, are widely used among older persons. In the B-PROOF study, 16% of the participants used supplements at baseline that contained B-vitamins (median folic acid dose 200 µg/d, median vitamin B12 dose 1.1 µg/d), both prescribed or non-prescribed, and 37% of the participants of the LASA in the 2009 data collection wave used any vitamin supplement. Baseline data from the B-PROOF study indicated that only a small percentage of the participants was deficient in folate or vitamin B12, despite mildly elevated homocysteine levels: 3% was folate deficient (concentration <10 nmol/L) and 4% was vitamin B12 deficient (holotranscobalamin <32 pmol/L and methylmalonic acid >0.45 µmol/L). Deficient levels should be treated, because vitamin B12 is essential for the development and myelination of the central nervous system and maintenance of its normal function, and folate plays a crucial role in the one-carbon metabolism, so that many critical cellular pathways depend on folate. However, people often believe that 'there is no harm in trying'. This might not be applicable to B-vitamin use, as suggested by our B-PROOF findings on cancer. The findings from future research on the potential risks of B-vitamin use in relation to its benefits will hence be very relevant.

In many countries, among which the United States and Canada, mandatory fortification of bread and/or flour with folic acid is applied. This has led to a sharp decrease in the number of neural tube defects.[65-67] In 2008, the debate as to whether folic acid should be added to bread took place in the Netherlands as well.[68] As most women in the Netherlands plan their pregnancy and the implementation of folic acid supplements among these women was successful, it was decided not to mandatory fortify bread with folic acid. Currently in the Netherlands, folic acid is added to some foods, for instance some kinds of margarines, breakfast cereals, and juice drinks. The findings from the B-PROOF study are not likely to change the policy on food fortification around the world. But if future research

will confirm the finding that high folic acid intake may be harmful for some people, such as persons with cancer or subclinical cancer, public health policies should then be reconsidered.

The dose of folic acid provided in the B-PROOF study (400 µg) was relatively low and well below the tolerable upper intake level for folic acid that has been established at 1 mg per day in Europe.[69] In the Netherlands, the recommendation for folic acid as formulated by the Health Council of the Netherlands is 300 µg.[68] Although we only studied a dose of 400 µg, our applied dose was close to the recommended level of intake.

Conclusion

To conclude this thesis, our B-PROOF trial suggests that there is no effect of vitamin B12 and folic acid supplementation on osteoporotic fractures among elderly with mildly elevated homocysteine levels. Some evidence for a beneficial effect on fracture incidence was observed in a subgroup of compliant persons >80 years. In additional observational studies, we observed an inverse association of plasma homocysteine levels with physical performance in women, but B-vitamin supplementation did not have an effect on physical performance in the total study sample. A small beneficial effect on bone elasticity (ultrasound attenuation) was observed in the intervention group compared to placebo among compliant persons >80 years, while no effect was found in the total study sample. The intervention had no effect on handgrip strength, falling, bone mineral density, or speed of sound. A higher incidence of cancer was reported in the intervention group, which was most pronounced in persons >80 years. Future research should confirm our findings and balance the possible benefits of B-vitamin supplementation in older persons against the possible harms.

References

1 Sawka AM, Ray JG, Yi Q, Josse RG, Lonn E. Randomized clinical trial of homocysteine level lowering therapy and fractures. Arch Intern Med 2007;167(19):2136-2139.

2 Gommans J, Yi Q, Eikelboom JW, Hankey GJ, Chen C, Rodgers H. The effect of homocysteine-lowering with B-vitamins on osteoporotic fractures in patients with cerebrovascular disease: substudy of VITATOPS, a randomised placebo-controlled trial. BMC Geriatr 2013;13:88.

3 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):1082-1088.

4 van Wijngaarden JP, Dhonukshe-Rutten RAM, van Schoor NM et al. Rationale and design of the B-PROOF study, a randomized controlled trial on the effect of supplemental intake of vitamin B12 and folic acid on fracture incidence. BMC Geriatr 2011;11:80.

5 van Meurs JBJ, Dhonukshe-Rutten RAM, Pluijm SMF et al. Homocysteine levels and the risk of osteoporotic fracture. N Engl J Med 2004;350(20):2033-2041.

6 Sambrook P, Cooper C. Osteoporosis. Lancet 2006;367(9527):2010-2018.

7 Homocysteine Lowering Trialists' Collaboration. Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. Am J Clin Nutr 2005;82(4):806-812.

8 van Oort FVA, Melse-Boonstra A, Brouwer IA et al. Folic acid and reduction of plasma homocysteine concentrations in older adults: a dose-response study. Am J Clin Nutr 2003;77(5):1318-1323.

9 Chung M, Lee J, Terasawa T, Lau J, Trikalinos TA. Vitamin D with or without calcium supplementation for prevention of cancer and fractures: an updated meta-analysis for the U.S. Preventive Services Task Force. Ann Intern Med 2011;155(12):827-838.

10 Karasik D, Demissie S, Cupples LA, Kiel DP. Disentangling the genetic determinants of human aging: biological age as an alternative to the use of survival measures. J Gerontol A Biol Sci Med Sci 2005;60(5):574-587.

11 Borkan GA, Norris AH. Assessment of biological age using a profile of physical parameters. J Gerontol 1980;35(2):177-184.

12 Benetos A, Okuda K, Lajemi M et al. Telomere length as an indicator of biological aging: the gender effect and relation with pulse pressure and pulse wave velocity. Hypertension 2001;37(2 Pt 2):381-385.

13 Aviv A. Hypothesis: pulse pressure and human longevity. Hypertension 2001;37(4):1060-1066.

14 Sell DR, Lane MA, Johnson WA et al. Longevity and the genetic determination of collagen glycoxidation kinetics in mammalian senescence. Proc Natl Acad Sci U S A 1996;93(1):485-490.
15 Seeman TE, McEwen BS, Rowe JW, Singer BH. Allostatic load as a marker of cumulative biological risk: MacArthur studies of successful aging. Proc Natl Acad Sci U S A 2001;98(8):4770-4775.
16 Bulpitt CJ, Rajkumar C, Cameron JD. Vascular compliance as a measure of biological age. J Am Geriatr Soc 1999;47(6):657-663.
17 Fisher AL. Of worms and women: sarcopenia and its role in disability and mortality. J Am Geriatr Soc 2004;52(7):1185-1190.
18 de Ruijter W, Westendorp RG, Assendelft WJ et al. Use of Framingham risk score and new biomarkers to predict cardiovascular mortality in older people: population based observational cohort study. BMJ 2009;338:a3083.
19 Qiu C, Winblad B, Fratiglioni L. The age-dependent relation of blood pressure to cognitive function and dementia. Lancet Neurol 2005;4(8):487-499.
20 Kado DM, Bucur A, Selhub J, Rowe JW, Seeman T. Homocysteine levels and decline in physical function: MacArthur Studies of Successful Aging. Am J Med 2002;113(7):537-542.
21 Kuo HK, Liao KC, Leveille SG et al. Relationship of homocysteine levels to quadriceps strength, gait speed, and late-life disability in older adults. J Gerontol A Biol Sci Med Sci 2007;62(4):434-439.
22 Soumare A, Elbaz A, Ducros V, Tavernier B, Alperovitch A, Tzourio C. Cross-sectional association between homocysteine and motor function in the elderly. Neurology 2006;67(6):985-990.
23 Rolita L, Holtzer R, Wang C, Lipton R, Derby C, Verghese J. Homocysteine and Mobility in Older Adults. J Am Geriatr Soc 2010;58:545-550.
24 Avlund K, Schroll M, Davidsen M, Løvborg B, Rantanen T. Maximal isometric muscle strength and functional ability in daily activities among 75-year-old men and women. Scand J Med Sci Sports 1994;4:32-40.
25 Viitasalo J, Era P, Leskinen A, Heikkinen E. Muscular strength profiles and anthropometry in random samples of men aged 31–35, 51–55 and 71–75 years. Ergonomics 1985;28:1563-1574.
26 van Meurs JB, Pare G, Schwartz SM et al. Common genetic loci influencing plasma homocysteine concentrations and their effect on risk of coronary artery disease. Am J Clin Nutr 2013;98(3):668-676.
27 Lewerin C, Matousek M, Steen G, Johansson B, Steen B, Nilsson-Ehle H. Significant correlations of plasma homocysteine and serum methylmalonic acid with movement and cognitive performance in elderly subjects but no improvement from short-term

vitamin therapy: a placebo-controlled randomized study. Am J Clin Nutr 2005;81(5):1155-1162.
28 Wyckoff KF, Ganji V. Proportion of individuals with low serum vitamin B-12 concentrations without macrocytosis is higher in the post folic acid fortification period than in the pre folic acid fortification period. Am J Clin Nutr 2007;86(4):1187-1192.
29 Clarke R, Halsey J, Bennett D, Lewington S. Homocysteine and vascular disease: review of published results of the homocysteine-lowering trials. J Inherit Metab Dis 2011;34(1):83-91.
30 Zhou YH, Tang JY, Wu MJ et al. Effect of folic acid supplementation on cardiovascular outcomes: a systematic review and meta-analysis. PLoS One 2011;6(9):e25142.
31 Smulders YM, Blom HJ. The homocysteine controversy. J Inherit Metab Dis 2011;34(1):93-99.
32 Integraal Kankercentrum Nederland. 2014. http://cijfersoverkanker.nl.
33 Vollset SE, Clarke R, Lewington S et al. Effects of folic acid supplementation on overall and site-specific cancer incidence during the randomised trials: meta-analyses of data on 50,000 individuals. Lancet 2013;381(9871):1029-1036.
34 Wien TN, Pike E, Wisloff T, Staff A, Smeland S, Klemp M. Cancer risk with folic acid supplements: a systematic review and meta-analysis. BMJ Open 2012;2(1):e000653.
35 Qin X, Cui Y, Shen L et al. Folic acid supplementation and cancer risk: a meta-analysis of randomized controlled trials. Int J Cancer 2013;133(5):1033-1041.
36 Ebbing M, Bonaa KH, Nygard O et al. Cancer incidence and mortality after treatment with folic acid and vitamin B12. JAMA 2009;302(19):2119-2126.
37 Smith AD, Kim YI, Refsum H. Is folic acid good for everyone? Am J Clin Nutr 2008;87(3):517-533.
38 Ulrich CM, Potter JD. Folate supplementation: too much of a good thing? Cancer Epidemiol Biomarkers Prev 2006;15(2):189-193.
39 Kim YI. Folate: a magic bullet or a double edged sword for colorectal cancer prevention? Gut 2006;55(10):1387-1389.
40 Holick MF, Binkley NC, Bischoff-Ferrari HA et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 2011;96(7):1911-1930.
41 Gezondheidsraad. Naar een toereikende inname van vitamine D. 2008. Den Haag: Gezondheidsraad.
42 Institute of Medicine. Dietary Reference Intakes for Calcium and Vitamin D. 2011. Washington, DC: The National Academies Press.
43 van Schoor NM, Visser M, Pluijm SMF, Kuchuk N, Smit JH, Lips P. Vitamin D deficiency as a risk factor for osteoporotic fractures. Bone 2008;42(2):260-266.

44 Avenell A, Gillespie WJ, Gillespie LD, O'Connell D. Vitamin D and vitamin D analogues for preventing fractures associated with involutional and post-menopausal osteoporosis. Cochrane Database Syst Rev 2009;(2):CD000227.
45 Abrahamsen B, Masud T, Avenell A. Patient level pooled analysis of 68 500 patients from seven major vitamin D fracture trials in US and Europe. BMJ 2010;340:b5463.
46 Trivedi DP, Doll R, Khaw KT. Effect of four monthly oral vitamin D3 (cholecalciferol) supplementation on fractures and mortality in men and women living in the community: randomised double blind controlled trial. BMJ 2003;326(7387):469.
47 Reid IR, Bolland MJ, Grey A. Effects of vitamin D supplements on bone mineral density: a systematic review and meta-analysis. Lancet 2014;383(9912):146-155.
48 Muir SW, Montero-Odasso M. Effect of vitamin D supplementation on muscle strength, gait and balance in older adults: a systematic review and meta-analysis. J Am Geriatr Soc 2011;59(12):2291-2300.
49 Bischoff-Ferrari HA, wson-Hughes B, Staehelin HB et al. Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials. BMJ 2009;339:b3692.
50 Avenell A, MacLennan GS, Jenkinson DJ et al. Long-term follow-up for mortality and cancer in a randomized placebo-controlled trial of vitamin D(3) and/or calcium (RECORD trial). J Clin Endocrinol Metab 2012;97(2):614-622.
51 Verhagen AP, de Vet HC, de Bie RA et al. The Delphi list: a criteria list for quality assessment of randomized clinical trials for conducting systematic reviews developed by Delphi consensus. J Clin Epidemiol 1998;51(12):1235-1241.
52 Huisman M, Poppelaars J, van der Horst M et al. Cohort Profile: The Longitudinal Aging Study Amsterdam. Int J Epidemiol 2011;40:868-876.
53 Kwon S, Perera S, Pahor M et al. What is a meaningful change in physical performance? Findings from a clinical trial in older adults (the LIFE-P study). J Nutr Health Aging 2009;13(6):538-544.
54 Perera S, Mody SH, Woodman RC, Studenski SA. Meaningful change and responsiveness in common physical performance measures in older adults. J Am Geriatr Soc 2006;54(5):743-749.
55 Crosby RD, Kolotkin RL, Williams GR. Defining clinically meaningful change in health-related quality of life. J Clin Epidemiol 2003;56(5):395-407.
56 de Vet HC, Ostelo RW, Terwee CB et al. Minimally important change determined by a visual method integrating an anchor-based and a distribution-based approach. Qual Life Res 2007;16(1):131-142.
57 Guralnik JM, Branch LG, Cummings SR, Curb JD. Physical performance measures in aging research. J Gerontol 1989;44(5):M141-M146.
58 Myers AM, Holliday PJ, Harvey KA, Hutchinson KS. Functional performance measures: are they superior to self-assessments? J Gerontol 1993;48(5):M196-M206.

59 Kempen GI, Steverink N, Ormel J, Deeg DJ. The assessment of ADL among frail elderly in an interview survey: self-report versus performance-based tests and determinants of discrepancies. J Gerontol B Psychol Sci Soc Sci 1996;51(5):254-260.
60 Wang R, Lagakos SW, Ware JH, Hunter DJ, Drazen JM. Statistics in medicine--reporting of subgroup analyses in clinical trials. N Engl J Med 2007;357(21):2189-2194.
61 Sun X, Ioannidis JP, Agoritsas T, Alba AC, Guyatt G. How to use a subgroup analysis: users' guide to the medical literature. JAMA 2014;311(4):405-411.
62 Davey Smith G, Ebrahim S. 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? Int J Epidemiol 2003;32(1):1-22.
63 Minelli C, Thompson JR, Tobin MD, Abrams KR. An integrated approach to the meta-analysis of genetic association studies using Mendelian randomization. Am J Epidemiol 2004;160(5):445-452.
64 van Schoor NM, Smit JH, Twisk JW, Lips P. Impact of vertebral deformities, osteoarthritis, and other chronic diseases on quality of life: a population-based study. Osteoporos Int 2005;16(7):749-756.
65 Honein MA, Paulozzi LJ, Mathews TJ, Erickson JD, Wong LY. Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. JAMA 2001;285(23):2981-2986.
66 Williams LJ, Mai CT, Edmonds LD et al. Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. Teratology 2002;66(1):33-39.
67 Canfield MA, Collins JS, Botto LD et al. Changes in the birth prevalence of selected birth defects after grain fortification with folic acid in the United States: findings from a multi-state population-based study. Birth Defects Res A Clin Mol Teratol 2005;73(10):679-689.
68 Gezondheidsraad. Naar een optimaal gebruik van foliumzuur. 2008. Den Haag: Gezondheidsraad.
69 European Food Safety Authority. Tolerable upper intake levels for vitamins and minerals. 2006. EFSA.