

# Folic Acid and Vitamin B12 Supplementation and the Risk of Cancer: Long-term Follow-up of the B Vitamins for the Prevention of Osteoporotic Fractures (B-PROOF) Trial



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## Abstract

**Background:** Folic acid and vitamin B12 play key roles in one-carbon metabolism. Disruption of one-carbon metabolism may be involved in the risk of cancer. Our aim was to assess the long-term effect of supplementation with both folic acid and vitamin B12 on the incidence of overall cancer and on colorectal cancer in the B Vitamins for the Prevention of Osteoporotic Fractures (B-PROOF) trial.

**Methods:** Long-term follow-up of B-PROOF trial participants ( $N = 2,524$ ), a multicenter, double-blind randomized placebo-controlled trial designed to assess the effect of 2 to 3 years daily supplementation with folic acid (400  $\mu\text{g}$ ) and vitamin B12 (500  $\mu\text{g}$ ) versus placebo on fracture incidence. Information on cancer incidence was obtained from the Netherlands cancer registry (Integraal Kankercentrum Nederland), using the International Statistical Classification of Disease (ICD-10) codes C00–C97 for all can-

cers (except C44 for skin cancer), and C18–C20 for colorectal cancer.

**Results:** Allocation to B vitamins was associated with a higher risk of overall cancer [171 (13.6%) vs. 143 (11.3%); HR 1.25; 95% confidence interval (CI), 1.00–1.53,  $P = 0.05$ ]. B vitamins were significantly associated with a higher risk of colorectal cancer [43 (3.4%) vs. 25 (2.0%); HR 1.77; 95% CI, 1.08–2.90,  $P = 0.02$ ].

**Conclusions:** Folic acid and vitamin B12 supplementation was associated with an increased risk of colorectal cancer.

**Impact:** Our findings suggest that folic acid and vitamin B12 supplementation may increase the risk of colorectal cancer. Further confirmation in larger studies and in meta-analyses combining both folic acid and vitamin B12 are needed to evaluate whether folic acid and vitamin B12 supplementation should be limited to patients with a known indication, such as a proven deficiency.

## Introduction

A large proportion of the population globally, especially older people, use dietary supplements to promote good health (1). Studies of National and International Food Consumption Surveys, reported for example in the United States, United Kingdom, and the Netherlands that 56%, 39%, and 27% of older adults, respectively, use dietary supplements (2–5). However, supplements may not always be favorable, and in certain cases or doses, they may even have adverse health effects (6). In addition, potential effects need to be put into perspective according to different fortification policies in countries (e.g., mandated vs. voluntary folic acid fortification). Together with vitamin B12, folic acid plays a key role in one-carbon metabolism being involved in DNA methylation and DNA synthesis (7, 8). Several studies have suggested that altered DNA methylation is associated with a higher risk of certain cancers, including breast, prostate, and colorectal cancer. Until recently, it was believed that folate and folic acid supplementation may have a protective effect on the risk of malignancies (9, 10). However, over the past decade, there have been some concerns that folic acid supplementation may actually increase the risk of cancer (11–15), possibly by promoting the progression of preneoplastic and undiagnosed neoplastic lesions (8, 16).

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Trial registration: The B-PROOF study is registered with the Netherlands Trial Register (NTR NTR1333) and with ClinicalTrials.gov (NCT00696514).

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**doi:** 10.1158/1055-9965.EPI-17-1198

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Although some countries, including the United States, South Africa, and Australia, have introduced population-wide folic acid fortification to prevent neural tube defects in the fetus (17–21), mandatory folic acid fortification has not been implemented in New Zealand or in several Western European countries partly because of these concerns about potential adverse effects on cancer incidence or progression (21, 22).

A recent meta-analysis of 10 studies ( $n = 19, 106$ ; age range 26–69 years), reported no significant excess risk of folic acid (0.4–1 mg) supplementation on overall cancer incidence (23). However, previous results from the B Vitamins for the Prevention of Osteoporotic Fractures (B-PROOF) study, a randomized controlled trial on vitamin B12 and folic acid supplementation on fracture risk in older persons, reported a higher incidence of self-reported cancer in the intervention group relative to the control group after a follow-up of 2 to 3 years [HR 1.56; 95% confidence interval (CI), 1.04–2.31]. Additional subgroup analysis revealed that the excess risk was predominantly explained by a higher colorectal cancer incidence, and that the effect appeared to be strongest in people aged older than 80 years (24).

Because the adverse effect of folic acid and vitamin B12 supplementation on self-reported (colorectal) cancer was previously observed within 2 to 3 years in the B-PROOF study (24), the objective of this study was to validate these findings with data on confirmed cancer diagnosis and assess the long-term effects of folic acid and vitamin B12 cosupplementation on the risk of overall cancer incidence and on colorectal cancer using prolonged follow-up of trial participants. As such, this secondary analysis of the B-PROOF study will contribute to current understanding of the biological plausibility of the effect of folic acid and vitamin B12 cosupplementation on cancer (colorectal cancer) risk, which will contribute to the ongoing fortification debate in several countries.

## Materials and Methods

The B-PROOF study is a large multi-center [Erasmus MC (Rotterdam, the Netherlands), VU University Medical Center (VUmc; Amsterdam, the Netherlands), and Wageningen University (WUR; Wageningen, the Netherlands)], randomized, placebo-controlled, and a double blind study, investigating the effect of daily oral vitamin B12 and folic acid supplementation over a period of 2 to 3 years on fracture incidence.

Recruitment of participants took place between September 2008 and March 2011. A detailed description and study protocol of the trial has been reported elsewhere (25). Participants ( $n = 2,919$ ) aged 65 years and over with an elevated homocysteine level (Hcy 12–50  $\mu\text{mol/L}$ ) were included. Participants were excluded if they had a renal insufficiency (creatinine level  $> 150 \mu\text{mol/L}$ ) or history of a malignancy (excluding non-melanoma skin cancer) in the past 5 years or if they used high dosages of B vitamins (intramuscular injections of vitamin B12 and/or folic acid intake  $> 300 \mu\text{g/day}$ ; this was reported at the time of recruitment and was asked again by the questionnaire at the baseline).

Written informed consent was obtained before allocated treatment for all the participants. For this analysis, we used only the information of participants who gave permission to contact health institutes and medical doctors for their health details and medical history ( $n = 2,524$ ).

The B-PROOF study was registered in the Netherlands Trial Register (NTRNTR1333) and ClinicalTrials.gov (NCT00696514). The Ethics Committee approval for the study protocol was obtained from the Medical Ethics committees of Erasmus MC, VUmc, and WU, according to the declaration of Helsinki (25).

The intervention group received a daily tablet with 500  $\mu\text{g}$  vitamin B12 and 400  $\mu\text{g}$  folic acid. In addition, both the control and intervention groups received 15  $\mu\text{g}$  (600 IU) of vitamin D3 daily to ensure a normal vitamin D status. The intervention and placebo tablets, produced by Orthica, are indistinguishable in taste, smell, and appearance. The duration of intervention was 2 years, and to increase power, individuals who finished their participation extended their participation for 1 more year ( $n = 339$  had 3-year intervention; ref. 25).

The primary outcome of this study was the incidence of any cancer defined on the basis of the International Statistical Classification of Disease (ICD-10) codes, C00–C97. Individual data were obtained by linkage to the Netherlands cancer registry, Integraal Kankercentrum Nederland (IKNL), from baseline until May, 2017. The Netherlands Cancer Registry is linked to the International Agency for Research on Cancer (IARC) and delivers pseudonymous data to the European database of the European Network of Cancer Registries (ENCR). Hence, employees of the cancer registry were unaware of treatment allocation of the participants. We used C00–C97 ICD codes for overall cancer (except C44 for skin cancer), and C18–C20 for colorectal cancer (26).

At baseline, height was measured using a stadiometer in duplicate to the nearest 0.1 cm and weight by using a calibrated weighing device (SECA 761) to the nearest 0.5 kg, both without wearing shoes (25). Body mass index (BMI) was calculated as weight in kg/height in  $\text{m}^2$ . A structured questionnaire was used to assess self-reported medical history (cardiovascular disease and diabetes mellitus), current use of medication and supplements, alcohol intake, and smoking habits. Blood was collected, plasma homocysteine (Hcy), serum folate, vitamin B12, holotranscobalamin (HoloTC), 25(OH)D, and methylenetetrahydrofolate reductase (MTHFR) genotype were determined; details of the methods used have been described previously (25).

## Statistical analyses

We extended the follow-up of the original B-PROOF study to study the incidence of pathology-proven solid cancers. For all variables, mean with SD or percentages were reported for each group. Differences between groups were tested with the *t* test for continuous variables, Mann–Whitney *U* test for normally skewed variables, and  $\chi^2$  test for categorical variables. The cumulative event-free survival for cancer was analyzed by a Kaplan–Meier event curve. We calculated follow-up time as the number of months from the baseline measurement until the first diagnosis of incident cancer, death, loss-to-follow-up, or end-of-the-study period, whichever occurred first. The incidence rate ratio was calculated on the incidence-rate of cancer for both treatment groups, which is defined as the number of cancer cases divided by the total sum of the follow-up in each group (cases/persons years). To avoid bias, primary analysis was based on the intention-to-treat (ITT) principle, where participants were analyzed on the basis of the initial treatment allocation. Unadjusted Cox proportional hazard analyses were conducted with treatment (intervention vs. control group) as the independent variable and the cancer diagnosis as the dependent outcome variable. Multivariable

Cox proportional hazard regression analyses were applied adjusted for serum HoloTC, because this variable differed significantly between the intervention and the control group despite randomization. All other potential confounders were equally distributed between both groups. In addition, subgroup analyses were performed to assess whether the treatment effect was different in strata of sex, age, plasma Hcy, and MTHFR polymorphism, interaction with these variables were evaluated in the multivariable model for both overall cancer and colorectal cancer. When  $P$  for interaction was  $<0.1$ , subgroup analyses were performed. Second, per protocol (PP) analyses were performed that included data only from subjects who were compliant ( $>80\%$  of pills consumed) to the study protocol, details have been described previously (25) and also a sensitivity analysis was performed in participants who were not using folic acid and vitamin B12 supplements. Exploratory analysis has been done by duration of treatment (2 years vs. 3 years). Furthermore, for comparison purposes, we also calculated the risk ratio (RR) and log-rank observed–expected statistic. Values of  $P < 0.05$  were considered to be statistically significant. Analyses were performed using IBM SPSS 21.

## Results

A flow chart of 2,524 participants (86.5% of the initial 2,919 participants) is shown in Fig. 1. Baseline characteristics were similar for the participants with and without informed consent for medical follow-up ( $n = 2,524$  vs.  $n = 395$ ).

Table 1 presents the selected baseline characteristics of the B-PROOF population, by allocated treatment. Mean (SD) age was 74 years (6.2) in both treatment and control groups and mean values for all other baseline characteristics were similar for treatment ( $n = 1,257$ ) and control groups ( $n = 1,267$ ), except for serum HoloTC concentration, which was slightly higher in the treatment group [mean 74.3 (44.5 SD) vs. 70.9 (42.5 SD);  $P < 0.05$ ].

ITT analyses showed that 314 persons were diagnosed with any cancer [171 cases (13.6%) in the intervention group vs. 143 cases

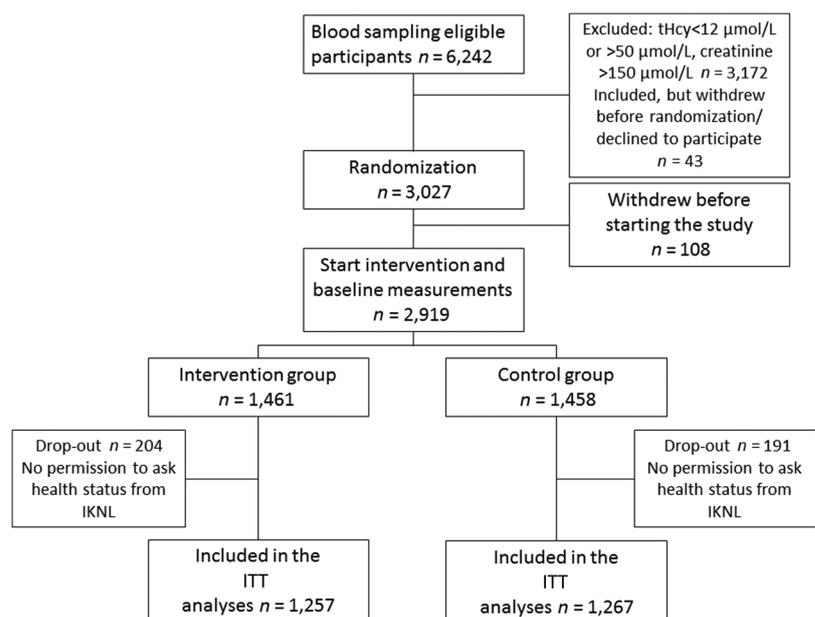
(11.3%) in the control group] and 68 persons were diagnosed with colorectal cancer [43 cases (3.4%) in the intervention group vs. 25 cases (2.0%) in the control group] during a median follow up of 78 months; IQR: 74–83. Crude Cox proportional hazards models showed that persons in the intervention group did not have a significantly higher risk of any cancer than persons in the control group (HR 1.23; 95% CI, 0.98–1.53; Table 2). However, the risk of colorectal cancer was significantly higher in the intervention group than persons in the control group (HR 1.76; 95% CI, 1.07–2.88; Table 2; Figs. 2 and 3). After additional adjustment for baseline HoloTC, the risk of any cancer tended to be higher in the intervention group than in the control group (HR 1.25; 95% CI, 1.00–1.57) and a significant increased risk remained for colorectal cancer (HR 1.77; 95% CI, 1.08–2.90; Table 2).

Interaction analyses revealed that the effect of the intervention did not significantly differ by age ( $<80$  vs.  $>80$  years), sex, plasma Hcy, and MTHFR polymorphism ( $P > 0.10$ ) for overall cancer and colorectal cancer.

Per protocol (PP) analysis was conducted in compliant participants ( $n = 2,330$ ). After PP analyses, the HR on any cancer weakened but the HR on colorectal cancer became stronger relative to the ITT analyses (HR 1.00; 95% CI, 0.99–1.00 and HR 2.17; 95% CI, 1.26–3.75, respectively, in the adjusted model; Table 3). Sensitivity analysis in participants who were not using folic acid and/or vitamin B12 supplements, showed that the HR for any cancer and colorectal cancer became stronger relative to the ITT analyses (HR 1.30; 95% CI, 1.01–1.66 and HR 2.10; 95% CI, 1.21–3.63 respectively in the adjusted model).

Exploratory analysis stratified by duration of the treatment (2 years vs. 3 years) showed that the HR on colorectal cancer were slightly weaker for participants with 2 years of intervention relative to the ITT analyses, but was still significant (HR = 1.72; 95% CI, 1.03–2.88 in the adjusted model).

We compared the results from the Cox proportional hazard model with the risk ratio (RR) and log-rank statistics and found similar results.



**Figure 1.** Flowchart of the B-PROOF trial based on the CONSORT 2010 statement. A total of 2,524 participants were included in the ITT analyses.

**Table 1.** Selected characteristics of the trial population ( $n = 2,524$ )

	<b>Control: Vitamin D (<math>n = 1,267</math>)</b>	<b>Missing (<math>n</math>)</b>	<b>Intervention: Folic acid, vitamin B12, and vitamin D (<math>n = 1,257</math>)</b>	<b>Missing (<math>n</math>)</b>	<b>P</b>
Age(years) <sup>a</sup>	74.0 (6.2)	0	73.9 (6.6)	0	0.63
Sex (% women)	48.6	0	50.4	0	0.36
Education years (%)		2		0	0.30
5-10	67.3		65.3		
11-18	32.7		32.7		
Alcohol consumption (%)		1		0	0.57
Light	66.7		66.9		
Moderate	28.8		29.6		
Excessive	4.0		3.0		
Very excessive	0.4		0.5		
Smoking status (%)		0		0	0.80
Never	33.9		33.5		
Current	9.4		10.2		
Former	56.7		56.3		
BMI <sup>a</sup>	27.2 (4.0)	8	27.1 (4.0)	10	0.67
Homocysteine ( $\mu\text{mol/L}$ ) <sup>b</sup>	14.5 (13.0-16.7)	0	14.3 (13.0-16.5)	0	0.40
MTHFR (%)		161		145	0.27
CC	42.8		44.2		
CT	41.2		39.3		
TT	12.6		12.3		
Folic acid supplement use (%)		0		0	0.94
Yes	14.8		14.5		
No	83.3		83.8		
When necessary	1.9		1.8		
Vitamin B12 supplement use (%)		0		0	0.96
Yes	15.1		14.5		
No	83.2		79.3		
When necessary	1.9		1.8		
Vitamin D supplement use (%)		0		0	0.85
Yes	19.7		18.8		
No	78.5		79.3		
When necessary	1.7		1.8		
Serum 25(OH)D (nmol/L) <sup>a</sup>	55.8 (23.9)	30	56.0 (25.9)	26	0.82
Serum folate (nmol/L) <sup>a</sup>	20.1 (7.3)	44	20.3 (7.4)	45	0.47
Serum vitamin B12 (pmol/L) <sup>a</sup>	283.6 (115.0)	17	289.7 (116.2)	11	0.19
Serum HoloTC (pmol/L) <sup>a</sup>	70.9 (42.5)	11	74.3 (44.5)	7	0.05 <sup>c</sup>

<sup>a</sup>Mean (SD).<sup>b</sup>Median (IQR).<sup>c</sup> $P < 0.05$ .

## Discussion

The findings of this study showed that allocating older persons with mildly elevated homocysteine levels to receive combined folic acid and vitamin B12 supplementation was associated with a slight excess risk of overall cancer, but a statistically significant increased risk for colorectal cancer when compared with placebo. The effect on colorectal cancer risk was even more extreme in compliant participants (>80%). As difference in cancer risk was already apparent within the first years of follow-up, these findings are consistent with evidence that folic acid (combined with vitamin B12) may promote the growth of early precursor mucosal lesions (8, 16). However, on the basis of the previous observations

and the results of the B-PROOF study, we cannot yet ascertain whether this is due to an individual effect of folic acid or vitamin B12, or an interactive effect of both folic acid and vitamin B12 combined. The current findings confirm previous observations from the primary analyses of the B-PROOF trial (using self-reported cancer data), which showed an increased cancer risk in participants using folic acid and vitamin B12 supplementation (24). A major strength of this study is the extended follow-up of the B-PROOF study combined with the use of pathology-proven malignancies as an outcome measure. This is particularly important because of the long latency period between dietary risk-factors and cancer as well as the timeframe from premalignant lesions to cancer diagnosis in the elderly. However, the findings

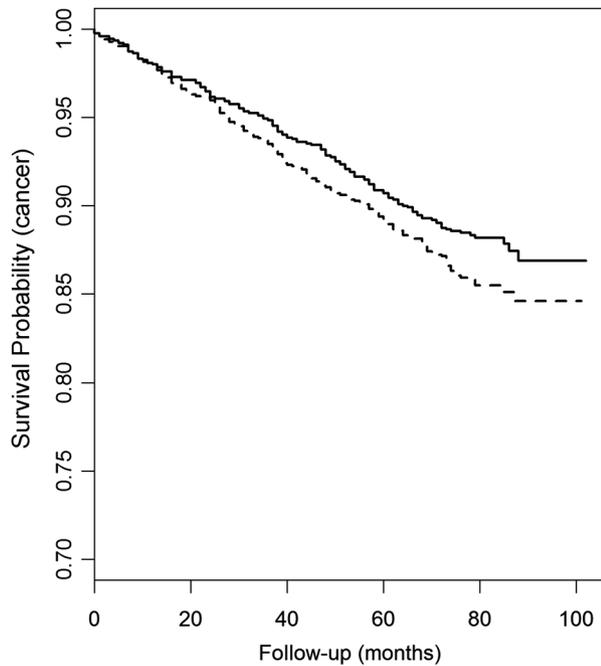
**Table 2.** Effect of folic acid and vitamin B12 on overall cancer, and on colorectal cancer incidence

<b>Type of cancer (cases treatment vs. control group)</b>	<b>Cases/100 PY treatment versus control group</b>	<b>HR (95%CI)<sup>a</sup></b>	<b>P</b>	<b>HR (95% CI)<sup>b</sup></b>	<b>P</b>
Any cancers (171 vs. 143)	2.3 vs. 1.9	1.23 (0.98-1.53)	0.07	1.25 (1.00-1.57)	0.05
Colorectal cancer (43 vs. 25)	0.6 vs. 0.3	1.76 (1.07-2.88) <sup>c</sup>	0.03	1.77 (1.08-2.90) <sup>c</sup>	0.02

NOTE: Cox proportional hazard analysis of risk of cancers (ITT) in total group

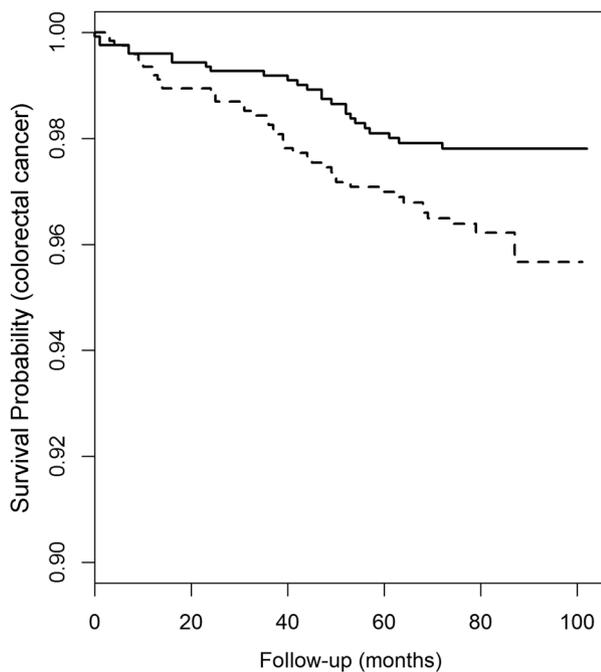
Abbreviation: PY, person years.

<sup>a</sup>Crude model.<sup>b</sup>Adjusted for HoloTC (significant difference between intervention and control group).<sup>c</sup> $P < 0.05$ .



**Figure 2.** Kaplan-Meier curve of survival analysis of any cancers for the intervention (dashed line) and the control group (continuous line) and the follow-up time in months.

differ from three recent meta-analyses, with mostly overlapping trials, which studied the effects of folic acid on cancer risk. Qin and colleagues found no significant overall effect of folic acid



**Figure 3.** Kaplan-Meier curve of survival analysis of colorectal cancer for the intervention (dashed line) and the control group (continuous line) and the follow-up time in months.

supplementation (mean dosage 1.64 mg) on cancer and colorectal cancer during a mean follow-up time of 5.3 years (mean age: 62.5 years; ref. 27). Vollset and colleagues demonstrated no significant effect on cancer incidence including colon cancer in a time-frame of 1.8 to 7.4 years with a mean dosage of 4.7 mg (mean age: 64 years; ref. 28). In contrast, Baggott and colleagues (2012) reported a higher cancer risk in participants receiving folic acid supplementation during 3 to 8 years (mean dosage 1.3 mg), in a meta-analysis of a subset of these trials (mean age: 62 years; ref. 29). The results of this study differ from the three meta-analyses, probably because these studies addressed a younger population, as well as different dosages of supplementation and different outcome measures. It should also be noted that most studies included individual folic acid supplementation without vitamin B12. Besides B-PROOF, only two other randomized controlled trials (RCTs) studied the effect of both folic acid and vitamin B12. Although these trials included a selected population of people with ischemic heart disease, they also observed a significantly higher overall cancer risk (HR 1.21; 95% CI, 1.03–1.41;  $P=0.02$ ; ref. 11), which is consistent with our findings. Most of the previous trials had a shorter follow-up, albeit they had a longer duration of treatment than B-PROOF. Whether folic acid, vitamin B12, or both explain the results, cannot yet be confirmed, especially because the studies of the effect of dietary, supplements, and plasma levels of folate and vitamin B12 on cancer risk showed opposing results and none of these were RCTs (30–33). For example, Matejic and colleagues (2017) found that overall, folate, and vitamin B12 status was not clearly associated with breast cancer risk in their prospective cohort study. They did, however, find potential interactions between vitamin B12 and folate on the risk of breast cancer and suggested that low plasma folate concentrations (mainly 5-methyltetrahydrofolate), as a consequence of high vitamin B12 status, may impair DNA methylation (32). Price and colleagues (2016) reported a small increased risk of prostate cancer with higher folate and vitamin B12 concentrations, in data from six cohorts (33). Another recent study, reported a 30% to 40% increase in lung cancer risk in men using vitamin B12 supplements (not from multivitamins). They found no association of use of folic acid supplements in men and women in risk of lung cancer (31).

The previous analysis of the B-PROOF study showed that curves for cancer incidence separated shortly after the start of the intervention, which may imply that the effect of the treatment was on cancer progression rather than cancer induction. Because our previous analysis of the B-PROOF study showed a higher risk in persons aged >80 y (24), and given that the results of this study showed that 18 of the 28 excess cancers were colorectal cancer, it may be argued that the older age group has a higher prevalence of latent colorectal neoplastic cells (8) because the risk of colorectal cancer increases with advanced age (34) and older individuals may, therefore, be more prone to the effects of folic acid and vitamin B12 supplementation. However, we did not have data on the presence of early neoplastic lesions in the colorectal mucosa to confirm this hypothesis.

The intervention dosage was 500  $\mu\text{g}$  vitamin B12 and 400  $\mu\text{g}$  folic acid per day. Although the dosage of folic acid was close to the recommended daily intake and well below the tolerable upper intake level for folic acid of 1 mg/day in Europe (35), the dosage of vitamin B12 was almost 200 times higher than the recommended intake. For vitamin B12, no systematic toxicologic effects have been reported so far (35), but we cannot rule out that the high

**Table 3.** Effect of folic acid and vitamin B12 on overall cancer, and on colorectal cancer incidence

Type of cancer (cases treatment vs. control group)	Cases/PY treatment versus control group	HR (95%CI) <sup>a</sup>	P	HR (95% CI) <sup>b</sup>	P
Any cancers (160 vs. 124)	2.3 vs. 1.7	1.32 (1.05–1.67) <sup>c</sup>	0.02	1.00 (0.99–1.00)	0.10
Colorectal cancer (40 vs. 19)	0.6 vs. 0.3	2.15 (1.25–3.72) <sup>c</sup>	0.01	2.17 (1.26–3.75) <sup>c</sup>	0.01

NOTE: Cox proportional hazard analysis of risk of cancers (PP) in compliance participants >80%.

<sup>a</sup>Crude model.

<sup>b</sup>Adjusted for HoloTC (significant difference between intervention and control group).

<sup>c</sup> $P < 0.05$ .

Abbreviation: PY, person years.

dosage of vitamin B12 supplementation influenced the risk of colorectal cancer in our study.

There may be several plausible mechanisms by which folic acid and vitamin B12 supplementation increase the risk of colorectal cancer in particular. First, the epithelial cells of the colorectal mucosa have the most rapid turnover rate of any tissue in the body. Hence, it may be speculated that this tissue may be particularly sensitive to nutrients involved in cell growth such as B vitamins. Folic acid and vitamin B12 play a key role in one-carbon metabolism and cells require one-carbon units for DNA synthesis and methylation (36). Thus, these nutrients may influence pathways enhancing proliferation of cancer cells and modulate the DNA and therefore, the chance of developing a neoplastic cell (36). Folate has been demonstrated to affect neoplastic cells by enhancing growth in both animal and *in vitro* models in DNA synthesis (36). Both folic acid and vitamin B12 are essential for the synthesis of methionine and S-adenosyl methionine (SAM), which are required as the common methyl donor for the regulation of DNA methylation patterns in DNA-influencing gene expression (36–38). DNA methylation occurs mainly in CpG dinucleotides, concentrated in short CpG-rich DNA fragments so-called CpG islands (39, 40). In normal cells, CpG island in active promoters can be methylated, which lead to long-term silencing of transcription. However, gene expression may be inactivated in genes that are hypermethylated at their CpG island-containing promoters, through which a neoplastic cell can develop (41). Currently, little is known about the possible relation between vitamin B12 and cancer risk. However, because vitamin B12 has a key role in one-carbon metabolism and cells require one-carbon units for DNA synthesis, methylation as well as redox and reductive metabolism, vitamin B12 may influence pathways enhancing the proliferation of cancer cells (42).

A second potentially relevant mechanism for colorectal cancer specifically may be via the gut microbiome. Several studies have shown that microbial imbalance of *Fusobacterium spp.* and *Streptococcus gallolyticus susp. gallolyticus* may play a role in colorectal cancer etiology (43–45). Vitamin B12 and folate can be synthesized by human gut microbes as a valuable resource in the gut (46). It has been suggested by others that competition and exchange of vitamin B12 and cofactors from both dietary intake and gut microbes affect the gut microbial community (46). Thus, there may be an interaction between the gut microbiome and B vitamins but further exploration of this hypothesis is needed.

This study has several strengths as well as potential limitations. The main strengths of this study were the randomized controlled study design, the pathology-proven cancer, the large sample size of elderly subjects, and the prolonged follow-up relative to other trials. A limitation of this study is that it presents secondary

analyses of a randomized controlled trial primarily designed to study the effect on fracture risk. As a result, significant results of such analysis have to be interpreted in the context of other evidence in the literature. However, with a sample size of 2,524, an alpha of 5%, and a power of 80%, our study was able to detect a HR of 0.85/1.18 on overall cancer (47). In addition, the decision to study colorectal cancer in the B-PROOF study and other (nonsite specific) was made on the basis of prior results on self-reported cancer as adverse event of the trial. We did not include other gastrointestinal cancers due to the limited power. It can be argued that this approach may increase the probability of type I errors because we did not adjust for multiple comparisons. For possible type I errors, stringent interpretation of  $P$  values, especially for the results on all (nonsite specific) cancers ( $P = 0.05$ ) should be made with caution. The initial B-PROOF study included 2,919 participants, but for the current extended follow-up analyses, we collected data from a subgroup of 2,524 participants, which could introduce a source of bias. However, there was no difference between the intervention and control group in baseline characteristics between the participants with and without informed consent for medical follow-up.

Another source of potential bias was that the allocation to the intervention and control group was no longer blinded to the researchers. However, because the data collection of cancer was derived from the independent national cancer registry, and the physicians involved in the cancer diagnosis were blinded to the allocation of the intervention, observer bias is unlikely. Another possible limitation is that we only included Caucasian participants aged 65 years and over with elevated homocysteine levels in a country where no mandated folic acid fortification has been implemented. Therefore, the results may not be generalizable to other populations. Nonetheless, this trial is one of the few that were done in a population without mandated folic acid fortification and relatively low supplement use. As a result we were able to clearly discern the effect of supplementation in a population with limited intake of folic acid above the tolerable upper intake level.

## Conclusion

This study reported a higher risk of colorectal cancer among those allocated to folic acid and vitamin B12 compared with placebo, which persisted over time (6–9 years). This was observed in older ambulant persons with mildly elevated homocysteine concentrations. The primary analyses of the B-PROOF trial did not show any protective effect of folic acid and vitamin B12 supplementation on fracture, falls, and cardiovascular disease (with the exception of cerebrovascular accident). However, because secondary analyses of this trial showed potential adverse effects on cancer, careful monitoring of long-term hazards of B vitamins is required before making any recommendations for public health related to the

implementation of fortification policies. To clarify the role of combined supplementation with B vitamins on colorectal cancer, further confirmation, for example by individual meta-analyses of existing, large RCT of folic acid and vitamin B12, with additional information on the presence of early neoplastic lesions in the colorectal mucosa, is needed.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Disclaimer

The sponsors and patients had no role in the design or implementation of the study, data collection, data management, data analysis, data interpretation, or in the preparation, review, or approval of the manuscript.

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### Acknowledgments

The authors are grateful to all study participants and coworkers who helped in the organization of the B-PROOF trial. We would particularly like to acknowledge the support of Ms. A. Ham, Dr. S. Smit, R.N.; Ms. P.H. in 't Veld, MSc., and Prof. P. Lips, for practical help in the trial and Prof. R. Clarke for providing advice on the article. The initial B-PROOF study has received funding so far by the Netherlands Organization for Health Research and Development (ZonMw, Grant 6130.0031), The Hague; unrestricted grant from NZO (Dutch Dairy Association), Zoetermeer; Orthica, Almere; Netherlands Consortium Healthy Ageing (NCHA) Leiden/Rotterdam; Ministry of Economic Affairs, Agriculture and Innovation (project KB-15-004-003), The Hague; Wageningen University, Wageningen; VUmc, Amsterdam; Erasmus Medical Center, Rotterdam.

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Received January 5, 2018; revised March 21, 2018; accepted October 4, 2018; published first October 19, 2018.

### References

- Rock CL. Multivitamin-multimineral supplements: who uses them? *Am J Clin Nutr* 2007;85:277S–9S.
- Gahche J, Bailey R, Burt V, Hughes J, Yetley E, Dwyer J, et al. Dietary supplement use among U.S. adults has increased since NHANES III (1988–1994). *NCHS Data Brief* 2011;61:1–8.
- Ocke MC, Buurma-Rethans EJM, de Boer EJ, Wilson-van den Hooven C, Etemad-Ghameslou Z, Drijvers JJMM, et al. Diet of community-dwelling older adults: Dutch National Food Consumption Survey Older adults 2010–2012. RIVM Rijksinstituut voor Vplksgezondheid en Milieu; 2013. Contract No.: RIVM Rapport 050413001. Available from: <https://www.rivm.nl/bibliotheek/rapporten/050413001.pdf>.
- van Rossum CTM FH, Verkaik-Kloosterman J, Buurma-Rethans EJM, Ocke MC. Dutch National Food Consumption Survey 2007–2010: Diet of children and adults aged 7 to 69 years. RIVM Rijksinstituut voor Volksgezondheid en Milieu; 2011. Contract No.: RIVM Rapport 350050006. Available from: <https://www.rivm.nl/bibliotheek/rapporten/350050006.pdf>.
- Department of Health and the Food Standards Agency GOV.UK (2012). National diet and nutrition survey headline results from years 1, 2 and 3 (combined) of the rolling programme 2008/2009–2010/11. [cited 2018 8 May]. Available from: [https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment\\_data/file/207708/NDNS-Y3-report\\_All-TEXT-docs-combined.pdf](https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/207708/NDNS-Y3-report_All-TEXT-docs-combined.pdf)
- Boyles AL, Yetley EA, Thayer KA, Coates PM. Safe use of high intakes of folic acid: research challenges and paths forward. *Nutr Rev* 2016;74:469–74.
- Friso S, Udali S, De Santis D, Choi SW. One-carbon metabolism and epigenetics. *Mol Aspects Med* 2017;54:28–36.
- Miller JW, Ulrich CM. Folic acid and cancer—where are we today? *Lancet* 2013;381:974–6.
- Kim YI. Role of folate in colon cancer development and progression. *J Nutr* 2003;133:3731S–9S.
- Burr NE, Hull MA, Subramanian V. Folic acid supplementation may reduce colorectal cancer risk in patients with inflammatory bowel disease: a systematic review and meta-analysis. *J Clin Gastroenterol* 2017;51:247–53.
- Ebbing M, Bonna KH, Nygard O, Arnesen E, Ueland PM, Nordrehaug JE, et al. Cancer incidence and mortality after treatment with folic acid and vitamin B12. *JAMA* 2009;302:2119–26.
- Neuhouser ML, Nijhout HF, Gregory JF 3rd, Reed MC, James SJ, Liu A, et al. Mathematical modeling predicts the effect of folate deficiency and excess on cancer-related biomarkers. *Cancer Epidemiol Biomarkers Prev* 2011;20:1912–7.
- Figueiredo JC, Grau MV, Haile RW, Sandler RS, Summers RW, Bresalier RS, et al. Folic acid and risk of prostate cancer: results from a randomized clinical trial. *J Natl Cancer Inst* 2009;101:432–5.
- Chau R, Dashti SG, Ait Ouakrim D, Buchanan DD, Clendenning M, Rosty C, et al. Multivitamin, calcium and folic acid supplements and the risk of colorectal cancer in Lynch syndrome. *Int J Epidemiol* 2016;45:940–53.
- Moazzzen S, Dolatkah R, Tabrizi JS, Shaarbafti J, Alizadeh BZ, de Bock GH, et al. Folic acid intake and folate status and colorectal cancer risk: A systematic review and meta-analysis. *Clin Nutr* 2018;37:1926–34.
- Kim YI. Folate and colorectal cancer: an evidence-based critical review. *Mol Nutr Food Res* 2007;51:267–92.
- Centers for Disease Control and Prevention. Trends in wheat-flour fortification with folic acid and iron—worldwide, 2004 and 2007. *MMWR Morb Mortal Wkly Rep* 2008;57:8–10.
- Jacques PF, Selhub J, Bostom AG, Wilson PW, Rosenberg IH. The effect of folic acid fortification on plasma folate and total homocysteine concentrations. *N Engl J Med* 1999;340:1449–54.
- Sayed AR, Bourne D, Pattinson R, Nixon J, Henderson B. Decline in the prevalence of neural tube defects following folic acid fortification and its cost-benefit in South Africa. *Birth Defects Res A Clin Mol Teratol* 2008;82:211–6.
- Lopez-Camelo JS, Castilla EE, Orioli IM, INAGEMP (Instituto Nacional de Genética Médica Populacional), ECLAMC (Estudio Colaborativo Latino Americano de Malformaciones Congénitas). Folic acid flour fortification: impact on the frequencies of 52 congenital anomaly types in three South American countries. *Am J Med Genet A* 2010;152A:2444–58.

21. The Australian Institute of Health and Welfare (2011). Mandatory folic acid and iodine fortification in Australia and New Zealand: baseline report for monitoring. Available from: <https://www.aihw.gov.au/getmedia/1a4bb10d-dba2-479e-99fabefcfaf62d1/10787.pdf.aspx?inline=true>.
22. The European Food Safety Authority (EFSA) (2010). Folic Acid: An update on scientific developments. EFSA meeting summary report. Uppsala, Sweden. Available from: <https://www.livmedelsverket.se/globalassets/matvanor-halsa-miljo/kostrad-matvanor/gravida/folic-acid—an-update-on-scientific-developments.-rapport.-efsa-european-food-safety-authority.-2009..pdf?amp;epslanguage=sv>.
23. 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:e000653.
24. van Wijngaarden JP, Swart KM, Enneman AW, Dhonukshe-Rutten RA, van Dijk SC, Ham AC, et al. Effect of daily vitamin B-12 and folic acid supplementation on fracture incidence in elderly individuals with an elevated plasma homocysteine concentration: B-PROOF, a randomized controlled trial. *Am J Clin Nutr* 2014;100:1578–86.
25. van Wijngaarden JP, Dhonukshe-Rutten RA, van Schoor NM, van der Velde N, Swart KM, Enneman AW, 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.
26. World Health Organisation (WHO) (2015). International statistical classification of diseases and related health problems 10th revision (ICD-10). Version 2015. Available at: <http://apps.who.int/classifications/icd10/browse/2015/en#/C00-C97>.
27. 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;133:1033–41.
28. Vollset SE, Clarke R, Lewington S, Ebbing M, Halsey J, Lonn E, 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;381:1029–36.
29. Baggott JE, Oster RA, Tamura T. Meta-analysis of cancer risk in folic acid supplementation trials. *Cancer Epidemiol* 2012;36:78–81.
30. Arendt JF, Farkas DK, Pedersen L, Nexø E, Sørensen HT. Elevated plasma vitamin B12 levels and cancer prognosis: a population-based cohort study. *Cancer Epidemiol* 2016;40:158–65.
31. 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;35:3440–8.
32. Matejčić 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;140:1246–59.
33. Price AJ, Travis RC, Appleby PN, Albanes D, Barricarte Currea A, Borge T, et al. Circulating folate and vitamin B12 and risk of prostate cancer: a collaborative analysis of individual participant data from six cohorts including 6875 cases and 8104 controls. *Eur Urol* 2016;70:941–51.
34. Nolen SC, Evans MA, Fischer A, Corrada MM, Kawas CH, Bota DA. Cancer - incidence, prevalence and mortality in the oldest-old. A comprehensive review. *Mech Ageing Dev* 2017;164:113–26.
35. European Food Safety Authority (EFSA), Scientific Committee on Food Scientific Panel on Dietetic Products, Nutrition and Allergies (2006). Tolerable upper intake levels for vitamins and minerals. Available from: [http://www.efsa.europa.eu/sites/default/files/efsa\\_rep/blobserver\\_assets/ndatolerableuil.pdf](http://www.efsa.europa.eu/sites/default/files/efsa_rep/blobserver_assets/ndatolerableuil.pdf).
36. Williams EA. Folate, colorectal cancer and the involvement of DNA methylation. *Proc Nutr Soc* 2012;71:592–7.
37. Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis* 1997;18:869–82.
38. Wagner C. Biochemical role of folate in cellular metabolism. *Clin Res Regul Aff* 2001;18:161–80.
39. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, Rebhan M, et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* 2007;39:457–66.
40. Yamada Y, Watanabe H, Miura F, Soejima H, Uchiyama M, Iwasaka T, et al. A comprehensive analysis of allelic methylation status of CpG islands on human chromosome 21q. *Genome Res* 2004;14:247–66.
41. Chen QW, Zhu XY, Li YY, Meng ZQ. Epigenetic regulation and cancer (review). *Oncol Rep* 2014;31:523–32.
42. Newman AC, Maddocks ODK. One-carbon metabolism in cancer. *Br J Cancer* 2017;116:1499–504.
43. Pagnini C, Corleto VD, Mangoni ML, Pillozzi E, Torre MS, Marchese R, et al. Alteration of local microflora and alpha-defensins hyper-production in colonic adenoma mucosa. *J Clin Gastroenterol* 2011;45:602–10.
44. Sobhani I, Amiot A, Le Baleur Y, Levy M, Auriault ML, Van Nhieu JT, et al. Microbial dysbiosis and colon carcinogenesis: could colon cancer be considered a bacteria-related disease? *Therap Adv Gastroenterol* 2013;6:215–29.
45. Tjalsma H, Boleij A, Marchesi JR, Dutilh BE. A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. *Nat Rev Microbiol* 2012;10:575–82.
46. Degnan PH, Taga ME, Goodman AL. Vitamin B12 as a modulator of gut microbial ecology. *Cell Metab* 2014;20:769–78.
47. Schoenfeld DA. Sample-size formula for the proportional-hazards regression model. *Biometrics* 1983;39:499–503.

# BLOOD CANCER DISCOVERY

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*Cancer Epidemiol Biomarkers Prev* Published OnlineFirst October 19, 2018.

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